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Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells

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The reprogramming of somatic cells into induced pluripotent stem (iPS) cells upon overexpression of the transcription factors Oct4, Sox2, Klf4 and cMyc is inefficient. It has been assumed that the somatic differentiation state provides a barrier for efficient reprogramming; however, direct evidence for this notion is lacking. Here, we tested the potential of mouse hematopoietic cells at different stages of differentiation to be reprogrammed into iPS cells. We show that hematopoietic stem and progenitor cells give rise to iPS cells up to 300 times more efficiently than terminally differentiated B and T cells do, yielding reprogramming efficiencies of up to 28%. Our data provide evidence that the differentiation stage of the starting cell has a critical influence on the efficiency of reprogramming into iPS cells. Moreover, we identify hematopoietic progenitors as an attractive cell type for applications of iPS cell technology in research and therapy.

Transcription factor–induced reprogramming of somatic cells into iPS cells has been achieved in mouse[1–4], rat[5,6], monkey[7] and human[8–11]. It involves introducing the four transcription factors Oct4, Sox2, cMyc and Klf4, or the alternative set Oct4, Sox2, Lin28 and Nanog, into cells by retrovirus-mediated gene delivery, giving rise to pluripotent cells that are highly similar to embryonic stem (ES) cells. However, a major bottleneck of induced pluripotency in research and therapy is its extremely low efficiency: upon infection of diverse primary adult cells such as fibroblasts, keratinocytes, liver cells and pancreatic β cells with retroviruses or lentiviruses expressing Oct4, Sox2, cMyc and Klf4, only 0.01–0.2% of transduced cells give rise to iPS cells[12]. Reprogramming efficiencies are even lower when using transient gene delivery approaches[13–15]. The development of so-called 'secondary systems' has enabled the generation of iPS cells at higher efficiencies without the need for direct infection. In this approach, 'primary' iPS cells that have been generated with doxycycline-inducible versions of the reprogramming factors are first differentiated into somatic cells. Exposure of these cells to doxycycline then results in the re-expression of the factors and the generation of 'secondary' iPS cells[16–18].

During reprogramming, the epigenetic state of a somatic cell must be reset to a state compatible with pluripotentiality[19]. Therefore, it has been assumed that the differentiation state of the starting cell might influence the efficiency of reprogramming by providing a barrier to efficient epigenetic remodeling of the genome. Consistent with this notion, the treatment of differentiated cells undergoing reprogramming with compounds that inhibit DNA or histone methylation as well as histone deacetylation give rise to iPS cells more efficiently[6,20–23]. However, in a potential therapeutic setting, small compounds may have unpredictable long-term consequences. Identifying cell types that are most amenable to reprogramming would provide an alternative to the use of chemicals and teach us about the molecular barriers underlying nuclear reprogramming.

Somatic stem and progenitor cells in the adult share some features with pluripotent stem cells, such as the capacity to differentiate into different cell types and the presence of certain transcriptional regulators[24,25]. Therefore, it is conceivable that the genome of adult progenitor cells is more amenable to reprogramming than that of differentiated cells. Indeed, results from nuclear transfer experiments have indicated that neural and keratinocyte progenitors give rise to cloned embryos or mice more efficiently than differentiated cell types[26,27]. Notably, in the hematopoietic system, an inverse correlation has been reported between the differentiation stage of donor cells and cloning efficiency[28,29]: differentiated granulocytes were found to be more susceptible to reprogramming into cloned blastocysts than hematopoietic progenitor and stem cells. However, these data were a subject of controversy, as scoring the number

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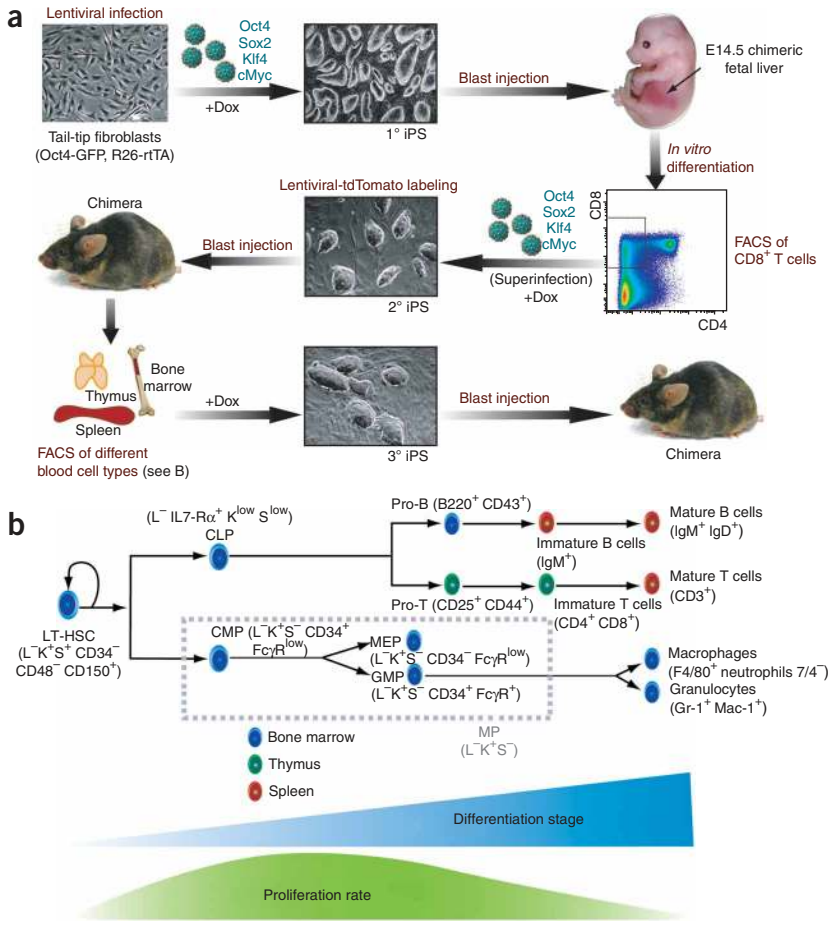

Figure 1 Development of a transgenic system for inducible expression of Oct4, Sox2, Klf4 and cMyc in the mouse hematopoietic system. (**a**) Strategy used to reprogram hematopoietic cells. Tail-tip fibroblasts heterozygous for the ROSA26-M2rtTA and Oct4-GFP knock-in alleles were infected with four different doxycycline-inducible lentiviruses encoding Oct4, Sox2, Klf4 and cMyc. Resulting primary (1°) iPS cells were injected into mouse blastocysts. Fetal liver was isolated from E14.5 embryos, *in vitro* differentiated by stromal co-culture and sorted for emerging $CD8^+$ T cells by FACS. $CD8^+$ T cells were optionally superinfected with the four doxycycline-inducible lentiviruses to ensure the strongest possible transgene expression and were subsequently cultured on doxycycline for 12 d. The resulting secondary (2°) iPS cells were labeled with a lentivirus constitutively expressing tdTomato and injected into blastocysts to generate adult chimeras. $tdTomato^+$ hematopoietic cells at different stages of differentiation were isolated by FACS from spleen, thymus and bone marrow and treated with doxycycline. The resulting tertiary (3°) iPS cell lines were used for molecular and functional analyses, including rearrangement and pluripotency assays. (**b**) Schematic illustration of hematopoiesis, with emphasis on the cell populations used in this study. Height of blue triangle indicates degree of differentiation (from HSCs to progenitors to terminally differentiated cells). Green curve illustrates proliferation rate (highest in progenitors; lowest in quiescent HSCs and most terminally differentiated cells). Surface markers used for FACS purification are given in parentheses. HSCs, hematopoietic stem cells; MP, myeloid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; CLP, common lymphoid progenitor; L, lin; K, c-Kit; S, Sca-1.

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of cloned preimplantation embryos can be misleading and may not reflect true reprogramming potential[30].

The role of the differentiation stage of the starting cell during transcription factor–mediated reprogramming has not yet been rigorously addressed. A recent study suggested that neural progenitors give rise to iPS cells at a reasonably high efficiency (3.6%) using direct viral infection[31]. However, another study did not find any major differences in efficiency of iPS cell formation by neural progenitors compared with fibroblasts[32], and a third independent report found that neural progenitors were actually less efficient than fibroblasts[33]. Notably, the expression of the four reprogramming factors in terminally differentiated B lymphocytes has been found to be insufficient for the generation of iPS cells, which requires the viral introduction of an additional transcription factor, C/EBPα (ref. 34). In contrast, mature pancreatic β cells have been converted into iPS cells by the ectopic expression of the four reprogramming factors alone[35]. Thus, it remains unclear whether the differentiation stage of the starting cell has an influence on the efficiency of reprogramming into iPS cells.

Here, we have addressed this question by generating iPS cells from prospectively isolated hematopoietic stem cells (HSCs), myeloid and lymphoid progenitors and several mature blood cell types using a genetically homogeneous 'secondary system' to express the four reprogramming factors (Oct4, Sox2, cMyc and Klf4). Our results show that all hematopoietic cell types tested, including terminally differentiated lymphocytes, can be reprogrammed into iPS cells by expression of the four transcription factors alone. Moreover, we demonstrate that the differentiation stage of cells has a strong impact on the efficiency and kinetics of reprogramming.

RESULTS

Development of 'secondary system' to generate iPS cells

To determine the reprogramming potential of different blood cells, we generated a 'secondary system' that allows expression of reprogramming factors in a controllable fashion in a genetically homogenous population of cells[16–18]. Specifically, we derived iPS cells from neonatal mouse tail-tip fibroblasts carrying an Oct4-GFP reporter[36] as well as the ROSA–reverse tetracycline-controlled transactivator (rtTA)[37] (**Fig. 1a**). Infecting these fibroblasts with lentiviruses expressing Oct4, Sox2, Klf4 and cMyc under the control of doxycycline-inducible promoters[38] gave rise to primary iPS cells that activated Oct4-GFP and could be propagated in the absence of doxycycline, indicating reprogramming into a transgene-independent pluripotent state (data not shown).

To assess whether defined mature hematopoietic cells derived from these iPS cells could be reprogrammed into secondary iPS cells, we attempted differentiation into T cells, whose maturation stage can be identified prospectively by surface markers and retrospectively by T cell receptor rearrangement analyses. First, we generated embryonic day 14.5 (E14.5) chimeric fetuses by blastocyst injection of primary iPS cells and isolated fetal liver cells known to contain hematopoietic progenitors (**Fig. 1a**). We co-cultured fetal liver cells with OP9-DL1

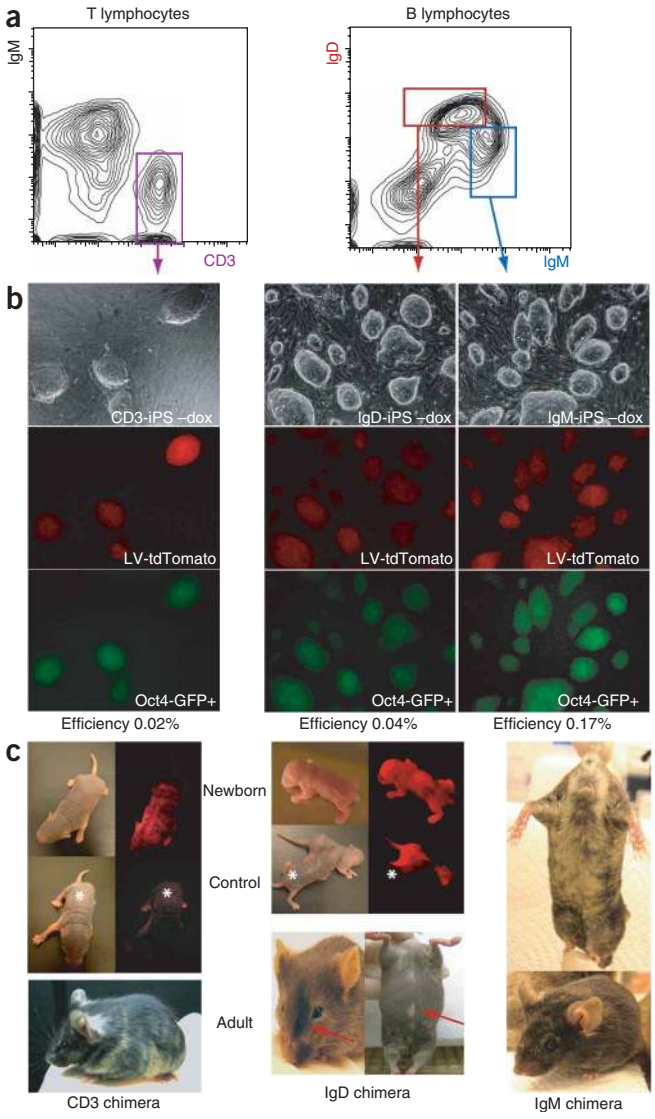

Figure 2 Generation and characterization of iPS cells derived from mature B and T lymphocytes by expression of the four transcription factors (Oct4, Sox2, Klf4 and cMyc) using secondary system. (**a**) Sorting strategy of tdTomato$^+$ (gate not shown) mature CD3$^+$ splenic T lymphocytes, immature IgM$^+$ and mature IgM^{low} IgD^{high} B lymphocytes from 10-week-old CD8-iPS chimeras by FACS. (**b**) ES-like morphology of tdTomato$^+$ Oct4-GFP$^+$ doxycycline-independent (–dox) iPS cell colonies derived from mature B and T cells. (**c**) Viable newborn chimeras (top) derived from CD3-iPS and IgD-iPS cell lines show red fluorescence originating from the labeled iPS cell lines. Shown are tdTomato$^+$ chimeric pups and nonchimeric littermates (marked by *) under regular light (left) and UV light (right). Adult chimeras (bottom) derived from CD3-iPS, IgD-iPS and IgM-iPS cells show obvious coat color chimerism.

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stromal feeder cells to induce T cell differentiation[39]. After 13 d, mature CD8$^+$ T cells emerged, which we sorted by FACS and explanted on regular irradiated mouse embryonic fibroblasts (MEFs) in the presence of doxycycline to reactivate the viral transgenes. As previous attempts to reprogram mature lymphocytes by expression of the four factors alone have been unsuccessful[34], we subjected cultured cells to another round of viral infection with the four factors to ensure the strongest possible transgene expression. Secondary iPS cell colonies that reactivated Oct4-GFP became detectable after 12 d and could be passaged in the absence of doxycycline (**Fig. 1a** and data not shown). Of note, viral superinfection was not essential for generating iPS cells from mature CD8$^+$ T cells, as we could also derive them without an additional round of infection (**Supplementary Fig. 1**). These results show that fetal liver–derived CD8$^+$ T cells remain amenable to reprogramming into iPS cells by expression of the four factors alone. We refer to these T cell-derived iPS cells as 'CD8-iPS' cells.

B and T cells reprogrammed by only four factors

Next, we wanted to determine whether adult-derived lymphocytes remain equally amenable to reprogramming into iPS cells by the expression of the four reprogramming factors. To this end, we labeled the CD8-iPS cell clone with a lentivirus constitutively expressing tdTomato[14] and injected cells into blastocysts to produce chimeric mice (**Fig. 1a**). CD8-iPS cells gave rise to high-degree chimeras, as shown by widespread red fluorescence of newborn pups and broad coat color chimerism of surviving adult mice (data not shown).

First, we attempted to reprogram mature T cells. We isolated mature peripheral splenic T lymphocytes from CD8-iPS chimeric mice using the surface marker CD3 (**Figs. 1b** and **2a**). Upon plating of CD3$^+$ T lymphocytes on feeder cells in ES medium in the presence of doxycycline and the T cell cytokines interleukin-2 (IL-2) and concanavalin A (Online Methods), Oct4-GFP–expressing tertiary iPS colonies emerged after 12 d in culture and could be passaged upon discontinuation of doxycycline (**Fig. 2b**, left); we refer to these cells as 'CD3-iPS' cells. The efficiency of CD3-iPS formation was roughly 0.02%, which is lower than that observed for heterogeneous tail fibroblasts isolated from the same animal (0.74%).

Because previous attempts to generate iPS cells from mature B lymphocytes by expressing the four factors have been unsuccessful[34], we next assessed whether immature, splenic B cells can give rise to iPS cells. Indeed, plating of IgM$^+$ immature B cells (**Figs. 1b** and **2a**) on feeder cells in the presence of doxycycline and the B cell stimulants CpG and lipopolysaccharide (LPS) gave rise to Oct4-GFP$^+$ iPS cell ('IgM-iPS') colonies at an average efficiency of 0.17% (**Fig. 2b**, right). Notably, even terminally differentiated B lymphocytes from spleen or lymph nodes, identified by IgM and IgD positivity (IgM^{low} IgD^{high}; **Figs. 1b** and **2a**) and cultured under the same conditions as IgM$^+$ cells, gave rise to Oct4-GFP$^+$ iPS colonies (which we refer to as 'IgD-iPS') at an average frequency of 0.04% (**Fig. 2b**, center). To exclude the possibility that secondary cells, which have already gone through one round of reprogramming, are more susceptible to reprogramming than primary cells, we also generated iPS cells from terminally differentiated IgM^{low} IgD^{high} B cells isolated from mice carrying the ROSA-rtTA transactivator and directly infected with a polycistronic doxycycline-inducible lentivirus expressing Oct4, Sox2, Klf4 and cMyc[40] (data not shown). Together, these results indicate that adult terminally differentiated B and T lymphocytes remain amenable to reprogramming into pluripotent stem cells by only four transcription factors. All iPS cell lines gave rise to well-differentiated teratomas consisting of cell types from all three germ layers (**Supplementary Fig. 2a**) and produced chimeric neonatal and adult mice (**Fig. 2c**), thus demonstrating their pluripotency. Moreover, a CD8-iPS derived high-degree chimera gave rise to germline offspring at 100% efficiency when mated with wild-type females (**Supplementary Fig. 3a**).

A previous attempt to reprogram mature B cells required the overexpression of the transcription factor C/EBPα in addition to the expression of the four factors to produce iPS cells[34]. To test whether

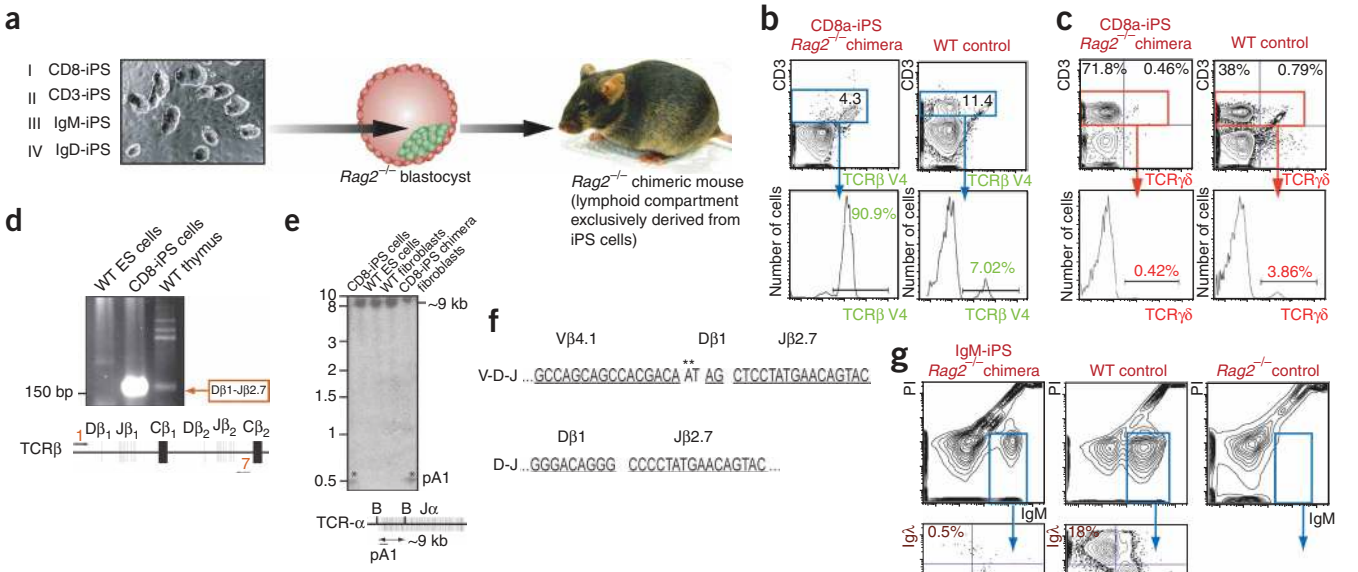

Figure 3 Monoclonal immune system in *Rag2*$^{-/-}$ chimeras produced with lymphocyte-derived iPS cells. (**a**) Schematic illustration of Rag2 complementation assay. (**b**) The majority of CD3^{+} cells derived from CD8-iPS chimeras express Vβ4.1 T cell receptor (TCR), versus 7.02% in wild-type (WT) control mice. (**c**) CD3^{+} splenocytes from CD8-iPS chimeras lack TCRγδ-expressing T cells (0.42%, versus 3.86% in WT controls). (**d**) Detection of TCRβ D-J rearrangement by PCR on DNA isolated from the CD8-iPS cell line. D-J locus and primer pair (1,7) are shown below the gel. (**e**) Top: V-D-J rearrangement band (*) at the TCRα locus, as determined by DNA blot analysis of BamHI (B)-digested genomic DNA isolated from CD8a-iPS cells and purified tail-tip cells from CD8-iPS chimeras. Bottom: schematic map of the TCRα locus, including the DNA blot probe pA1. (**f**) Sequenced joints of V-D-J and D-J rearrangements of both TCRβ alleles of the CD8-iPS cell line, as determined by genomic PCR with specific primers. Single elements are underlined and labeled. * indicates P/N nucleotides not encoded in the germline. Results were identical in ten different bacterial clones. (**g**) Detection of Igλ and Igκ light chains on IgM^{+} splenocytes of IgM-iPS *Rag2*$^{-/-}$ chimeras. Most iPS cell–derived IgM^{+} B cells express Igκ light chain (93.6%, versus 71.6% of WT control cells) but lack Igλ chain expression (0.5%, versus 18% in WT). Representative graphs of two experiments are shown. (**h**) Top: DNA blot analysis on genomic DNA from IgD-iPS cell lines to detect rearrangements at the IgH locus, using BamHI (B) with probe pH1, or HindIII (H) with probe pH2. Bottom: schematic map of IgH locus. Black bars denote JH1 to JH4 elements.

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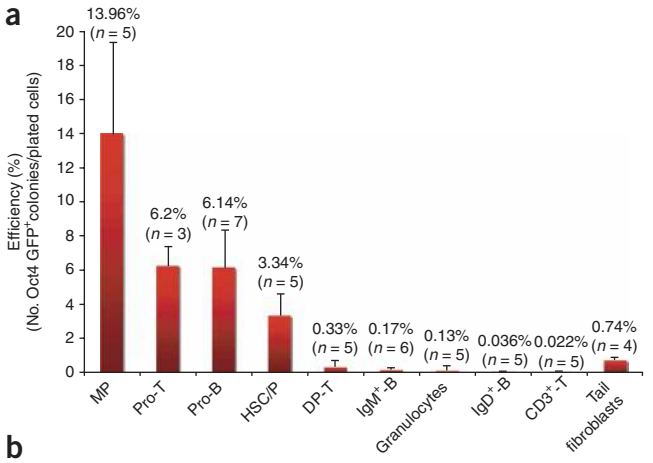
C/EBPα expression could further enhance the low reprogramming efficiency of B cells in our system, we infected tdTomato-positive immature IgM^{+} and mature IgM^{low} IgD^{high} B cells from a CD8-iPS chimera with a retrovirus-expressing C/EBPα and a human CD4 epitope to allow isolation of C/EBPα-expressing cells by FACS (**Supplementary Fig. 4a**). We observed up to a 20-fold increase in the number of Oct4-GFP^{+} colonies in IgM^{+} or IgM^{low} IgD^{high} B cell cultures overexpressing C/EBPα compared with untransduced cells or with cells infected with an empty control virus, indicating that the expression of C/EBPα indeed enhances the overall reprogramming efficiency of B lymphocytes (**Supplementary Fig. 4b,c**). In addition, we noticed that C/EBPα-expressing cultures formed faster, grew into larger colonies and downregulated the B cell marker B220 and upregulated Oct4-GFP sooner compared with untransduced cells (**Supplementary Fig. 4d,e**).

Lymphocyte iPS cells generate monoclonal immune system

Pluripotent cells derived from terminally differentiated lymphocytes by nuclear transfer have been shown to give rise to a monoclonal immune system[41]. To determine whether lymphocyte-derived iPS cells also produce a monoclonal lymphoid compartment, we injected IgM-iPS, IgD-iPS, CD3-iPS and CD8-iPS cells into Rag2-deficient blastocysts (**Fig. 3a**). Rag2 deficiency in mice results in a complete absence of all mature B and T lymphocytes, and thus any B and T cells detected in iPS cell chimeras are exclusively derived from the injected iPS cells[42]. *Rag2*$^{-/-}$ chimeras produced with the CD8-iPS clone had normal spleen, thymus and lymph nodes, indicating that iPS cells can entirely reconstitute different hematopoietic organs. Notably, T cells expressed only one out of 25 possible viable TCRβ receptors (Vβ4.1), demonstrating that the donor T cell had undergone a functional rearrangement that is homogeneously expressed on T cells of *Rag2*$^{-/-}$ chimeric mice, giving rise to an apparently monoclonal T cell compartment (**Fig. 3b**). Germline offspring from CD8-iPS chimeras also expressed the Vβ4.1 rearrangement on the vast majority of their T cells (**Supplementary Fig. 3b**). Consistent with this, we saw an absence of TCRγ/δ^{+} T cells in these mice (**Fig. 3c**). In addition to the functionally rearranged TCRβ allele, CD8-iPS cells contained a partially rearranged TCRβ allele comprising a Dβ1 to Jβ2.7 rearrangement (**Fig. 3d**), and DNA blot analysis confirmed that the TCRα locus had also been rearranged (**Fig. 3e**). Sequence analyses of both rearranged TCR alleles from genomic DNA of CD8-iPS cells corroborated the finding that these cells had been derived from a functional mature T cell (**Fig. 3f**).

Rag2$^{-/-}$ chimeric animals produced from IgM-iPS cells also contained a monoclonal T cell compartment, as they were originally derived from CD8-iPS cells (data not shown). In addition, these mice contained an apparently monoclonal B cell compartment, as suggested by the absence of Igλ and a stronger bias toward Igκ light



b

Cell type	Cytokines	Wells plated	iPS colonies (Oct4-GFP+, dox-independent)	Efficiency (%)	Cell viability on day 5
HSCs	KL,T,FL,3,6	120	19	15.8%	91.6%
HSC/P	KL,T,FL,3,6	276	31	11.2%	68.5%
MP	KL,T,FL,3,6	276	62	22.5%	73%
		120	33	27.5%	
CMP	KL,T,FL,3, 11,E,GM	240	26	10.8%	78.3%
GMP	KL,T,FL,3, 11,E,GM	240	60	25%	100%
MEP	KL,T,FL,3, 11,E,GM	120	11	9.16%	95%
CLP	KL,FL,7	51	8	15.7%	–
Pro-B	KL,FL,7	368	28	7.6%	52.1%
		60	7	11.6%	
DP-T cells	ConA,2	368	–	–	–
CD3+-T cells	ConA,2	368	–	–	–
IgM+-B cells	C,L,7	552	–	–	–
Granulocytes	GM,G	460	–	–	–
Macrophages I	M	360	2	0.55%	70%
Macrophages II	M	280	1	0.35%	63%

c

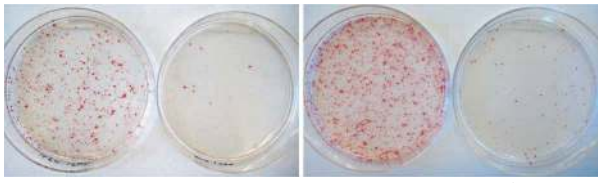


Figure 4 Potential of hematopoietic cell types to be reprogrammed into iPS cells. (**a**) Average efficiency of reprogramming hematopoietic cells into iPS cells. *x* axis shows cell types (see **Fig. 1b**). Cells were explanted on 10-cm dishes on feeder cells in the presence of ES medium supplemented with doxycycline and cytokines. Efficiencies were determined by dividing the number of Oct4-GFP$^+$ colonies that grew in the absence of doxycycline by the number of seeded cells (Online Methods). The percentage above each bar represents the mean value for that sample. Bars represent mean ± s.d. *n* denotes the number of independent experiments for a cell type. The progenitors from hematopoietic lineages show higher reprogramming potentials than any differentiated cell type shown here. (**b**) Reprogramming efficiencies of different cell types after single-cell sorting into 96-well plates. Reprogramming efficiencies were determined by counting Oct4-GFP$^+$ colonies at day 18, 3 d after doxycycline withdrawal. Cell viabilities of individual populations were determined by scoring uninduced cells sorted in 96-well or Terasaki plates. 'Macrophage I' cells were sorted directly from bone marrow into 96-well plates. 'Macrophage II' cells arose from total bone marrow cultures for 5 d in ES medium with macrophage colony-stimulating factor (M-CSF). KL, Kit-ligand; T, TPO; FL, Flt3 ligand; E, erythropoietin; GM, granulocyte-macrophage CSF (GM-CSF); G, G-CSF; M, M-CSF; C, CpG; L, LPS; 3, IL-3; 6, IL-6; 7, IL-7; 11, IL-11; Con A, concanavalin A. (**c**) Alkaline phosphatase staining of iPS-like colonies obtained from selected progenitors and mature cell types. Bone marrow myeloid progenitors (MPs; 1.5×10^3 cells per 10-cm dish), pro–B cells (2×10^3 cells per 10-cm dish), granulocytes (1.5×10^3 cells per 10-cm dish) and spleen IgD$^+$ mature B cells (1×10^6 cells per 10-cm dish) were plated on feeder cells in ES medium supplemented with doxycycline and cytokines. After 12 d, doxycycline was removed, and plates were stained for alkaline phosphatase activity at day 14.

chains on the great majority of peripheral B cells (**Fig. 3g**). Accordingly, we detected the presence of rearrangement bands at the immunoglobulin heavy-chain locus by DNA blot analysis of IgM-iPS cell DNA (**Fig. 3h**). Collectively, these results provide unambiguous genetic and phenotypic evidence for the derivation of iPS cells from terminally differentiated B and T cells by expression of the four factors alone.

High-efficiency formation of iPS cells from lymphoid progenitors

Our observation that IgM$^+$ immature B cells are more efficiently reprogrammed into iPS cells than mature IgM^{low} IgD^{high} B cells (**Fig. 2b**) prompted us to test whether more primitive hematopoietic cell types are even more amenable to reprogramming. To address this question, we sorted several progenitors from the bone marrow and thymus of chimeras produced by injection of the CD8-iPS cell clone (described above) into wild-type blastocysts.

First, we determined the efficiencies of deriving iPS cells from bone marrow–derived progenitor (pro-) B and thymic progenitor (pro-) T cells, which are the immediate precursors of B and T cells and can be isolated based on the surface marker combinations B220$^+$CD43$^+$ for pro–B cells and CD4$^-$CD8$^-$CD44$^+$CD25$^+$ for pro–T cells (also termed the double-negative (DN)-2 stage of T cell development; **Fig. 1b** and **Supplementary Fig. 2b**). Indeed, pro–T cells gave rise to iPS cells at a frequency of ~6.2%, which is almost 20-fold higher than the efficiency of immature CD4$^+$CD8$^+$ double-positive (DP) T cells (0.3%) and more than 300-fold higher than the efficiency of mature peripheral T cells (0.02%) (**Fig. 4a**). Likewise, the derivation of iPS cells from pro–B cells was ~6.1%, a 35-fold increase over immature IgM$^+$ B cells (0.17%) and a 150-fold increase over mature IgM^{low}IgD^{high} B cells (0.04%) (**Fig. 4a**). Our results show that at least in these two major lymphoid lineages, cells progressively lose the potential to give rise to iPS cells with increasing differentiation stage.

To address the possibility that differences in the expression of the viral transgenes between different cell populations might account for the observed differences in reprogramming efficiency, we compared the expression of the four doxycycline-induced viral transgenes in pro–B cells and IgM^{low}IgD^{high} B cells, which showed a 150-fold difference in reprogramming efficiency (**Fig. 4a**). We did not observe any significant differences in the pattern of transgene reactivation between these cell types (**Supplementary Fig. 5a**).

As most hematopoietic cells initially grew in suspension culture, some reprogrammed colonies might have originated from identical founder cells that spread and formed satellite colonies on the plate. To rule out this possibility, we sorted single mature T and B cells (spleen), pro–T cells and pro–B cells and their common precursor cells, common lymphoid progenitors (CLPs) (bone marrow), into single wells of multiple 96-well plates and counted the number of emerging Oct4-GFP$^+$ colonies after 18 d (3 d after doxycycline withdrawal). The efficiency of reprogramming T and B cells was too low to be detected in this experiment (with 200–500 wells analyzed per cell type), but pro–B cells gave rise to iPS cells at a similar frequency (7.6%–11.6%) as when plated on 10-cm plates (3.1%–8.8%). Notably,

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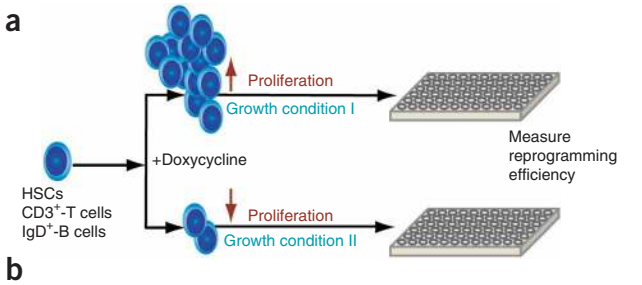

b

Cell type	Growth condition	Cytokines	Cycling cells (%)	Efficiency (%)
HSC	I	KL,T,FL,3,6	40.9%	15.8%
	II	Low dose T	9.09%	18%
$CD3^+$-T cells	I	CD3,CD28,2,15	34.6%	–
	II	CD3,15	9.5%	0.03%
IgD^+-B cells	I	C,L,4,6,7,15	41.8%	0.03%
	II	L,15	12.5%	0.05%

Figure 5 Effect of hematopoietic cell proliferation rate on reprogramming potential. (**a**) HSCs, B cells and T cells were grown under either proliferation-inducing or survival-promoting conditions at the time of transgene expression. The formation of iPS colonies was scored 18 d later, 3 d after doxycycline discontinuation. (**b**) Summary of the growth factor conditions for HSCs, B cells and T cells, the resultant fraction of cycling cells (measured by Hoechst or propidium iodide staining) and the efficiency of iPS cell formation. Reprogramming potential correlates with differentiation stage but not with proliferation rate. The TPO concentration differed in the high (20 ng/ml) and low (0.5 ng/ml) proliferation conditions. Efficiencies of HSCs were determined by single-cell sorting into multiple 96-well plates. Efficiencies of B and T cells were assessed by plating 1×10^5 cells into 12-well dishes in duplicates or triplicates. KL, Kit-ligand; T, TPO; FL, Flt3 ligand; C, CpG; L, LPS; CD3, antibody to CD3; CD28, antibody to CD28; 2, IL-2; 3, IL-3; 4, IL-4; 6, IL-6; 7, IL-7; 11, IL-11; 15, IL-15.

the more primitive CLPs yielded colonies at an efficiency of 15.7%, which is higher than that observed for pro–B and pro–T cells (**Fig. 4b**).

HSCs and myeloid progenitors produce iPS cells more efficiently

Next, we evaluated whether our observations in the lymphoid lineage could be extended to other cell types of the hematopoietic system and whether myeloid progenitors would give rise to iPS cells more efficiently than mature granulocytes and macrophages. Specifically, we isolated myeloid progenitor cells from CD8-iPS chimeras using the surface marker combination $lin^-c\text{-}Kit^+Sca\text{-}1^-$ (**Fig. 1b** and **Supplementary Fig. 2b**), as well as mature granulocytes using the $Gr\text{-}1^+Mac\text{-}1^+$ surface antigens and macrophages isolated by the $F4/80^+$ neutrophil $7/4^-$ marker combination. We plated defined numbers of cells on feeders in the presence of doxycycline. Myeloid progenitor cells gave rise to iPS cells at an average frequency of ~14% (**Fig. 4a**), and single-cell sorting into 96-well plates yielded an even higher efficiency of up to 27.5% (**Fig. 4b**). Myeloid progenitors are a heterogeneous population of cells comprising common myeloid progenitors (CMPs), derivative megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) (**Fig. 1b**). Single-cell sorting of CMPs, GMPs and MEPs into 96-well plates on feeder cells in ES cell medium supplemented with doxycycline and cytokines (**Fig. 4b**) gave rise to iPS cells at efficiencies of 10.8%, 25% and 9.2%, respectively, demonstrating that each progenitor cell type within the myeloid progenitor population has a high reprogramming potential. In contrast, mature granulocytes and macrophages gave rise to iPS cells at frequencies of only 0.13% (more than a 200-fold difference compared with myeloid progenitors) and 0.45% (more than a 60-fold difference compared with myeloid progenitors), respectively. We obtained similar results when we infected myeloid progenitors and granulocytes from ROSA26-rtTA/Oct4-GFP animals directly with concentrated lentivirus expressing Oct4, Sox2, Klf4 and cMyc from a polycistronic construct[40], thus excluding the possibility that the observed differences were specific to the secondary system used (**Supplementary Fig. 5b**). The slightly lower efficiencies of the whole plate–counting approach over the single-cell sorting approach are probably due to the conservative scoring approach; we counted only large iPS cell colonies that appeared on the 10-cm plate and ignored small colonies, as they could have been satellite colonies (Online Methods).

We did not observe any significant differences when studying the reprogramming potential of a mixed, more primitive population of cells containing HSCs and progenitor cells (HSC/Ps), identified by $lin^-c\text{-}Kit^+Sca1^+$ surface markers (**Supplementary Fig. 2b**). iPS cells from HSC/Ps were generated at efficiencies of 3.3% and 11.2% when explanted on whole plates or 96-well dishes, respectively, in ES cell medium supplemented with doxycycline and the cytokines Kit ligand, thrombopoietin (TPO), Flt3 ligand, IL-3 and IL-6 (**Fig. 4a–c**). Similarly, pure HSCs isolated with the most stringent marker combination available ($lin^-c\text{-}Kit^+Sca1^+CD48^-CD150^+CD34^-$)[43,44] and grown under the same culture conditions gave rise to iPS cell colonies at a comparable efficiency (15.8%) (**Fig. 4b**). Both myeloid progenitor– and HSC/P-derived iPS cells produced differentiated teratomas, indicating their pluripotency (**Supplementary Fig. 2a**). Together, these data show that immature cell populations (HSCs, HSC/Ps and myeloid progenitors) in general give rise to iPS cells at higher efficiencies than terminally differentiated cell types.

To test whether human hematopoietic progenitors are equally amenable to reprogramming into iPS cells, we infected $CD34^+$ cord blood progenitors with a human polycistronic vector expressing Oct4, Sox2, Klf4 and cMyc under doxycycline control as well as with a lentiviral construct expressing rtTA (**Supplementary Fig. 6**). iPS cell colonies emerged after roughly 12 d, which could be passaged in the absence of doxycycline. These cells stained positive for the pluripotency markers Tra1-81 and Sox2, differentiated into alpha-fetoprotein (AFP)–positive endodermal and $Tuj1^+$ ectodermal derivatives within embryoid bodies and gave rise to teratomas consisting of ectodermal, mesodermal and endodermal derivatives. This experiment provides a proof of principle for the derivation of iPS cells from human hematopoietic progenitors.

Reprogramming does not correlate with proliferation rate

Proliferation has been assumed to be critical for successful reprogramming[45]. HSCs are in a low-proliferation state and differentiated blood cells in a postmitotic state, whereas progenitor cells are highly proliferative[44] (**Fig. 1b**, **Supplementary Fig. 7** and data not shown). To test directly whether the differentiation stage or the actual proliferation rate of a given cell type affects reprogramming, we evaluated the reprogramming potential of HSCs and mature IgD^+ B cells and $CD3^+$ T cells grown under conditions that either slow or induce proliferation (**Fig. 5a**). Culturing of HSCs in a low dose of TPO (0.5 ng/ml) kept the majority of cells in a quiescent, undifferentiated state[46], whereas exposure to the full cytokine cocktail (Kit ligand, TPO, IL-3, IL-6 and Flt3 ligand) resulted in the induction of vigorous proliferation

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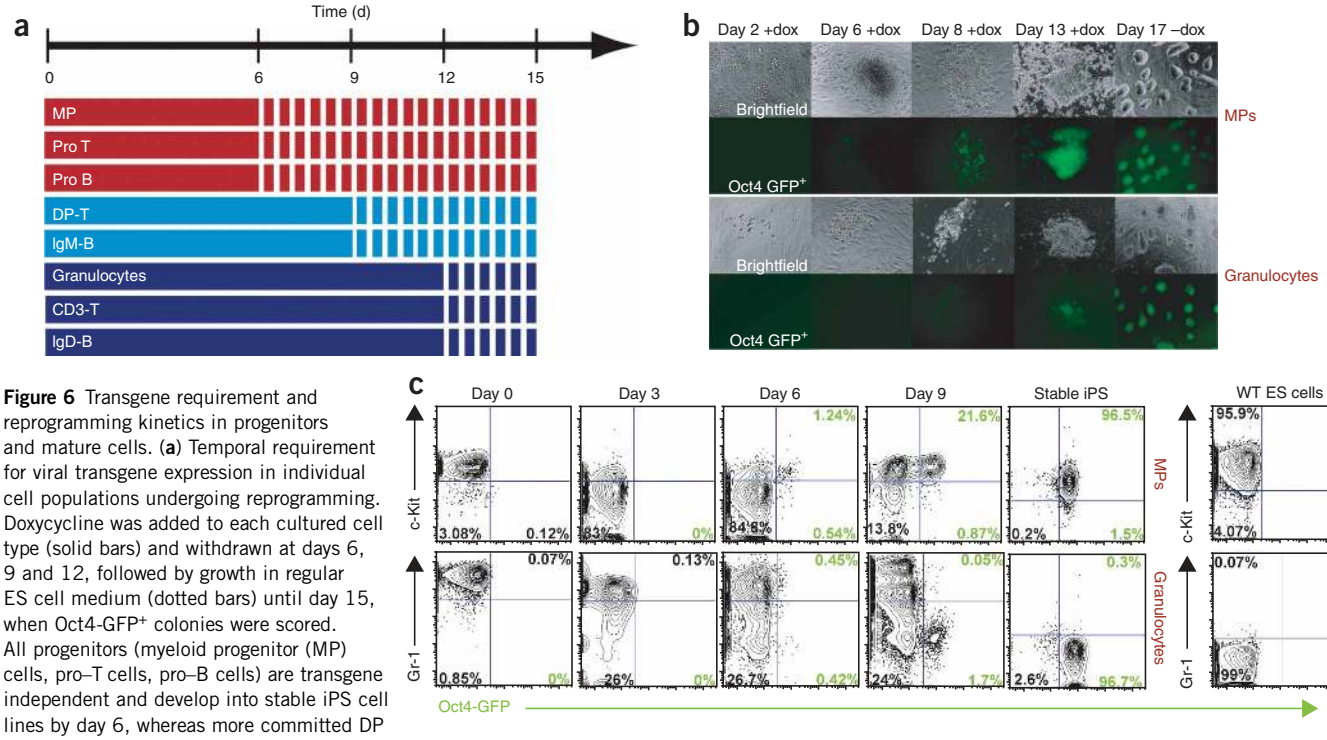

Figure 6 Transgene requirement and reprogramming kinetics in progenitors and mature cells. (**a**) Temporal requirement for viral transgene expression in individual cell populations undergoing reprogramming. Doxycycline was added to each cultured cell type (solid bars) and withdrawn at days 6, 9 and 12, followed by growth in regular ES cell medium (dotted bars) until day 15, when Oct4-GFP$^+$ colonies were scored. All progenitors (myeloid progenitor (MP) cells, pro–T cells, pro–B cells) are transgene independent and develop into stable iPS cell lines by day 6, whereas more committed DP thymocytes and IgM$^+$ splenocytes become independent of transgene expression by day 9, and mature granulocytes, IgD$^+$-B cells and CD3$^+$-T cells by day 12. (**b**) Time course of morphological changes and Oct4-GFP expression in myeloid progenitors and granulocytes undergoing reprogramming in the presence (+) or absence (–) of doxycycline (dox). Note the appearance of weak Oct4-GFP$^+$ cells by day 6 in myeloid progenitor cultures compared with day 8 in granulocytes. (**c**) Time course of surface marker expression and Oct4-GFP activity in myeloid progenitors and granulocytes undergoing reprogramming by FACS analysis. Note that myeloid progenitors reactivate Oct4-GFP sooner (day 6) than granulocytes (day 9). Oct4-GFP$^+$ cells emerge from within the c-Kit$^+$ population. Granulocytes gradually downregulate Gr-1 before activating Oct4-GFP within the Gr-1$^-$ population. Representative graphs of three experiments are shown. Numbers shown in quadrants are percentages of gated populations.

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(**Fig. 5b**). We did not observe any differences in the potential to produce iPS cells under these two growth conditions, suggesting that the differentiation stage, rather than the actual proliferation rate, of HSCs influences reprogramming. Likewise, the reprogramming potentials of B and T cells cultured under conditions that promote proliferation (for B cells, CpG, IL-4, IL-6, IL-7, IL-15 and LPS, and for T cells, antibodies to CD3, antibodies to CD28, IL-15 and IL-2) or under conditions that slow proliferation (for B cells, LPS and IL-15, and for T cells, antibodies to CD3 and IL-15) were comparable. Together, these data indicate that differentiation stage, rather than proliferation rate, is a critical parameter influencing reprogramming potential of immature and mature hematopoietic cells.

Progenitors reprogram faster than differentiated cells

Next, we wanted to test whether differences in reprogramming efficiencies manifest as differences in reprogramming kinetics. First, we evaluated whether progenitor-derived iPS cells became independent of the viral transgenes sooner than differentiated cells. We withdrew doxycycline from the individual cultures at 6, 9 and 12 d after induction and scored for Oct4-GFP$^+$ colonies at day 15. Myeloid progenitors, pro–B cells and pro–T cells gave rise to stable iPS cell colonies after only 6 d of transgene expression, whereas mature granulocytes, B cells and T cells required 12 d to become stably reprogrammed into a pluripotent state. Immature B (IgM$^+$) and CD4$^+$CD8$^+$ DP T cells required 9 d of transgene expression (**Fig. 6a**), suggesting a direct link between differentiation stage and the length of transgene requirement.

Next, we followed the temporal changes in surface marker expression on myeloid progenitors and granulocytes undergoing reprogramming (**Fig. 6b,c**). Consistent with results from the requirements of transgene expression, explanted myeloid progenitors gave rise to ES cell–like colonies containing Oct4-GFP$^+$ cells as early as 6 d after transgene induction, whereas granulocytes required at least 9 d to assume ES cell–like morphology and Oct4-GFP expression (**Fig. 6b,c**). FACS analysis for the myeloid progenitor marker c-Kit, which is also expressed on ES cells, showed that a subpopulation of c-Kit$^+$ cells reactivated Oct4-GFP as early as 6 d after transgene activation and rapidly expanded over the next few days (**Fig. 6c**). In contrast, granulocytes gradually downregulated the granulocyte marker Gr-1 and then reactivated Oct4-GFP beginning at day 9, indicating that reprogramming is delayed in granulocytes compared with myeloid progenitors (**Fig. 6c**). Consistent with this, the number of Oct4-GFP$^+$ cells was tenfold higher in myeloid progenitor–derived cultures than in granulocyte-derived cultures at day 9 (**Fig. 6c**). Tail-tip fibroblasts obtained from chimeric animals showed similar Oct4-GFP reactivation kinetics as differentiated hematopoietic cells (**Supplementary Fig. 8**).

To test whether differences in the reprogramming potentials of progenitors and mature cell populations persisted at later stages of reprogramming, we sorted individual Oct4-GFP$^+$ cells derived from either myeloid progenitors or granulocytes at day 9 into 96-well plates in the continuous presence of doxycycline and counted the number of wells with iPS cell colonies at day 20. We did not detect any significant differences in the potentials of Oct4-GFP$^+$ myeloid progenitor–derived and granulocyte-derived cells to produce iPS cell colonies (12% versus 9%), suggesting that upon reactivation of the endogenous Oct4 locus, both progenitors and mature cells have acquired the same abilities to become stable iPS cells (data not shown).

DISCUSSION

Using nine different primitive hematopoietic cell populations (long-term HSCs, HSC/Ps, myeloid progenitors, GMPs, MEPs, CMPs, CLPs, pro–B cells and pro–T cells) and their differentiated progeny (B cells, T cells, macrophages, granulocytes), we have shown that immature cells of the hematopoietic lineage are more amenable to reprogramming than differentiated cell types. This provides, to our knowledge, the first direct link between differentiation stage and reprogramming efficiency into iPS cells. The efficiencies at which progenitors converted into iPS cells were up to two orders of magnitude higher (7%–28%) than those of differentiated blood cell types (0.02%–0.6%) or fibroblasts (0.74%) and, to our knowledge, constitute the highest reprogramming efficiencies reported so far.

Our data are consistent with the notion that HSCs and hematopoietic progenitors are more efficiently reprogrammed because their epigenetic state is more amenable to transcription factor–induced remodeling. Accordingly, HSC/Ps, myeloid progenitors, pro–B cells and pro–T cells become independent of transgene expression sooner than any differentiated cell types tested. Moreover, stem and progenitor cells lack expression of lineage-specific genes, which can be inhibitory for reprogramming[22], and share expression of at least one marker, c-Kit, with ES cells, which may contribute to the enhanced reprogramming of immature blood cells.

Notably, we have found that reprogramming into iPS cells is independent of the proliferation rate of cells; rather, it correlates with their differentiation stage. That is, quiescent and cytokine-activated HSCs and proliferative progenitors reprogrammed most efficiently, whereas differentiated B and T lymphocytes reprogrammed least efficiently, regardless of their proliferation rate (**Fig. 1b**). We did not detect an increased reprogramming efficiency of HSCs compared with progenitor cells, suggesting that within immature cell populations, there may not be a strict correlation between differentiation stage and reprogramming efficiency. Alternatively, our failure to detect a higher reprogramming efficiency of stem cells over progenitor cells and of the more primitive CMPs over GMPs/MEPs may be due to technical issues such as sample size variations in transgene expression or plating efficiencies of individual cell populations.

The observation that HSCs and hematopoietic progenitors are more amenable to reprogramming into iPS cells than any tested differentiated cell type is consistent with nuclear transfer experiments of neural and keratinocyte stem cells[26,27]. However, our data are in contrast to results[29] that show that HSC and progenitor populations give rise to cloned blastocysts less efficiently than granulocytes. Differences in efficiency may be due to the different reprogramming methods used (nuclear transfer versus transcription factor–mediated reprogramming). Another reason may be differences in measuring reprogramming efficiency. In reference 29, the authors measured reprogramming efficiency by counting the number of cloned blastocysts derived from oocytes injected with individual nuclei, whereas we measured the number of transgene-independent, Oct4-GFP–expressing iPS cell colonies derived from a clonal population of viable cells.

Our results may provide an explanation for the previously reported failure to produce iPS cells from mature B cells by the expression of Oct4, Sox2, Klf4 and cMyc alone[34]. In that study, the myeloid transcription factor C/EBPα and the four reprogramming factors were ectopically expressed to generate B cell–derived iPS cells. As discussed by the authors of that study, their failure to generate iPS cells might have been due to the low overall efficiency of reprogramming B cells, which is overcome by C/EBPα expression. Our observation that C/EBPα expression further enhances the reprogramming efficiency of B cells is consistent with this idea.

These data also corroborate the previous observations that genetically defined, terminally differentiated cell populations remain amenable to reprogramming into iPS cells[34,35,47]. Because mature cell types appear slightly less efficient than heterogeneous cell populations such as fibroblasts and drastically less efficient than isolated progenitors, as shown here, our findings raise the possibility that some iPS cells produced from explanted tissues may in fact be derived from resident stem or progenitor cells rather than from differentiated cells, as assumed. It will be interesting to test whether somatic stem and progenitor cells from other tissues are more amenable to reprogramming than their mature progeny.

Our findings that adult progenitor cells are converted efficiently into iPS cells without additional chemical treatments or genetic manipulation have implications for research and medicine. The use of progenitor cells will make it feasible to perform biochemical and genetic analyses on cells undergoing reprogramming. Moreover, understanding the molecular differences between progenitors and differentiated cells can further teach us about the epigenetic and transcriptional barriers that seem inherent to nuclear reprogramming. On the clinical side, the use of somatic progenitors from adult tissues should make the derivation of patient-specific iPS cell lines more efficient and thus affordable. In addition, progenitor cells such as cord blood cells are likely to have accrued few, if any, genetic aberrations compared with differentiated cell types and may thus be a safer source for iPS cells.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. Mouse TCRβ sequences: GenBank AE000663, AE000664 and AE000665.

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

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AUTHOR CONTRIBUTIONS

S.E., A.F., H.H. and K.H. conceived and designed the study. S.E., M.S., N.M. and G.M. provided study materials. S.E., A.F., M.S. and T.A. collected and assembled the data. H.H. and K.H. provided financial support. K.H. provided administrative support. S.E., A.F., H.H. and K.H. analyzed and interpreted the data and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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ONLINE METHODS

Viral production and infection. Viral infections were performed with replication-defective doxycycline-inducible lentiviral vectors and a lentiviral vector constitutively expressing the rtTA, as described previously[38]. To produce infectious viral particles, 293T cells cultured on 10-cm dishes were transfected with the LV-tetO vectors (11 μg) together with the packaging plasmids VSV-G (5.5 μg) and Δ8.9 (8.25 μg) using FuGENE (Roche). Viral supernatants were harvested on three consecutive days starting 24 h after transfection, yielding a total of ~30 ml of supernatant per viral vector. Viral supernatant was concentrated approximately 100-fold by ultracentrifugation at 50,000*g* for 1.5 h at 4 °C and resuspension in 300 μl PBS. Infections were carried out in 1 ml ES cell medium (15% FBS, 1,000 U/ml LIF) using 5 μl of each viral concentrate per 35-mm plate.

To assess reprogramming efficiencies of myeloid progenitor cells and granulocytes after direct viral infection, 5×10^4 myeloid progenitors or granulocytes from a ROSA26-rtTA Oct4-GFP double heterozygous mouse were infected for 24 h in a round-bottomed 96-well plate with a concentrated polycistronic lentivirus expressing Oct4, Sox2, Klf4 and cMyc.

Differentiation of fetal liver cells into T cells. Mouse tail tip fibroblast–derived Oct4-GFP iPS clone no. 5 was injected into BDF1 blastocysts. Fetal liver was isolated from an E14.5 embryo, minced and co-cultured on OP9-DL1 (delta-like1) fibroblasts in αMEM medium (Invitrogen) containing 20% FBS (selected FBS for OP-9 cells) supplemented with penicillin and streptomycin, 10 ng/ml mouse Flt3 ligand and 10 ng/ml mouse IL-7 (R&D Systems) to induce differentiation into mature T cells, as described elsewhere[39]. After 13 d, cells were sorted based on CD8 and CD4 expression. Sorted cells were optionally superinfected with all four doxycycline-inducible lentiviral vectors in 96-well format overnight and then plated on irradiated MEFs in cytokine-conditioned medium (described below) in the presence of 2 μg/ml puromycin and 1 μg/ml doxycycline. After 15 d on doxycycline, emerging ES-like colonies were picked and cultured on MEFs in regular ES medium without doxycycline. iPS cell clone CD8 was expanded and used for blast injection to generate CD8-iPS chimeras.

Hematopoietic cell culture and induction of iPS cell formation. All cell types were cultured in 5% CO_2 at 37 °C in ES medium. Long-term (LT)-HSCs (lin$^-$ c-Kit$^+$ Sca1$^+$ CD34$^-$ CD48$^-$ CD150$^+$), HSC/Ps (lin$^-$ c-Kit$^+$ Sca1$^+$) and myeloid progenitors (lin$^-$ c-Kit$^+$ Sca1$^-$) were cultured in the presence of 50 ng/ml mouse Kit ligand, 20 ng/ml human TPO, 10 ng/ml mouse IL-3, 10 ng/ml mouse IL-6, and 10 ng/ml mouse Flt3 ligand (R&D Systems). To sustain LT-HSCs in a low-proliferation state, we cultured them in low TPO (0.5 ng/ml) to promote survival[46]. Granulocytes were cultured in the presence of 10 ng/ml granulocyte colony-stimulating factor (G-CSF) and 5 ng/ml granulocyte-macrophage CSF (GM-CSF). Primary and *in vitro*–differentiated macrophages were cultured in the presence of 5 ng/ml M-CSF. CMPs, MEPs and GMPs were cultured in the presence of 25 ng/ml mouse Kit ligand, 25 ng/ml human TPO, 10 ng/ml mouse IL-3, 25 ng/ml mouse IL-11, 25 ng/ml mouse Flt3 ligand, 2.5 U/ml EPO and 10 ng/ml GM-CSF (R&D Systems). CLPs, pro–B cells and DN-2 pro–T cells were cultured in the presence of 50 ng/ml mouse Kit ligand, 10 ng/ml mouse Flt3 ligand and 10 ng/ml mouse IL-7. Mature CD3$^+$ T cells and CD4$^+$CD8$^+$ DP T cell precursors were cultured in the presence of 10 ng/ml mouse IL-2 (R&D Systems) and 2.5 μg/ml concanavalin A (Sigma). IgM$^+$ and IgM$^+$IgD$^+$ mature B cells were cultured in the presence of 2 μM CpG (Invivogen), 10 ng/ml LPS (Sigma Aldrich) and 10 ng/ml mouse IL-7 (R&D Systems). Immediately after FACS, all cell types were counted and dead cells excluded by trypan blue staining. Next, cells were plated on irradiated MEFs and cultured in ES medium supplemented with the individual cytokines in the presence or absence of doxycycline (1 μg/ml). For the high- and low-proliferation T cell conditions, the Dynabeads CD3/CD28 T Cell Expander (Invitrogen) was used with a cell-to-bead ratio of 1:1.

***In vitro* differentiation of bone marrow cells into macrophages.** Total bone marrow cells were cultured for 6 d in the presence of 5 ng/ml M-CSF in ES medium and then sorted as single cells into the same medium with doxycycline into 96 wells. These cells correspond to the 'macrophage II' cells described in **Figure 4b**.

Calculation of iPS cell formation efficiency. Efficiencies were determined by dividing the number of Oct4-GFP$^+$ colonies after doxycycline withdrawal by the initial number of plated cells. HSC/Ps, myeloid progenitor cells, pro–B cells, pro–T cells, DP T cells and granulocytes were plated at a densities between 1×10^3 and 2.5×10^3 cells per 10 cm dish. IgM, IgD and CD3$^+$ cells were seeded at densities of 1×10^5 to 4×10^5 cells per six-well dish. Each cell type was plated in duplicate; the total number of experiments for each cell type is indicated in **Figure 4a**. To exclude the scoring of satellite colonies or non-iPS colonies, early ES cell–like colonies were marked on the dish and counted as an iPS colony only when positive for Oct4-GFP after doxycycline withdrawal. Efficiencies using the 96-well approach were determined by counting wells with iPS colonies on day 18, 3 d after doxycycline withdrawal. Efficiencies of iPS formation after direct infection were determined as described previously[32].

Transgene requirement assay. Each sorted hematopoietic cell type was plated immediately after sorting (in triplicate) into individual wells of 24-well plates on MEFs in ES cell medium supplemented with cell-specific cytokines and doxycycline (as described above). In detail, 1×10^3 myeloid progenitor cells, 1×10^3 pro–B cells, 1×10^3 pro–T cells, 2×10^4 CD4$^+$CD8$^+$ T cells, 4×10^4 CD3$^+$ T cells, 2×10^5 IgM$^+$ B cells, 4×10^5 IgD$^+$ B cells and 1×10^5 granulocytes were plated in triplicate on MEFs. To determine the length of transgene requirement for each cell type, doxycycline was withdrawn on days 6, 9 and 12, and medium was changed to normal ES medium without cytokines and doxycycline. Each well was scored for the presence of doxycycline-independent iPS cell colonies on day 15.

Flow cytometric analysis and cell sorting. Immunostaining was performed as described previously[48]. The following antibody conjugates and matched isotype control antibodies were used (obtained from BD Biosciences or eBioscience, unless otherwise indicated): antibodies to human CD4 (OKT4, allophycocyanin (APC), biotin), CD4 (GK1.5, biotin), CD8a (53-6, APC–Alexa Fluor 750), CD3ε (145-2C11, biotin, APC), TCRβ (H54-597, APC), TCRγδ (eBioGL3, biotin), mouse Vβ TCR screening panel (fluorescein isothiocyanate (FITC)), CD44 (IM7, FITC), CD25 (PC61, APC), B220 (RA3-6B2, APC), IgM (II/41, biotin), IgD (11-26, Alexa Fluor 647), Igκ light chain (187.1, FITC; BD Pharmingen), Igλ light chain (RML-42, APC; BioLegend), CD43 (S7, Alexa Fluor 488), Gr-1 (RB6-8C5, APC), Mac-1 (M1/70, FITC), F4/80 (MCA497, FITC; AbD Serotec), neutrophils 7/4 (MCAA771, Alexa Fluor 647; AbD Serotec), SSEA-1 (eBioMC-480), CD48 (HM48.1, APC), CD150 (TC15-12F12.2, PE, Alexa Fluor 488; BioLegend), CD34 (RAM34, FITC, Pacific Blue), CD117 (c-Kit, 2B8, APC, APC–Alexa Fluor 750), Sca-1 (D7, APC, Alexa Fluor 488; BioLegend). Biotinylated antibodies were further subjected to either streptavidin-APC or streptavidin–Pacific Blue (Molecular Probes). For cell sorting experiments of LT-HSCs HSC/Ps and myeloid progenitors, lineage-positive cells were depleted before flow cytometry using streptavidin-coupled Dynabeads (M-280; Invitrogen) after labeling with a cocktail consisting of biotinylated monoclonal antibodies to Gr-1, Mac-1, B220, CD4, CD8 and Ter-119. Subsequently, cells were stained with appropriate fluorochrome-conjugated lin$^-$ c-Kit$^+$ Sca1$^+$ markers as well as streptavidin-PE-Cy5 to detect residual biotinylated primary antibodies. Propidium iodide (1 μg/ml; Molecular Probes) was used to exclude dead cells at all times. A modified FACSAria flow cytometer (BD) with five lasers (UV 300 nm, violet 405 nm, blue 488 nm, green 532 nm, red 633 nm) running FACSDiVa software was used for sorting. Analyses were performed using FACSDiVa as well as FlowJo (Tree Star) software.

For single-cell sorting experiments into 96-well plates, MEFs were seeded into the inner 60 wells of a 96-well plate the day before FACS. The medium of the wells was exchanged to ES cell medium supplemented with cell-specific cytokines and doxycycline before FACS.

Variable-diversity-joining (VDJ) rearrangement analyses. VDJ rearrangements at the TCRα locus and rearrangements at the IgH locus were determined by DNA blot analysis as described previously[41]. DJ and VDJ rearrangements at the TCRβ locus of the CD8-iPS clone were amplified by PCR analysis[49]. PCR products were then cloned into the TOPO vector system (Invitrogen), and at least ten bacterial clones, corresponding to DJ and VDJ rearrangements, were sequenced. Sequences were analyzed using the NCBI BLAST/BLASTN suite

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and aligned to sequences of the mouse TCRβ locus obtained from GenBank accessions AE000663, AE000664 and AE000665.

Alkaline phosphatase staining. Alkaline phosphatase staining was performed with the Vector Red substrate kit (Vector Labs) according to manufacturer's instructions.

Generation of teratomas and chimeras. For teratoma induction, 2×10^6 cells of each iPS cell line were injected subcutaneously into the dorsal flank of isoflurane-anesthetized SCID mice. Teratomas were recovered 3–4 weeks post-injection, fixed overnight in 10% formalin, paraffin embedded and sectioned. Sections were stained with hematoxylin and eosin and imaged using a Leica DMI4000B camera. For chimera production, female BDF1 or *Rag2*$^{-/-}$ mice were superovulated with pregnant mare serum and human chorionic gonadotropin and mated to BDF1 stud males or *Rag2*$^{-/-}$ stud males, respectively. Zygotes were isolated from plugged females 24 h after human chorionic gonadotropin injection. After 3 d of *in vitro* culture in KSOM medium, blastocysts were injected with iPS cells and transferred into day 2.5 pseudo-pregnant recipient females. C-sections were performed 17 d later, and pups were fostered with lactating Swiss females.

C/EBPα retroviral infection. The mouse C/EBPα retrovirus has been described previously[50]. Retroviral stocks were prepared by transient transfection of PlatE-Eco cells using FuGENE (Roche), and supernatants were harvested 48 h later and concentrated by Centricon spin columns (Millipore). For infections, purified B cell subsets were resuspended in ES medium supplemented with CpG (2 μM, Invivogen), lipopolysaccharide (LPS) (10 ng/ml, Sigma Aldrich) and polybrene (8 μg/ml, Sigma). Next, 1-ml aliquots were plated on 24-well plates precoated with retronectin (Takara), and 50 μl of concentrated retrovirus was added to each well. The plates were incubated at 37 °C, and 24 h later the medium was replaced by supplemented B cell ES medium (described above). 48 to 72 h later, cells were sorted by FACS into those expressing C/EBPα and those not expressing C/EBPα. Both populations of cells were plated on MEFs in ES medium supplemented with cytokines and doxycycline (1 μg/ml).

Proliferation and apoptosis assays. All cell types were cultured in 5% CO_2 at 37 °C in ES medium supplemented with specific growth factors, as described above. After 5 d, aliquots of each sample were diluted with trypan blue, and viable cells were counted using a hemocytometer. To determine the rate of apoptosis, aliquots were stained with annexin V–FITC and 7-aminoactinomycin D according to the manufacturer's recommendations (BD Biosciences) and analyzed on a BD LSRII flow cytometer as described above. The proliferation rate of individual cell populations was measured either by Hoechst 33342 staining as described previously[44] or by propidium iodide–based cell cycle analysis. For propidium iodide–based analyses, cells were first permeabilized by fixation in 70% ice-cold ethanol for 30 min at 4 °C. After two washes in wash buffer (PBS supplemented with 0.2% BSA), pellets were resuspended in cell cycle buffer (3.8 mM sodium citrate, 40 μg/ml propidium iodide, 1 μg/ml RNase A) and stained for 30 min at 25 °C. Samples were run on a FACScan and analyzed with FlowJo.

Quantitative PCR. After cell sorting by flow cytometry, all cell types were cultured in the absence and presence of doxycycline in cell-specific medium as described above. After 3 d, cells were harvested for mRNA preparation. Real-time quantitative PCR reactions were set up in triplicate with the Brilliant II SYBR Green QPCR Master Mix (Stratagene) and run on a Mx3000P QPCR System (Stratagene) (**Supplementary Table 1**).

Derivation of human iPS cells from CD34$^+$ cord blood progenitors. Human umbilical CD34$^+$ cord blood cells were purchased from STEMCELL Technologies and cultured (after thawing) for 24 h in StemSpan serum-free expansion medium (SFEM) supplemented with StemSpan CC100 cytokine cocktail (STEMCELL Technologies). We infected 1×10^5 cells for 24 h in a round-bottomed 96-well plate with a concentrated polycistronic lentivirus expressing human cDNAs for Oct4, Sox2, Klf4 and cMyc, similar to the mouse polycistronic lentivirus vector described recently[40]. We then plated the cells on MEFs in StemSpan SFEM and StemSpan CC100 cytokine cocktail supplemented with human bFGF (10 ng/ml) and doxycycline. Human bFGF and doxycycline were added to the cultures daily. After doxycycline withdrawal on day 12, the medium was switched to human ES medium (see below).

Cell culture of human iPS cells on feeder cells and on Matrigel and chemical-defined medium mTESR. Irradiated fibroblasts were grown in DMEM with 10% FBS; human iPS cells were grown on feeder cells in knockout DMEM (KO-DMEM), knockout serum replacer (KOSR), nonessential amino acids, glutamine, β-mercaptoethanol and 10 ng/ml human bFGF, as described previously[51]. Human iPS cells were also grown in feeder-free conditions on culture dishes treated with Matrigel, in mTeSR medium.

***In vitro* differentiation of human iPS cells derived from human CD34$^+$ cord blood cells.** For *in vitro* differentiation, human iPS colonies were dissociated using dispase and placed in suspension culture with either DMEM supplemented with 10% FBS or with neuronal induction medium DMEM/F12 supplemented with GlutaMax, N2 and B27 to initiate embryoid body formation. After 1 week, embryoid bodies were plated to adherent conditions with gelatin and treated with either the supplemented DMEM or the neuronal induction medium. For teratoma formation, 1×10^7 human iPS cells were pelleted and injected into SCID mice, either subcutaneously or underneath the kidney capsule. Tumors were harvested after 7–13 weeks and processed for histological analysis.

Immunostaining. Immunostaining was performed using antibodies to Sox2 (Abcam ab15830), Tra-1-81 (MAB4381, Millipore), β-tubulin III (T2200, Sigma), and alpha-fetoprotein (sc-15375, Santa Cruz Biotech).

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