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A reprogrammable mouse strain from gene targeted embryonic stem cells

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Abstract

The derivation of induced pluripotent stem cells (iPSCs) usually involves the viral introduction of reprogramming factors into somatic cells. Here, we used gene targeting to generate a mouse strain with a single copy of an inducible, polycistronic reprogramming cassette, allowing for the induction of pluripotency in various somatic cell types. Since these “reprogrammable mice” can be easily bred, they provide a useful tool to study the mechanisms underlying cellular reprogramming.

Somatic cells can be reprogrammed into iPSCs by the transcription factors c-Myc, Klf4, Oct4 and Sox2[1]. The study of the molecular mechanisms underlying this conversion is hampered by the low efficiency of reprogramming and by the requirement to introduce the reprogramming factors into target cells. To circumvent these constraints, so-called “secondary systems” have been developed, which are based on iPSCs made with doxycycline-inducible lentiviruses[2,3]. Somatic cells derived from such primary iPSCs can be converted into secondary iPSCs by culture in doxycycline-containing media[4,5,6].

Secondary systems based on viral vectors have several limitations. For example, many iPSC lines show viral transgene silencing, leading to heterogeneous transgene expression and thus variable results. In addition, the presence of viral integrations at random genomic locations can alter endogenous gene expression. Further, the breeding of mice derived from such iPSCs is complicated by the need to screen their offspring for multiple viral transgenes. To develop an improved reprogramming system, we placed a doxycycline-inducible polycistronic cDNA encoding the four reprogramming factors Oct4, Klf4, Sox2 and c-Myc (OKSM)[7] in the 3′ untranslated region of the collagen type I, alpha 1 gene (Col1a1, herein referred to as Collagen)[8] (see Figure 1a). This gene targeting is mediated by Flp recombinase and was performed in embryonic stem cells (ESCs) that express the reverse

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Author contributions

M.S. and K.H. conceived the study, N.M. performed blastocyst injections, M.S. performed all other experiments with help from M.B., M.S. and K.H. analyzed the data and wrote the manuscript.

tetracycline-dependent transactivator (rtTA) from the ubiquitously expressed ROSA26 locus[8]. This system has previously been shown to achieve high-level and homogeneous expression of different transgenes in cultured cells as well as *in vivo*[8].

Integration of the OKSM cassette into the Collagen locus was successful in 12 out of 12 picked ESC clones (Supplementary Figure 1). To test the functionality of this transgenic system in comparison to a viral secondary system, we isolated mouse embryonic fibroblasts (MEFs) after blastocyst injections with either Collagen-OKSM ESCs (called Collagen-MEFs) or iPSCs derived from ROSA26-rtTA MEFs carrying two copies of a doxycycline-inducible lentiviral vector expressing the same polycistronic cassette (Lenti-MEFs)[7] (Figure 1b). Upon culture in the presence of doxycycline, both Collagen-MEFs and Lenti-MEFs formed colonies with ESC-like morphology that stained positive for pluripotency-associated markers such as alkaline phosphatase, Nanog and endogenous Oct4 (Figure 1c and Supplementary Figure 2). Such colonies could be expanded in the absence of exogenous factor expression and will be referred to as iPSCs. Collagen-MEFs produced iPSC colonies approximately 10-fold more efficiently than Lenti-MEFs (Figure 1d,e), consistent with the observation that Collagen-MEFs homogenously expressed exogenously Oct4 two days after induction while only patchy reactivation was seen in Lenti-MEFs (Figure 1f). Collagen-MEF iPSCs formed highly differentiated teratomas in SCID mice (Figure 1g) and contributed to adult coat color chimeras (Figure 1h). Pluripotency of iPSCs was further demonstrated by their ability to support the development of “all-iPSC” midgestation fetuses after injection into tetraploid blastocysts (Figure 1i).

To enable the tracking of cells undergoing reprogramming, we labeled Collagen-OKSM ESCs with a constitutively active lentiviral vector expressing the reporter gene tdTomato. MEF cultures were established from chimeric embryos and brightly red-fluorescent cells plated at single cell density onto irradiated feeder cells. Fluorescence microscopy showed that MEFs expressing the four reprogramming factors adopted a smaller cell size and proliferated faster within the first 48h of doxycycline administration compared to uninduced cells. This resulted in clusters of coalescent cells between days 4 6, from which iPSC colonies emerged between days 8 and 14 of culture (Supplementary Figure 3). Withdrawal of doxycycline at different time points showed that the first stably reprogrammed cells appeared by day 7, consistent with previous reports using lentiviral vectors[2,3] (Supplementary Figure 4a). When doxycycline was withdrawn before day 7, MEFs that had undergone morphological changes uniformly reverted back to a fibroblast-like shape and ceased proliferation (Supplementary Figure 4a). This suggests that the acquisition of cellular immortality, a crucial and rate-limiting step during iPSC formation[9], occurs late during the reprogramming process and coincides with the acquisition of pluripotency. This observation also indicates that previously reported stable intermediates or “partially reprogrammed cells”[10,11], are no common phenotype during factor-mediated reprogramming but rather the consequence of incomplete transgene silencing when using retroviral vectors to derive iPSCs. Interestingly, the re-exposure of cells to doxycycline after one week of withdrawal did not yield stable iPSC colonies from more than 100 regressed colonies assayed, suggesting that these cells had become refractory to reprogramming, possibly due to the exhaustion of their proliferative capacity[9].

We next attempted to establish a mouse strain carrying the inducible reprogramming cassette in all tissues. To this end, we bred adult Collagen-OKSM chimeras (Figure 2a) with mice that carried the gene for enhanced green-fluorescent protein (*EGFP*) in the Oct4 locus, which serves as a faithful indicator for the acquisition of pluripotency[12]. The resulting animals will be referred to as "reprogrammable mice" (Figure 2B). Notably, some reprogrammable mice developed a single aggressively growing tumor that histologically presented as a largely undifferentiated teratoma (Supplementary Figure 5a,b). This phenotype is likely due to leaky transgene expression in an undefined cell type and raises the possibility that somatic cells can be reprogrammed into pluripotent cells not only in vitro but also in vivo.

To validate the Collagen-OKSM/Oct4-GFP reprogramming system, we cultured postnatal tail-tip fibroblasts (TTFs) in the presence of doxycycline, which resulted in the formation of GFP^+ ESC-like colonies at a frequency of approximately 0.1% (Figure 2c, d). Of note, the lower reprogramming efficiency of TTFs compared with MEFs (see Figure 1e) was not due to incomplete reactivation of the reprogramming cassette (data not shown), indicating that fetal fibroblasts are more amenable to reprogramming than postnatal cells, as has been recently seen[13]. The efficiency of TTF reprogramming was increased approximately 2-fold when culturing the cells in media containing serum replacement (SR) (Figure 2d). These iPSCs adopted an ESC-like morphology faster than cells derived in serum-containing media, consistent with previous observations[14]. In the absence of feeder layers, TTFs grown in serum-free media had to be seeded at a high density (~10,000 cells/cm^2) to reach optimal reprogramming efficiencies compared to cells grown in serum-containing media, which required a sparse starting culture (~1,000 cells/cm^2) (Figure 2e). Together, these results suggest that the reprogramming process is not entirely cell-autonomous but is influenced by both inhibitory and supportive signals from the culture environment.

Interestingly, Collagen-TTFs homozygous for the ROSA26-rtTA allele reached reprogramming efficiencies of 0.5–1% (Figure 2d) and reprogrammed faster than heterozygous cells, as demonstrated by the earlier appearance of doxycycline-independent GFP^+ colonies (Figure 2f). This shows that higher levels of transgene expression are favorable for both reprogramming kinetics and efficiency.

To demonstrate reprogramming of a defined somatic cell lineage, we prospectively isolated different hematopoietic cell types from adult reprogrammable mice (Supplementary Figure 6). We consistently failed to obtain iPSC colonies from mature T cells, B cells and granulocytes heterozygous for both ROSA26-rtTA and Collagen-OKSM, although we occasionally observed colonies from monocytes with this genotype (Figure 2g). In contrast, Collagen-OKSM heterozygous cells that were homozygous for ROSA26-rtTA, reproducibly gave rise to GFP^+ iPSCs from all tested cell types, at frequencies ranging from 0.01% (B cells) to 0.3% (monocytes) (Figure 2g and Supplementary Figure 7). This suggests that a critical level of factor expression is required for reprogramming of terminally differentiated blood cells, which is only achieved in cells expressing rtTA from both ROSA26 alleles. In contrast, hematopoietic stem cells (HSCs) and granulocyte-macrophage progenitors (GMPs) from ROSA26-rtTA heterozygous mice generated iPSCs at high (5–10%) efficiencies (Figure 2h). Remarkably, we derived iPSCs from HSCs homozygous for ROSA26-rtTA at

frequencies of up to 40% (Figure 2h), which is to our knowledge the highest reprogramming efficiency for an adult somatic cell type. The overall higher reprogramming efficiency of immature hematopoietic cells compared with mature cells is consistent with our previous finding that reprogramming potential inversely correlates with differentiation stage[15].

Of note, reprogramming of blood cells was equally successful in the presence or absence of cell type-specific cytokines supporting hematopoietic cell survival and proliferation, with two exceptions. First, granulocytes did not form iPSCs in the presence of the growth factor Granulocyte macrophage-colony stimulating factor (GM-CSF) and second, ROSA26-rtTA heterozygous HSCs and GMPs reprogrammed 3–4 times less efficiently without cytokines (Figures 2g,h). The observation that ROSA26 homozygous HSCs and GMPs did not show this effect (Figure 2h) suggests that higher expression levels of reprogramming factors can substitute for the survival and/or proliferation signals provided by cytokines.

The mouse strain described here overcomes several of the limitations associated with secondary reprogramming systems and should be a useful tool to study the mechanisms underlying the conversion of somatic cell types into iPSCs. It may be especially attractive for dissecting the biochemistry of induced pluripotency in the context of adult stem and progenitor cells that seem to reprogram at high efficiency. Another crucial question that can be addressed with this system is whether ESCs and iPSCs are molecularly and functionally equivalent. Specifically, the possibility to compare Collagen-OKSM ESCs to genetically matched derivative iPSCs from different tissues will allow resolving this question.

Experimental procedures

Mice

Derivation, handling and genotyping of Oct4-GFP [12] and ROSA26-rtTA[8] mice were described previously. Collagen-OKSM reprogrammable mice will be deposited with The Jackson Laboratories once the colony has been expanded.

Cell culture

ESCs and iPSCs were cultured in ES medium (DMEM with 15% FBS, L-Glutamine, penicillin-streptomycin, non-essential amino acids, β-mercaptoethanol and 1000 U/ml LIF) on irradiated feeder cells. ESC-derived MEFs were isolated by trypsin-digestion of midgestation (E14.5) chimeric embryos followed by culture in fibroblast medium (DMEM with 10% FBS, L-Glutamine, penicillin-streptomycin, non-essential amino acids and β-mercaptoethanol) containing puromycin. TTF cultures were established from tail-tip biopsies of newborn (3–8 days of age) mice and maintained in fibroblast medium. Hematopoietic cells were isolated from peripheral blood and bone marrow as previously described[15].

Gene targeting of ESCs

A polycistronic cassette encoding Oct4, Klf4, Sox2 and c-myc[7] was cloned into the shuttle plasmid pBS31 using NotI/ClaI digestion. The resulting plasmid were electroporated into KH2 ESCs together with a plasmid driving expression of Flp recombinase[8]. Correct targeted

clones were selected for by hygromycin selection and confirmed by Southern blotting as previously described[8].

Lentiviral vectors

To produce infectious viral particles, 293T cells cultured on T75 flasks were transfected with a lentiviral vector expressing tdTomato from the ubiquitin promoter (11 μg) together with the packaging plasmids VSV-G (5.5 μg) and Δ8.9 (8.25 μg) using Fugene (ROCHE) transfection reagent. Viral supernatants were harvested on 3 consecutive days starting 24 hours after transfection, yielding a total of ~50 ml of supernatant per virus. Viral supernatant was concentrated approximately 200-fold by ultracentrifugation at 20,000 rpm for 1.5 hours at 4°C and resuspension in 250 μl PBS. Viral concentrates were stored at −80°C. Transduction of iPSCs was carried out in 0.2 ml ES medium containing 5μg/ml polybrene using 20–40 μl of viral concentrate on a 48well plate. Regular ES medium was added 24 hours after infection and red-fluorescent iPSCs isolated by FACS.

Immunofluorescence

iPSCs were cultured on pretreated cover slips, fixed with 4% PFA and permeabilized with 0.5% Triton X-100. The cells were then stained with primary antibodies against mOct4 (Santa Cruz, sc-8628) and mNanog (Abcam, ab21603), followed by staining with the respective secondary antibodies conjugated to either Alexa Fluor 488 or 546 (Invitrogen). Nuclei were counterstained with DAPI (Invitrogen). Cells were imaged using a Leica DMI4000B inverted fluorescence microscope equipped with a Leica DFC350FX camera. Images were processed and analyzed using Adobe Photoshop software.

Teratoma formation

iPSCs were harvested by trypsinization and injected into the flanks of NOD/SCID mice, using ~5 million cells per injection. Mice were sacrificed 3 weeks later and teratomas isolated and processed for histological analysis.

Blastocyst injections

Female BDF1 mice were superovulated by intraperitoneal injection of PMS and hCG and mated to BDF1 stud males. Zygotes were isolated from females with a vaginal plug 24 hour after hCG injection. Zygotes for 2n injections were *in vitro* cultured for 3 days in vitro in KSOM media, blastocysts were identified, injected with ESCs or iPSCs and transferred into pseudopregnant recipient females. For 4n injections, zygotes were cultured overnight until they reached the 2-cell stage, at which point they were electrofused. One hour later, 1-cell embryos were carefully identified and separated from embryos that had failed to fuse, cultured in KSOM for another 2 days and then injected.

Reprogramming

MEFs and TTFs were counted and seeded in fibroblast media at the desired density on either gelatin-coated plates or plates that also contained a layer of irradiated feeder cells. The next day, ES medium containing 2 μg/ml doxycycline was added and replenished every 3 days. Upon doxycycline withdrawn, cultures were washed twice with PBS and then continued in

standard ES medium until colonies were either picked, scored by fluorescence microscopy or stained for Alkaline phosphatase activity (Vector Red substrate kit, Vector Labs). Freshly isolated hematopoietic cells were FACS sorted and immediately plated on top of irradiated feeder layers in ES media containing doxycycline on either 35mm or 100mm plates. Cell densities were 200 cells/well for HSCs and GMPs and 5000 to 100,000 cells/well for all other cell types. Doxycycline was withdrawn from HSCs and GMPs after two weeks and from the slower reprogramming peripheral blood cells after between four and six weeks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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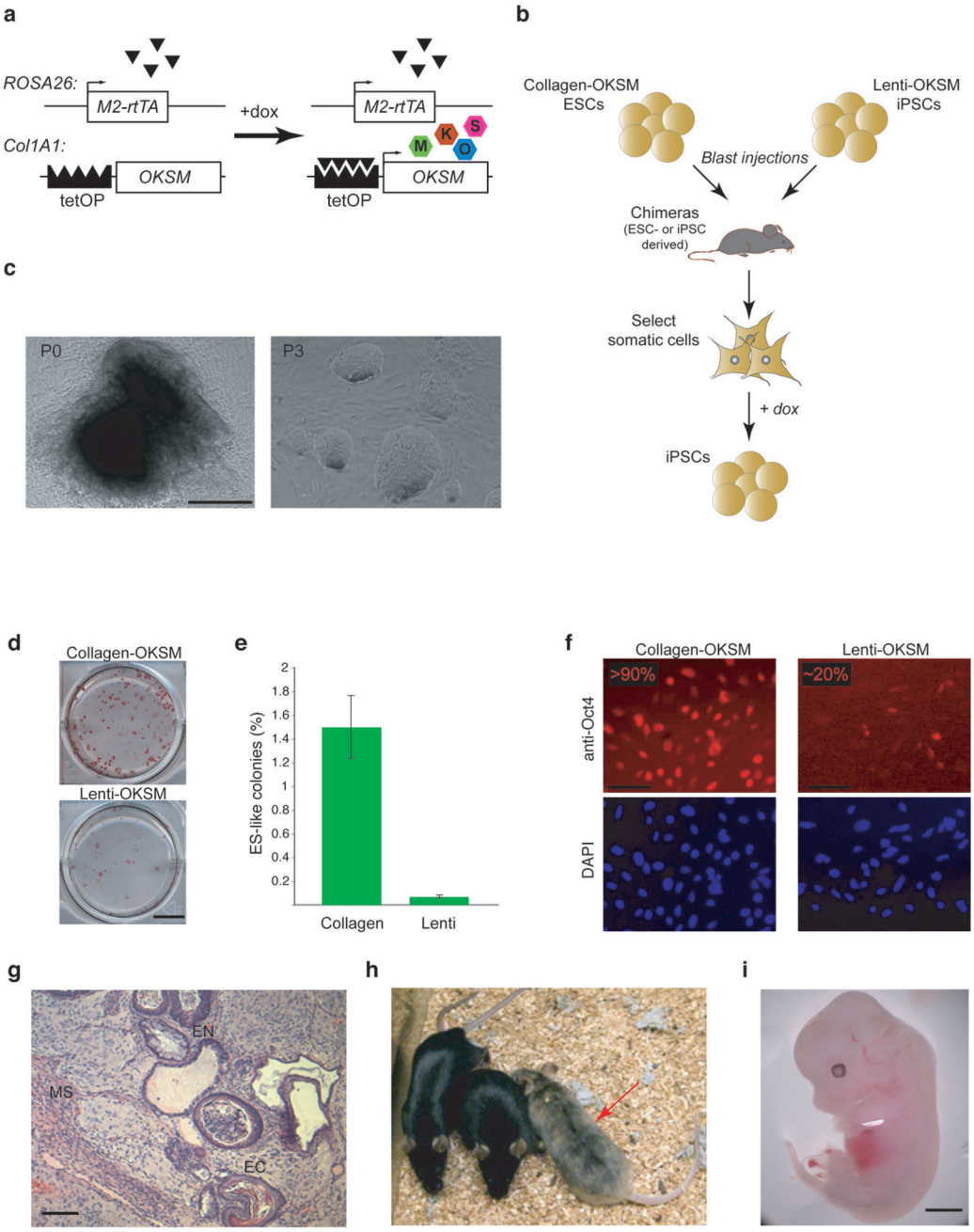

Figure 1. An ES-cell based reprogramming system

(**a**) Genomic configuration of Collagen-OKSM ESCs with the cDNA of the optimized reverse tetracycline-dependent transactivator (M2-rtTA, protein product represented by dark triangles) targeted to the constitutively active ROSA26 locus and a polycistronic cassette encoding Oct4 (O), Klf4 (K), Sox2 (S) and c-Myc (M) targeted to the Col1a1 locus under control of a tetracycline-dependent minimal promoter (tetOP). In the presence of doxycycline (dox) M2-rtTA binds to tetOP, thereby inducing expression of the four reprogramming factors. (**b**) Outline for a secondary reprogramming system based on Collagen-OKSM ESCs or Lenti-OKSM iPSCs. Chimeric animals are generated by

blastocyst injection and ESC-derived or iPSC-derived somatic cells are isolated and reprogrammed into iPSCs by culture in dox-containing media. (**c**) Brightfield images of a chimera-derived MEF-iPSC clone before picking (P0, left) and after passaging (P3, right). (**d**) Alkaline phosphatase staining of colonies derived from Collagen-OKSM MEFs or MEFs carrying two copies of a dox-inducible lentiviral vector encoding the same polycistronic cassette (Lenti-OKSM). (**e**) Graph showing the efficiency of iPSC formation from Collagen-OKSM MEFs (blue bar) and Lenti-OKSM MEFs (green bar), respectively. (**f**) Fluorescent images of Collagen-OKSM and Lenti-OKSM MEFs cultured for two days in the presence of doxycycline and stained with an antibody against Oct4 (red) and DAPI (blue). Numbers indicate the percentage of $Oct4^+$ cells. Scale bars = 100 μm. (**g**) Histological section through a teratoma derived from Collagen-OKSM MEF-iPSCs showing differentiation in ectodermal (EC, keratinized epithelium), endodermal (EN, glandular structures) and mesodermal (MS, muscle) derivatives. (**h**) Image of an adult coat color chimera (red arrow) derived from MEF-iPSC. Also shown are two non-chimeric BDF1 mice. (**i**) Image of an E13.5 embryo derived after injection of MEF-iPSCs into tetraploid blastocysts (“all iPSC” fetus). Scale bars, 500 μm (c), 10 mm (d), 100 μm (f), 50 μm (g), 2 mm (i).

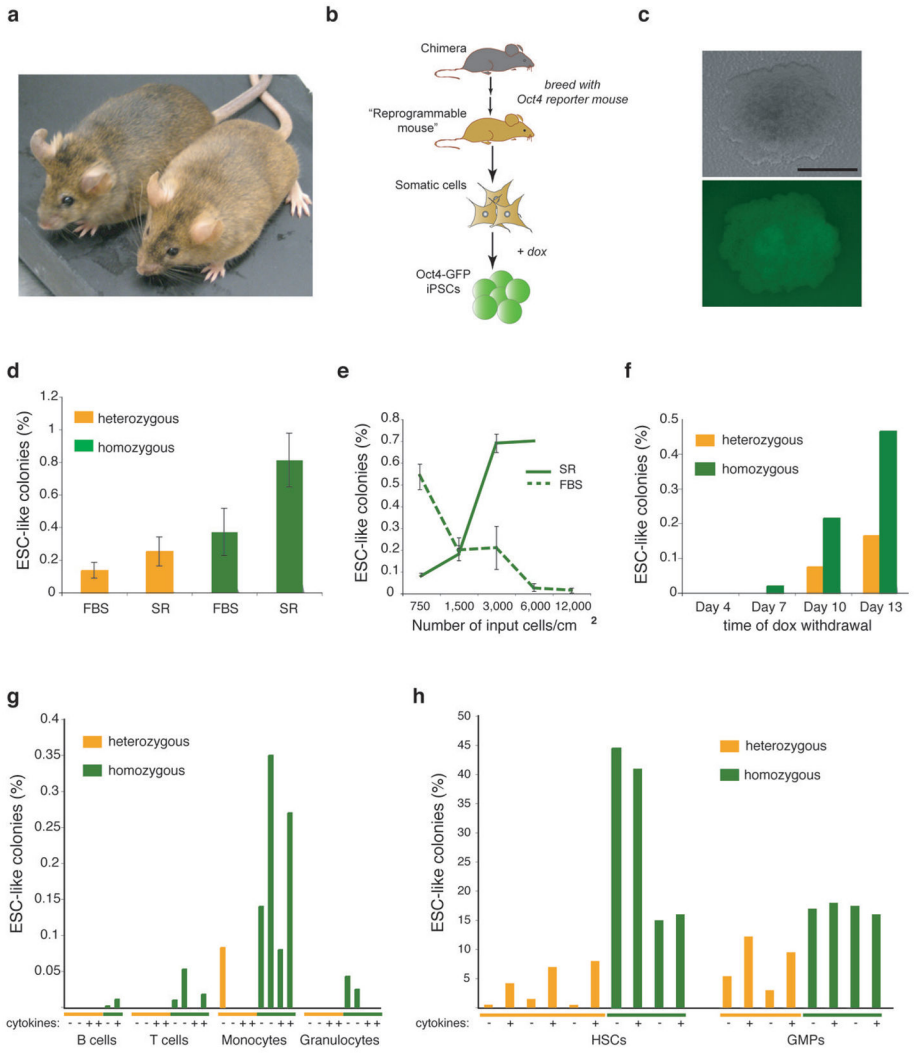

Figure 2. Derivation and characterization of reprogrammable mice

(**a**) Images of two representative adult chimeras derived from Collagen-OKSM ESCs. (**b**) Outline for the derivation of reprogrammable mice by crossing ESC chimeras with Oct4-GFP reporter mice. Acquisition of pluripotency in somatic cells isolated from these animals is revealed by GFP fluorescence. (**c**) Brightfield and GFP fluorescence image of an iPSC colony derived from postnatal tail-tip fibroblasts (TTFs) isolated from a reprogrammable mouse. (**d**) Effect of serum (FBS) and serum replacement (SR) on the efficiency of iPSC formation from ROSA26-rtTA heterozygous (het, yellow bars) or homozygous (homo, green bars) TTFs. GFP^+ transgene-independent colonies with ESC-like morphology were scored. Error bars indicate one standard deviation. (**e**) Effect on cell density on the reprogramming efficiency of homozygous TTFs cultured in either serum (FBS, dotted lines) or serum replacement (SR, solid lines). (**f**) Efficiencies of reprogramming after culture of ROSA26-rtTA heterozygous and homozygous TTFs in dox-containing medium for different lengths of time. Dox was withdrawn on the indicated days and colonies scored on day 20. (**g**) Reprogramming efficiencies of mature hematopoietic cell types isolated from the peripheral blood of reprogrammable mice heterozygous (yellow bars) or homozygous (green

bars) for ROSA26-M2rtTA. Cells were cultured either in the presence (+) or absence (−) of cell type-specific cytokines (see methods section). (**h**) Reprogramming efficiencies of hematopoietic stem cells (HSCs) and granulocyte/macrophage progenitor cells (GMPs) from bone marrow. Scale bar, 500 μm (c).