Supplementary Material For:

Transgenic Mice with Defined Combinations of Drug Inducible Reprogramming Factors

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Supplementary Methods

Mice, cell culture and viral infections

The derivation of iBiPS #9 cell line has been reported previously [1]. In short, the line was derived from reprogramming of sorted pro-B cells explanted from chimeras that were generated by injection of a MEF-derived iPS line that had in turn been infected with Dox-inducible lentiviruses. The genetic background of the iB-iPS #9 line was C57B6/129SvJae, ROSA26-M2rtTA+/– and Nanog-GFP+/–. To produce transgenic offspring, an iB-iPS#9 chimera that transmitted the transgenes through the germline in 100% of the offspring was crossed to wild-type females. In some cases iB-iPS9 chimera was mated with wild type C57B6 M2rtTA+/+ females (carrying no viral transgenes), however homozygote mice for the M2rtTA alleles were not included in our progeny analysis. Lentiviral preparation and infection with Dox-inducible lentiviruses encoding Oct4, Klf4, c-Myc and Sox2 cDNA driven by the TetO/CMV promoter has been described elsewhere [2]. Lentiviral stocks were prepared by transient transfection of 293T cells using Fugene (Roche), and supernatants were harvested 48 hr later. ESCs and established iPS lines were cultured on irradiated MEFs, in ESC medium containing LIF, 10% FBS, non-essential amino acids, L-glutamine, β-mercaptoethanol and penicillin/streptomycin. Isolation and reprogramming of intestinal epithelium, keratinocytes and liver cells were performed as previously described [2,3]. Primary colonies were then picked, dissociated and plated onto irradiated MEFs and further cultured in the absence of Dox until stable iPS lines were established. When Nanog-GFP knock-in allele was present or introduced into the somatic cells used in reprogramming experiments it was used as a marker for reprogramming.

Isolation and Culture of Whole Peripheral Blood

Whole, peripheral blood was isolated from 200µl of peripheral blood from 4-8 week old mice. Red blood cell (RBC) lysis was subsequently performed by mixing blood with 500 µl of HEPES Buffer and RBC lysing buffer Hybri-Max (Sigma-Aldrich). Lysis was allowed to take place for 5 min, at which point the mixture was centrifuged for 5 min at 3,000 rpm to remove RBCs. The cell pellet was resuspended in ESC medium supplemented with 2 µg/µl Dox, and hematopoietic cytokines IL-4, IL-7, Flt-3L, SCF, G-CSF, GM-CSF (10ng/ml each, Peprotech), anti-CD40 (0.1µg/ml, BD-Biosciences), LPS (10ng/ml, Sigma-Aldrich) and plated onto 6-well plates pre-coated with OP9 bone marrow stromal cells (ATCC). For separation of different blood cell types the MACS® separation columns (Miltenyi Biotech) were used together with MACS® magnetic beads conjugated to CD11b, CD11c or CD19 antibodies according to the manufacturer's

instruction. In Figure 1F, iB-iPS9 CD11b+ cells were isolated from chimera generated by injecting constitutive GFP labeled iB-iPS #9 line which allowed specific purification of transgenic CD11b+ cells from these chimeras.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 20 minutes at 25 °C, washed 3 times with PBS and blocked for 15 min with 5% FBS in PBS containing 0.1% Triton-X. After incubation with primary antibodies against Oct4 (Abcam), Nanog (Bethyl Laboratories) and SSEA1 (monoclonal mouse, Developmental Studies Hybridoma Bank) for 1 h in 1% FBS in PBS containing 0.1% Triton-X, cells were washed 3 times with PBS and incubated with fluorophore-labeled appropriate secondary antibodies. Specimens were analyzed on an Olympus Fluorescence microscope and images were acquired with a Zeiss Axiocam camera.

Analysis of viral integrations

Genomic DNA was digested with the indicated restriction enzymes for 6-10h. Electrophoresis and transfer was followed according to standard procedures. The blots were hybridized to radioactively labeled probes—Oct4: exon 1; c-Myc, Klf4 & Sox2: full-length cDNA. Endogenous band sizes: Oct4: 6.6 kb—XhoI, 7.3Kb—SphI/SpeI, 10.8Kb—Sph/MfeI; Sox2: 4.7 kb—EcoRV, 4.6 kb & 1.3 kb—PstI; Klf4: 5.3 kb—PstI, 7 kb—XbaI; c-Myc: 5.4 kb—PstI, 9.6 kb—XbaI. PCR primers used to detect lentiviral transgenes in genomic DNA samples were as follows: 5' TetO: AAAGTGAAAGTCGAGCTCGGTACC; 3' mOct4: CCTTCTCCAACTTCACGGCATT; 3' mSox2: GCCTCCGGGAAGCGTGTACTTA; 3' mc-Myc: ACTGAGGGGTCAATGCACTCGG: 3' mKlf4: CCTGGTGGGTTAGCGA GTTGGA. V6.5 ES cells (C57B6/129SvJae) or Balb/c ES cells were used as negative controls for determining background and endogenous bands.

Quantitative RT-PCR

By using previously described primers and protocol [2], 5 µg of total RNA extracted using Trizol reagent (Invitrogen) were treated with DNase I (Zymo Research). Subsequently, 1 µl of DNA-free RNA was reverse transcribed using First Strand Synthesis kit (Invitrogen) and quantitative PCR analysis was performed using ABI Prism 7000 (Applied Biosystems) with Platinum SYBR green Q-PCR SuperMix-UDG with ROX (Invitrogen). Equal loading was ensured by amplifying GAPDH mRNA and all reactions were performed in duplicates or triplicates.

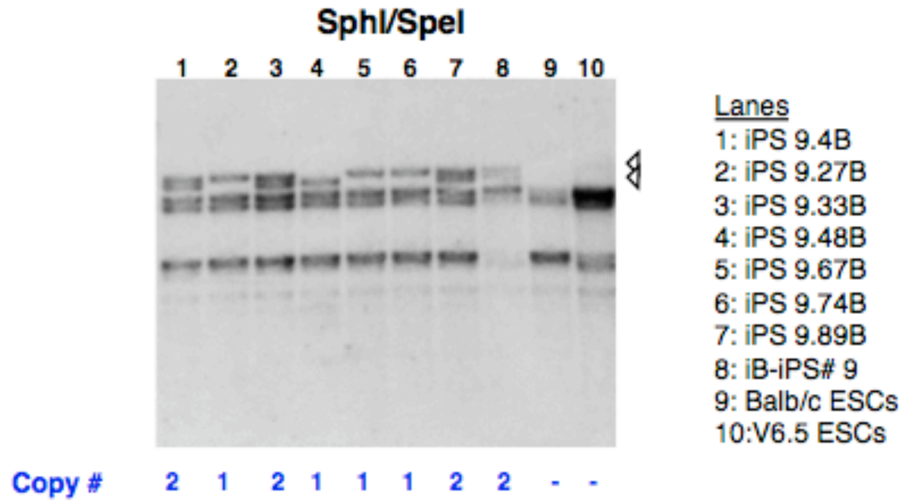
Chimera and Teratoma Formation

All animal studies and protocols were approved by an institutional review committee. Diploid blastocysts (94–98 h after hCG injection) were placed in a drop of Hepes-CZB medium under mineral oil. A flat tip microinjection pipette with an internal diameter of 16 μm was used for iPS cell injections. Each blastocyst received 8-10 iPS cells. After injection, blastocysts were cultured in potassium simplex optimization medium (KSOM) and placed at 37 °C until transferred to recipient females. About 10 injected blastocysts were transferred to each uterine horn of 2.5-day-postcoitum pseudo-pregnant B6D2F1 female. Pups were recovered at day 19.5 and fostered to lactating BALB/c mothers when necessary. Teratoma formation was performed by depositing 2x10^6 cells under the flanks of recipient SCID or Rag2-/- mice. Tumors were isolated 3-6 weeks later for histological analysis.

Methods references:

1) Hanna, J. et al. *Cell* **133**, 250-264 (2008)
2) Wernig, M. et al. *Nature biotechnology* **26**, 916-924 (2008)
3) Aoi, T. et al. *Science* **321**, 699-702 (2008)

Supplementary Figure 1: Southern Blot analysis to determine the Oct4 transgene copy number.
V6.5 (C57Bl/6x129) and Balb/c ESCs are used as controls. Open arrowheads indicate the proviral integrations. "B" indicates iPS line derived from peripheral blood.



Supplementary Figure 2: Transgene inheritance in adult offspring and reprogramming from peripheral blood cells of the founder male mouse.

A) PCR genotyping of all F1 (iB-iPS#9 chimera X WT F1) adult offspring.* One mouse carrying all four factors was not tested for blood reprogramming, as it died prior to completion of the screen. B) Experimental and predicted inheritance of at least one copy of each of viral transgenes in the F1 offspring. Predicted inheritance is the chance of inheriting at least one copy of a specific transgene, given that the iB-iPS9 cell line carries two copies of Oct4, two copies of Klf4, one copy of Sox2 and one copy of c-Myc. The single copy vectors c-Myc and Sox2 were transmitted to approximately 50% of the mice, whereas the two copy vectors Oct4 and Klf4 were found in approximately 75% of the offspring, which is consistent with the expected ratios from independent segregation of a single copy of c-Myc and Sox2 and of two copies of Oct4 and Klf4 proviruses, respectively. C) To establish a simple test that could be used to assay for reprogramming of somatic cells in a large number of animals, we bled the iB-iPS#9 chimera and cultured whole peripheral blood-derived cells on OP9 bone marrow stromal cells and Dox. Reprogramming of whole blood-derived cells from the iB-iPS#9 male chimera resulted in the formation of colonies with characteristic ES-like morphology that appeared following 8-14 days of Dox induction. When picked and expanded in ES media, the colonies gave rise to iPS cell lines that were maintained in the absence of Dox and stained positive for stem cell specific antigen 1 (SSEA-1), Oct-4 and reactivated endogenous Nanog-GFP allele (C). iPS colony formation (top three panels), endogenous Nanog-GFP expression (lower two panels) and staining for SSEA-1 and Oct4 pluripotency markers (middle four panels) are shown from reprogramming of iB-iPS9 peripheral blood. Arrows in top panels indicate typical colonies with ES-like morphology. The days depicted indicate the time from initiation of Dox treatment. D) Different peripheral blood cell fractions (based on indicated surface markers) were plated on OP9 bone marrow stroma with conditioned media and Dox. After 14 days plates were fixed and stained for alkaline phosphatase (AP) activity. Figure D shows that alkaline phosphatase (AP) positive cells were mostly derived from isolated CD11b+ myeloid subpopulations, but not from CD11c, or CD19 positive cells (D). This result suggests that CD11b+ cells are efficiently reprogrammed in our assay, but does not exclude the possibility that other cells types present in the peripheral blood might also be reprogrammed, though perhaps with lower efficiency and/or under different growth conditions and/or at different induction levels.

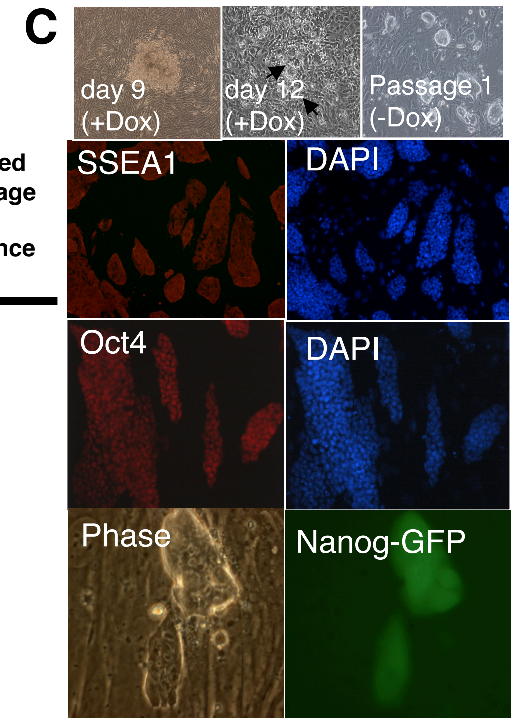
A

Viral Transgene Combination	Rosa26-M2rtTA +	Rosa26-M2rtTA -	n (%) total= 91 mice
None	1	0	1 (1.1%)
O	4	0	4 (4.4%)
S	1	0	1 (1.1%)
K	4	1	5 (5.5%)
M	0	2	2 (2.2%)
OS	5	0	5 (5.5%)
OK	7	4	11 (12.1%)
OM	2	0	2 (2.2%)
SK	5	0	5 (5.5%)
SM	1	1	2 (2.2%)
KM	2	1	3 (3.3%)
OSK	11	7	18 (19.8%)
OSM	1	0	1 (1.1%)
OKM	11	4	15 (16%)
SKM	3	2	5 (5.5%)
OSKM	8*	3	11 (12%)

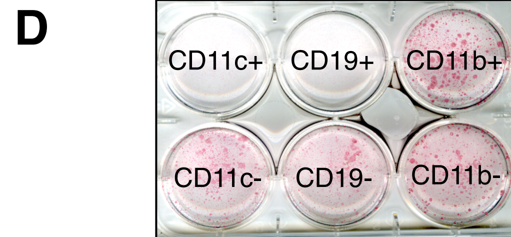
B

Factor	Transgene copy number determined by Southern Blot	Percentage of inheritance by PCR	Predicted Percentage of inheritance
Oct4	2	74% (67/91)	75%
Sox2	1	53% (48/91)	50%
Klf4	2	80% (73/91)	75%
c-Myc	1	45% (41/91)	50%

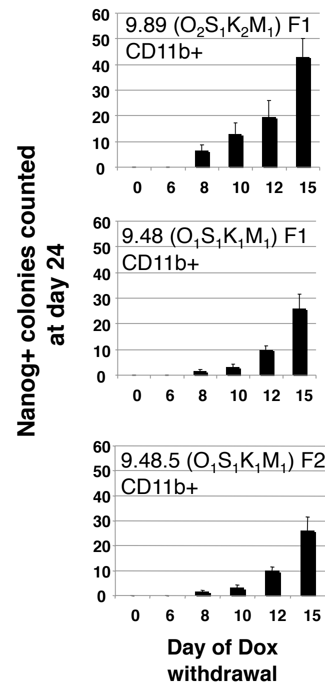
C



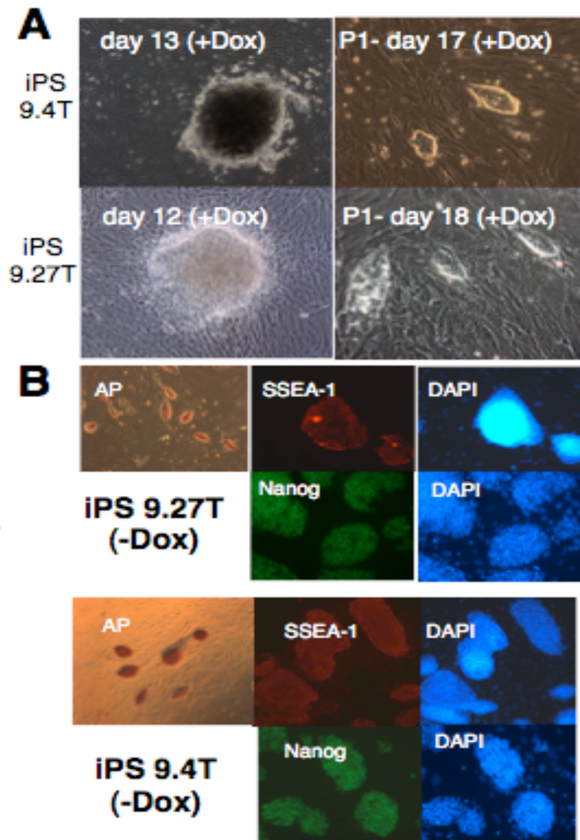
D

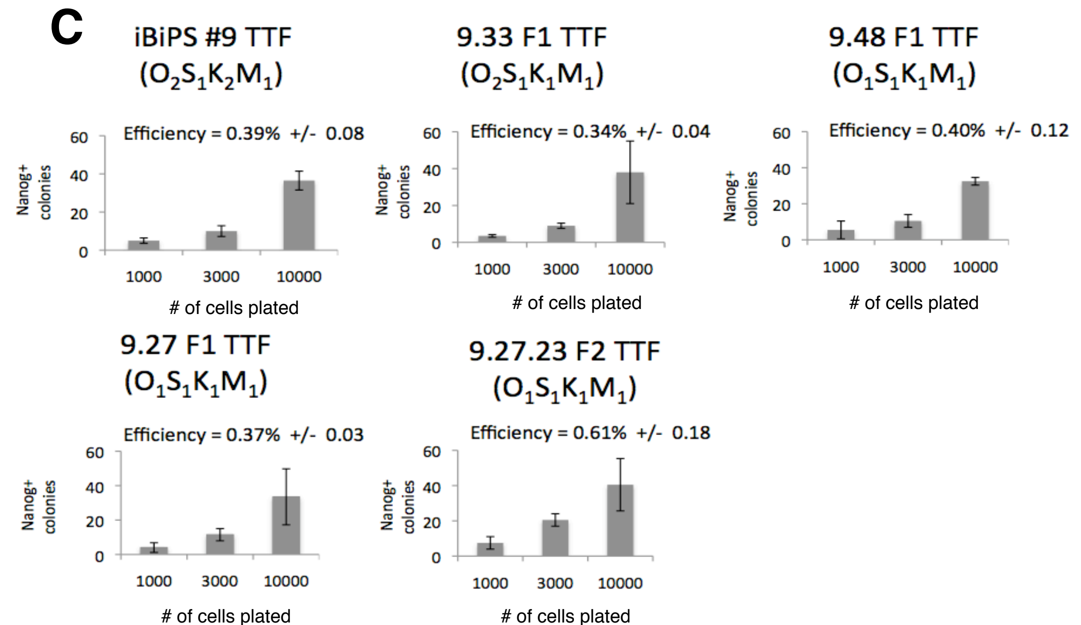


Supplementary Figure 3: Number of reprogrammed colonies from CD11b+ positive cells from peripheral blood at day 24 after Dox induction. Dox was withdrawn from the culture medium at the indicated time points. The number of colonies was determined by immuno-staining for Nanog at Day 24. Error bars: SEM generated from duplicate wells for each Dox withdrawal time point.

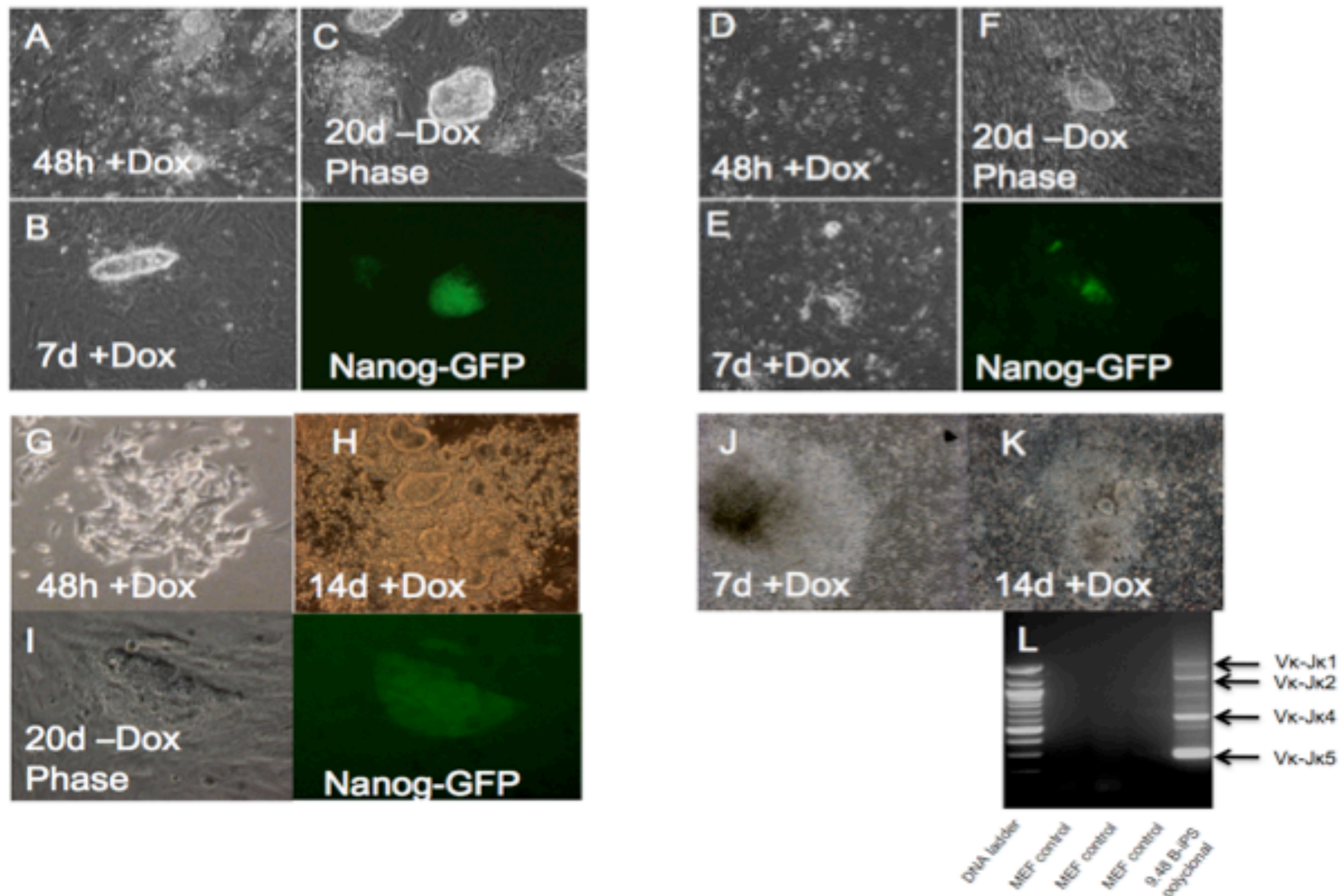


Supplementary Figure 4: Reprogramming of adult transgenic tail tip derived fibroblasts.
A) Derivation of representative tail tip fibroblasts (TTF) iPS line from 9.4 and 9.27 mice. (B) Staining for pluripotency markers AP, SSEA1 and Nanog. of 9.4T and 9.27T iPS lines (C) Efficiency of reprogramming of TTF cells, analyzed 25 days after Dox induction, as determined by immuno-staining for Nanog. Efficiencies were calculated as the fraction of Nanog positive colonies to cells seeded. Error bars indicated the SEM generated from duplicate wells used for each cell density. The offspring ID (offspring number and F1 or F2 generation) and their genotype (copy number for each transgene indicated by a subscript) are also shown above each graph. Importantly there was no detectable reduction in reprogramming efficiency between F1 and F2 transgenic mice carrying a single copy of each of the reprogramming factors (consistent with CD11b+ reprogramming efficiency results in Figure 1F). “T” indicates iPS lines derived from tail tip fibroblasts.

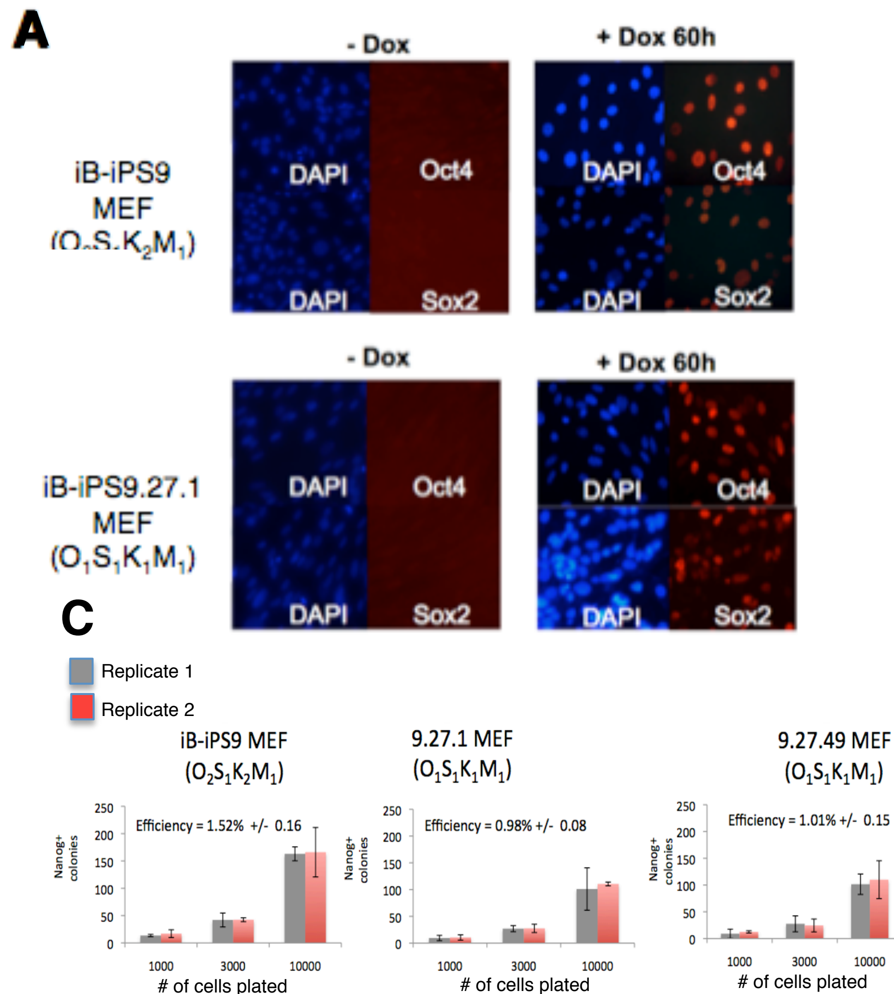


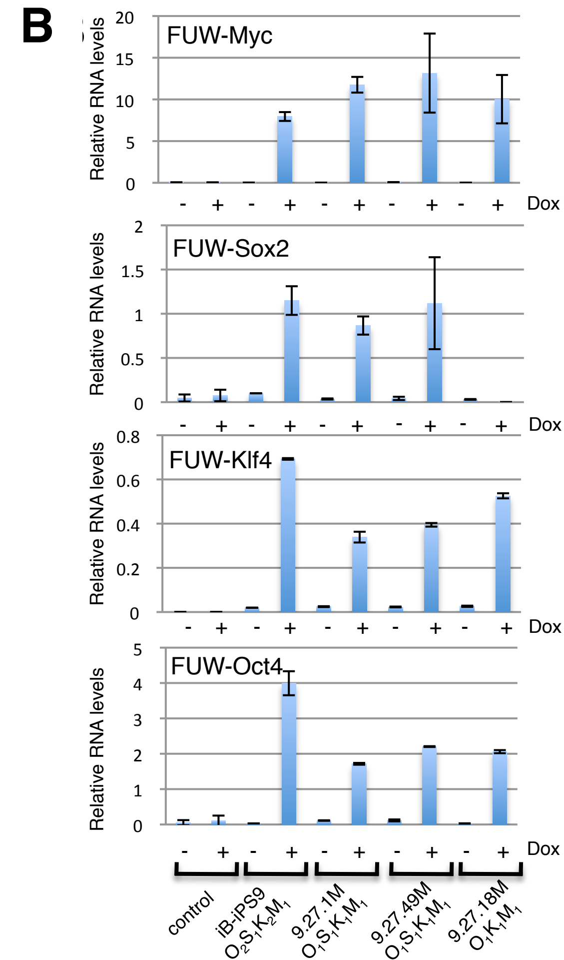


Supplementary Figure 5: Reprogramming multiple somatic cell types carrying one copy of each of the four reprogramming factors. (A-C) Reprogramming of intestinal epithelium. (A) Intestinal epithelial cells isolated and cultured on MEFs in the presence of Dox began form spheroid colonies in suspension within 48 hrs. (B) After 7 days of Dox treatment the spheroids had formed colonies with ES morphology. (C) Withdrawal of dox on D]day 14 resulted in fully reprogrammed iPS cultures that express Nanog-GFP readily detected at day 20. (D-F) Keratinocytes were isolated and cultured according to standard protocols. (E) Dox administration to those colonies for 7 days resulted in cell aggregation and the appearance of colonies with ES-like morphology. (F) Withdrawal of dox on day 14 resulted in stable iPS colonies expressing Nanog-GFP. (G-I) Reprogramming of liver cells. (G) Liver cell cultures showed multiple epithelioid aggregations in vitro 48h after Dox inductions. (H) ES-like cells were abundant in the cell cultures after 14d of Dox induction. I) Withdrawal of dox on day 14 resulted in stable iPS colonies expressing Nanog-GFP at day 20. (J-K) pLIB-C/EBPα infected mature CD19+ B cells grown (J) 7d and (K) 14d on Dox. (L) κ light chain rearrangements were specifically detected by PCR on genomic DNA from polyclonal B-iPS line obtained following 3 passages of the whole originally plated culture. All tissues in this experiment were derived from 9.74 F1 ($O_1S_1K_1M_1$) reprogrammable mouse.

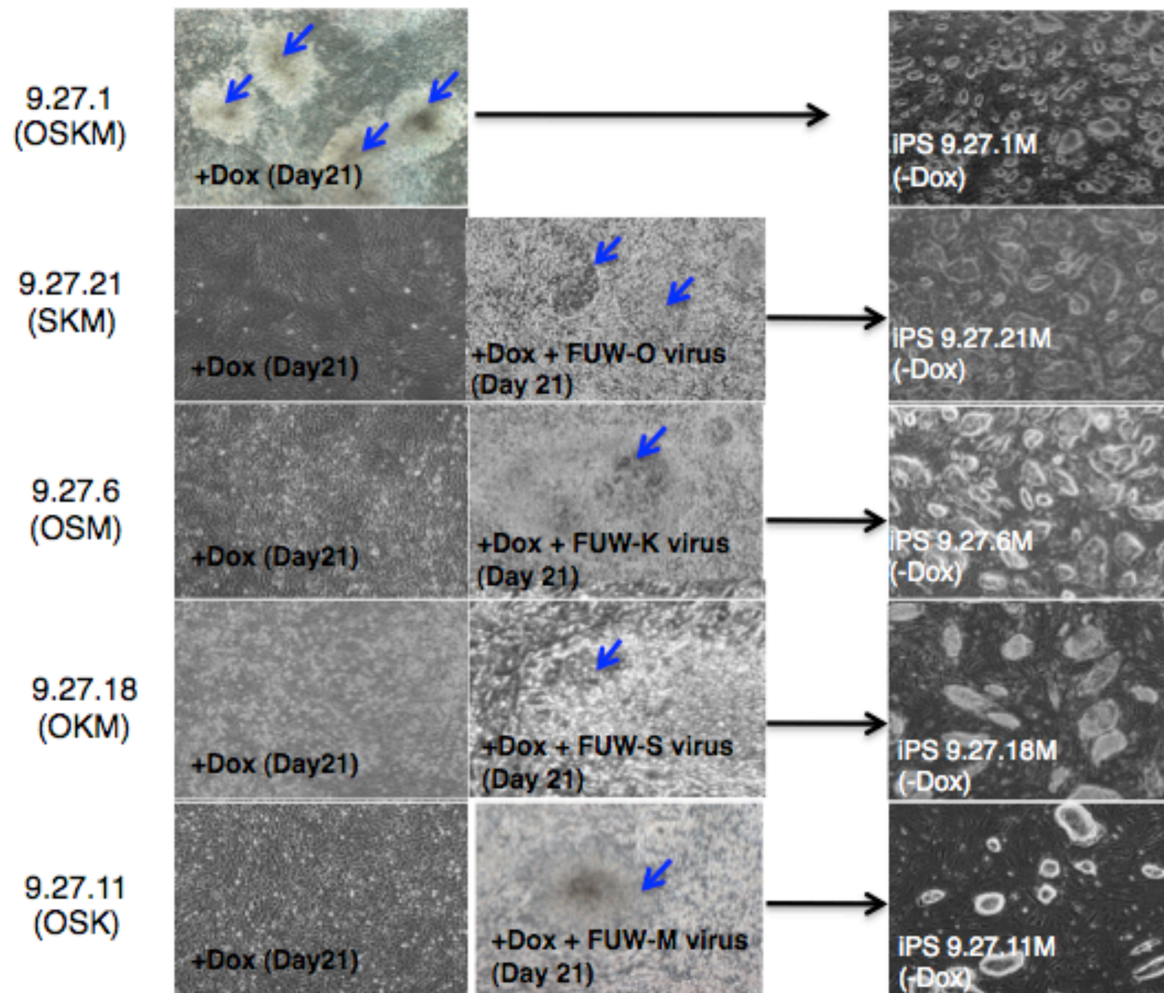


Supplementary Figure 6 : Transgene expression and efficiency of reprogramming in MEF lines after Dox induction. A) Immuno-fluorescent staining for detection of Oct4 and Sox2 in untreated MEF cell lines or following 60h of Dox induction. Dox induction resulted in activation of the transgenes that varied at the single cell levels. >90% of individual cells stained positive for Oct4 and Sox2. B) Quantitative RT-PCR for transgene specific expression of Oct4, Sox2, Klf4 and c-Myc in different MEF lines that were untreated or treated with Dox for 72 hours relative to GAPDH levels. The origin and genotype for each line tested is indicated. Error bars represent SD for duplicate measurements. MEFs carrying a single proviral copy of c-Myc and Sox2, regardless of whether they were derived from F1 or F0 generations, expressed comparable levels of transgenes upon Dox treatment suggesting that i) expression was comparable in different progeny with the same genotype and ii) no significant transgene silencing took place after germline transmission C) Efficiency of MEF reprogramming after 27 days of Dox induction as determined by immunostaining for Nanog. Efficiencies were calculated as the fraction of Nanog positive colonies to cells seeded. Error bars indicate the SEM generated from duplicate wells used for each cell density. Results from two replicate experiments are shown.

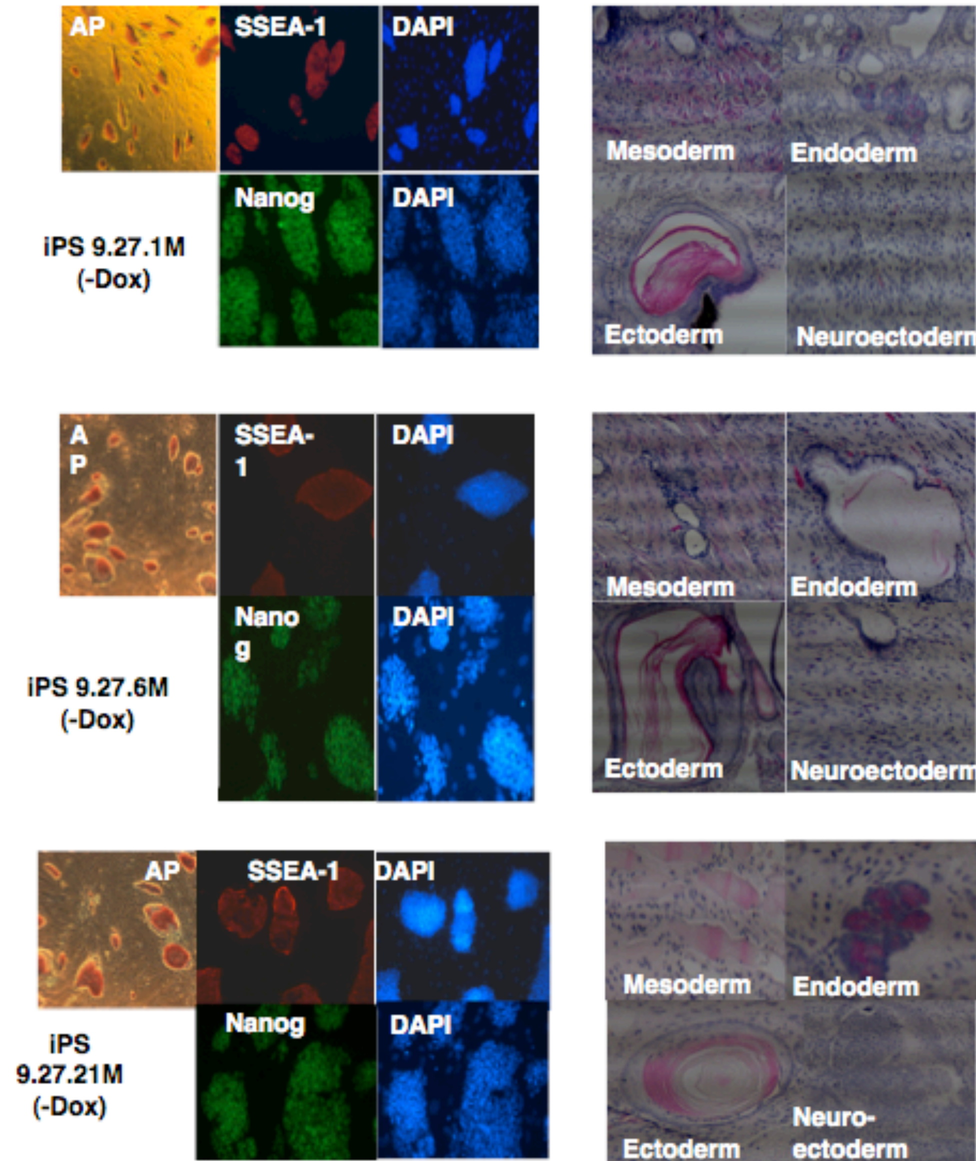




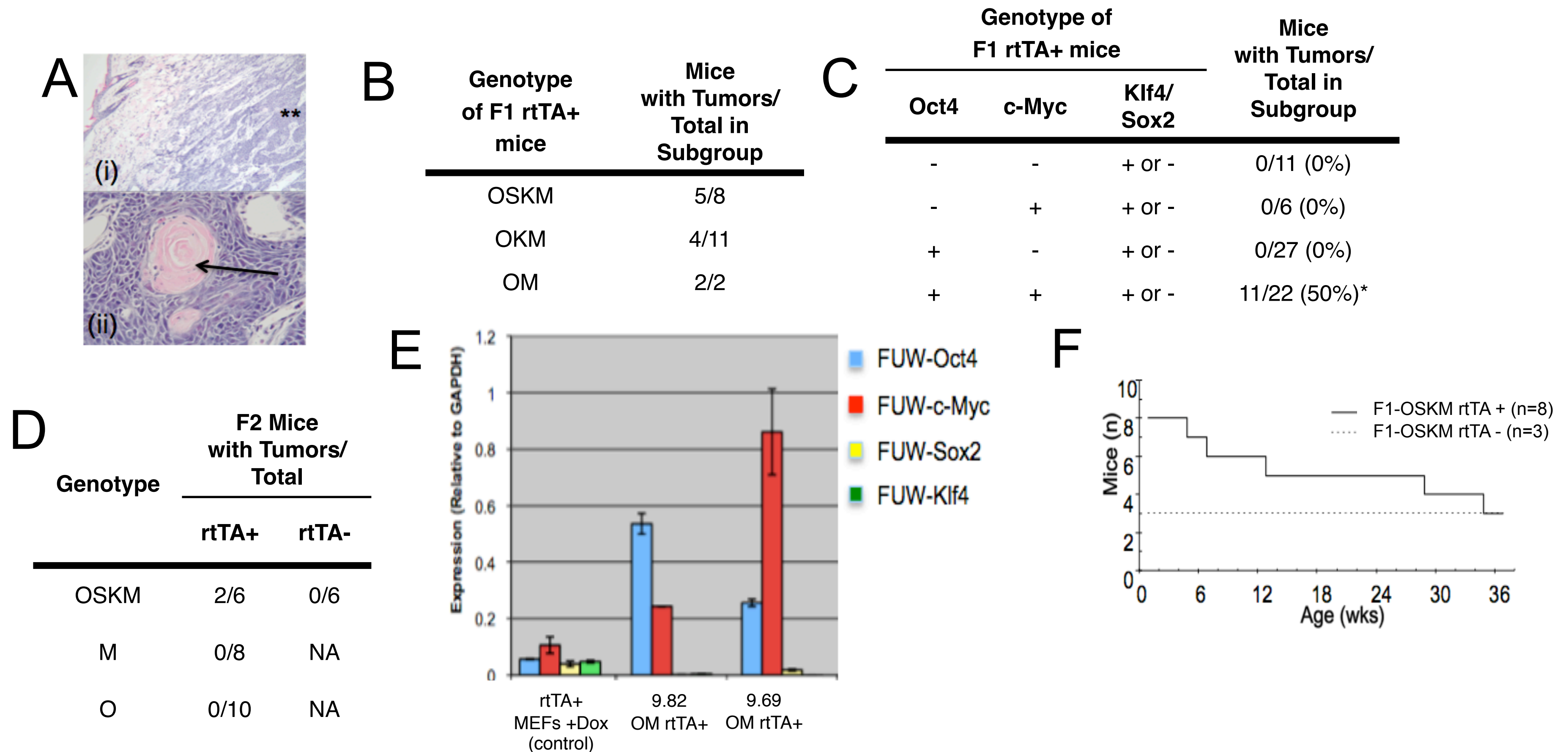
Supplementary Figure 7: Reprogramming of MEF library cell lines with different combinations of reprogramming factors. Representative images demonstrating ES-like colonies following 21 days of Dox induction in MEF lines carrying different combination of reprogramming factors (genotype for each line is indicated on the left of the panel). MEF lines that were missing one of the reprogramming factors following germline transmission were infected on day 0 of the experiment with teto-FUW Dox-inducible lentivirus (FUW) encoding the missing transcription factor as indicated in the middle panels. Blue arrows indicate examples of typical observed colonies that were further expanded and generated Dox independent iPS lines (shown in the right column) following 1-2 passages.



Supplementary Figure 8: Characterization of MEF derived iPS lines. Staining of representative iPS lines described in Fig. 2B and supplementary Figure 7 online for pluripotency markers AP, SSEA1 and Nanog. Additionally, we show hematoxylin and eosin staining of teratoma derived from the same lines. “M” indicates iPS lines derived from mouse embryonic fibroblasts (MEF).



Supplementary Figure 9: Tumor formation in the transgenic progeny. (**A**) Hematoxylin and Eosin staining of a representative skin epithelial tumor typically observed in some mice in the progeny. (i) asterisks point epithelial infiltrative cells with typical (keratinization (black arrow) observed among the invasive epithelial tumor infiltrates. (**B**) Genotype of F1 mice that developed tumors up to 36 weeks of age. (**C**) Tumor development in rtTa+ mice that carried Oct4 and c-Myc transgenes irrespective of the presence of Sox2 or Klf4 alleles.. (**D**) Tumor incidence in F2 progeny up to 22 weeks of age. NA: Data not available. The results in panels C-D suggest that epithelial tumors occur only in mice carrying both Oct4 and c-Myc transgenes (and not in mice carrying c-Myc or Oct4 only). * in C indicates Fisher's exact test to measure the probability that tumor incidence is dependent on Oct4. Analysis yielded P value <0.055 for F1 progeny data . When including the F2 progeny in panel (D) statistical significance was reached with P value was <0.002. (**E**) Specific expression of Oct4 and c-Myc transgene derived transcripts by RT-PCR in tumors obtained from two OM rtTa+/- mice. Dox treated rtTA+ MEFs that do not carry any factor transgenes were used as a negative control. (**F**) Survival curve for 4 factor F1 mice in our study (36 weeks follow up).
CONCLUSIONS: Firstly, rtTa- do not develop tumors (Panels D and F). Secondly, in rtTa+/- mice, c-Myc or Oct4 alone are not sufficient to result in tumor formation in our system, suggesting that leaky expression of c-Myc and Oct4 synergistically induced epithelial tumor formation. Thirdly, >30% OSKM RtTa+ mice survived 9 months or longer, which allowed further expansion of the mouse colony. $O_1S_1K_1M_1$ mice that lack the M2-rtTa allele are currently mated to homozygocity, because rtTA- mice do not develop tumors. Such mice can be bred to mice homozygous for the RtTa+ allele to generate drug inducible reprogrammable mice.



B

Genotype of F1 rtTA+ mice	Mice with Tumors/ Total in Subgroup
OSKM	5/8
OKM	4/11
OM	2/2

C

Genotype of F1 rtTA+ mice			Mice with Tumors/ Total in Subgroup
Oct4	c-Myc	Klf4/ Sox2	
-	-	+ or -	0/11 (0%)
-	+	+ or -	0/6 (0%)
+	-	+ or -	0/27 (0%)
+	+	+ or -	11/22 (50%)*

D

Genotype	F2 Mice with Tumors/ Total	
	rtTA+	rtTA-
OSKM	2/6	0/6
M	0/8	NA
O	0/10	NA

Supplementary Table 1. Summary of iPS cell derivation from whole blood-derived cells of four factor offspring.

Offspring ID	Genotype[a]	iPS Line	
		Blood	TTFs
9.4	$O_2S_1K_1M_1$	+	+
9.14	$O_2S_1K_1M_1$	ND[b]	+
9.27	$O_1S_1K_1M_1$	+	+
9.33	$O_2S_1K_1M_1$	+	+
9.48	$O_1S_1K_1M_1$	+	+
9.67	$O_1S_1K_1M_1$	+	ND[b]
9.74	$O_1S_1K_1M_1$	+	+
9.89	$O_2S_1K_2M_1$	+	+

[a] Genotype and copy number were analyzed by Southern Blot.

[b] Not done because the mouse died prior to respective reprogramming test.

Supplementary Table 2. Summary for iPS derivation from F1 offspring blood samples.

Factor Combination	Number of M2-rtTA+ mice screened for iPS generation from Peripheral Blood	Number of mice that yielded positive results for peripheral blood iPS generation [a]
OSKM [b]	7	7 (100%)
OSK	11	0 (0%)
OSM	1	0 (0%)
OKM	11	0 (0%)
SKM	3	0 (0%)
OS	5	0 (0%)
OK	7	0 (0%)
OM	2	0 (0%)
SK	5	0 (0%)
SM	1	0 (0%)
KM	2	0 (0%)
O	4	0 (0%)
S	1	0 (0%)
K	4	0 (0%)
M	0	0 (0%)
None	1	0 (0%)

[a] Identical results obtained in 2 independent screening experiments.
[b] An additional mouse carrying all four factors was not tested for blood reprogramming, as it died prior to completion of the screen.

Supplementary Table 3. Diploid blastocyst injections of iPS lines

iPS Line	Genotype [a]	Blastocysts Injected	Pups			
			Chimeric/ Total	Born Live Chimeric	Born Dead Chimeric	Adult Chimeric /Total
iPS 9.4B	$O_2S_1K_1M_1$	30	2/4	1[b]	1	0/2
iPS 9.27B	$O_1S_1K_1M_1$	41	8/9	8[c]	0	2/8
iPS 9.33B	$O_2S_1K_1M_1$	42	3/6	3[d]	0	1/3
iPS 9.48B	$O_1S_1K_1M_1$	62	4/13	4	0	4/4
iPS 9.67B	$O_1S_1K_1M_1$	20	2/3	2	0	2/2
iPS 9.14T	$O_2S_1K_1M_1$	22	1/6	1	0	1/1
iPS 9.27T	$O_1S_1K_1M_1$	45	3/10	3	0	3/3

[a] Subscripts indicate transgene copy number.
[b] Pup missing from its cage 5 days after birth.
[c] Six pups were delivered live by c-section, but they died shortly thereafter.
[d] Two pups missing from their cage 5 days after birth.

"B" indicates iPS line derived from peripheral blood.

"T" indicates iPS line derived from tail tip fibroblasts.