Cell Stem Cell, Volume *5*

Supplemental Data

A Small Molecule Inhibitor of Tgf-β Signaling

Replaces *Sox2* in Reprogramming by Inducing *Nanog*

Justin K. Ichida, Joel Blanchard, Kelvin Lam, Esther Y. Son, Julia E. Chung, Dieter Egli, Kyle M. Loh, Ava C. Carter, Francesco P. Di Giorgio, Kathryn Koszka, Danwei Huangfu, Hidenori Akutsu, David R. Liu, Lee L. Rubin, Kevin Eggan

Supplemental Experimental Procedures

Derivation of MEFs and Cell Culture

MEFs were derived as previously described (Takahashi et al., 2007a).

Derivation of Tail Tip Fibroblasts, Cell Culture, and Retroviral Infection

Fibroblasts were isolated from tails of 8-week old *Oct4*::GFP mice and cultured in DMEM supplemented with 40% fetal bovine serum and penicillin/streptomycin. For reprogramming experiments, P2 fibroblasts were infected by the same method as described for MEFs.

Lead Compound Titrations to Determine Optimal Dosage

Infections and VPA/compound addition was done as in the original chemical screen, and wells were scored for GFP+ colonies on day 25 after compound addition.

Karyotyping

Karyotype analysis was performed at Cell Line Genetics.

Antibody Staining for Sox2 and Nanog and Alkaline Phosphatase Staining

iPS cells were cultured on irradiated MEF feeders in chamber slides, fixed with 4% paraformaldehyde (PFA) and stained with primary antibodies against mSox2 (Santa Cruz, sc-17320), mNanog (CosmoBio, REC-RCAB0002PF), followed by staining with the appropriate secondary antibodies conjugated to Alexa Fluor 546 (Invitrogen). Nuclei were counterstained with Hoechst33342 (Sigma). iPS cells were assayed for alkaline phosphatase activity using the Vector Red alkaline phosphatase assay kit from Vector Laboratories.

Spontaneous Differentiation of iPS Cells *in Vitro*

iPS cells were grown to 70–80% confluence in 10-cm plates (Falcon) in mES cell medium. To form embryoid bodies, cells were washed once with PBS to eliminate mES cell medium and then incubated with 1 ml of 0.25% trypsin (GIBCO) for 5–10 min at room temperature (21-25 °C). Cells were then resuspended in 10 ml of DM1 medium (DMEM-F12, GIBCO), 10% knockout serum (GIBCO), penicillin, streptomycin, glutamine (GIBCO) and 2-mercaptoethanol (GIBCO), counted, and plated at a concentration of 200,000 cells per ml in Petri dishes (Falcon). Two days later, embryoid bodies were split from one dish into four Petri dishes containing DM1 medium and the medium was changed after 3–4 d. On day 10 the embryoid bodies were collected in a 15-ml Falcon tube, washed once with PBS and then fixed in PFA 4% at 4 degrees C for 1 hour. The EBs were then washed 4 times in PBS to remove the residual PFA and incubated overnight in a solution of 30% of sucrose. The next day, the cells were embedded in OCT and frozen at -80 degrees C. The block containing EBs were then

sectioned with a cryostat into 10 μm sections. The sections were stained with primary antibodies against Alpha-fetoprotein (AFP)(Dakocytomation, A0008), Skeletal Myosin (MF20)(Developmental Studies Hybridoma Bank, MF20), or Beta-III-tubulin (TUJ1)(Sigma, T2200), and visualized by staining with a secondary antibody conjugated to Alexa Fluor 546 (Invitrogen).

Directed Differentiation of iPS Cells Into Motor Neurons

iPS and mES (V6.5) cells were differentiated into motor neurons according to methods previously described for mouse ES cells differentiation [27]. The iPS and mES cells were grown to 70–80% confluence in 10-cm plates (Falcon) in mES cell medium. To form embryoid bodies, cells were washed once with PBS to eliminate mES cell medium and then incubated with 1 ml of 0.25% trypsin (GIBCO) for 5–10 min at room temperature (21-25 °C). Cells were then resuspended in 10 ml of DM1 medium (DMEM-F12, GIBCO), 10% knockout serum (GIBCO), penicillin, streptomycin, glutamine (GIBCO) and 2-mercaptoethanol (GIBCO), counted and plated at a concentration of 200,000 cells per ml in Petri dishes (Falcon). Two days later, embryoid bodies were split from one dish into four Petri dishes containing DM1 medium supplemented with RAc (100 nM; stock: 1 mM in DMSO, Sigma) and Shh (300 nM, R&D Systems). Medium was changed after 3–4 d. On day 7, the embryoid bodies were dissociated into single-cell suspensions. The suspensions were pelleted in a 15-ml Falcon tube, washed once with PBS, and incubated in Earle's balanced salt solution with 20 units of papain and 1,000 units of DNase I (Worthington Biochemical) for 30–60 min at 37 °C. The mixture was then triturated with

a 10-ml pipette and centrifuged for 5 min at 300 x *g*. The resulting cell pellet was washed with PBS and resuspended in F12 medium (F12 medium, GIBCO) with 5% horse serum (GIBCO), B-27 supplement (GIBCO), N2 supplement (GIBCO) with neurotrophic factors (GDNF and BDNF, 10 ng ml^{-1}, R&D Systems). The cells were counted and plated on poly-D-lysine/laminin culture slides (BD Biosciences) or on a layer of primary glial cells. 3-5 days later, the cultures were fixed with PFA and stained with primary antibodies against TUJ1 (Sigma, T2200) and HB9 (Developmental Studies Hybridoma Bank, 81.5C10), and visualized by staining with secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 546 (Invitrogen). For counting HB9+ cells, motor neurons were differentiated as above except in embryoid body culture without dissociation and plating. Embryoid bodies were sectioned as above and stained with the TUJ1 and HB9 antibodies along with the Alexa Fluor 488 and Alexa Fluor 546 secondary antibodies. Cultures were counterstained with Hoechst 33342 and HB9+ and total nuclei were counted. Numbers were derived from at least 3 different embryoid bodies per cell line.

Teratoma Production and Analysis

A confluent 10 cm dish of iPS cells was trypsinized, pelleted, resuspended in .2 mls of mES media, and injected subcutaneously into a CD1-Nude mouse. 3-4 weeks later, teratomas were harvested, fixed overnight with 4% paraformaldehyde, embedded in paraffin, sectioned, HE stained, and analyzed.

Production of Chimeric Mice

Female ICR mice were superovulated with PMS and hCG and mated to ICR stud males. 24-hours after hCG injection, zygotes were isolated from vaginally plugged females. After culture in KSOM media for 3 days, the resulting blastocysts were injected with ~5-10 iPS cells from a C57BL6 background pre-labeled with a lentivirus constitutively expressing the red fluorescent protein tdTomato and transferred into pseudopregnant females. Embryos were either harvested at day E13.5 or allowed to develop to term. Chimeric embryos were visualized on a Leica MZ16FA dissecting microscope using RFP and bright field channels. For 8-cell stage injections, zygotes were developed *in vitro* to the 8-cell stage, injected with iPS cells, further developed *in vitro* to the blastocyst stage, and visualized.

Genital Ridge Isolation and Visualization

The genital ridges of E13.5 embryos were mechanically isolated and visualized using a Leica dissection microscope.

Generation of iPS Cells

GFP+ P0 colonies were picked manually and incubated in .25% trypsin (Gibco) for 20 minutes at room temperature before plating on a feeder layer in mES cell media. This process was repeated until passage 3, at which time colonies were trypsinized and passaged in bulk and maintained on feeders in mES cell media.

Whole-genome Expression Analysis

For comparison to mES and iPS cells, cells reprogrammed with RepSox were grown to near confluence on an irradiated layer and RNA was isolated with Trizol (Invitrogen). In other experiments analyzing the effect of RepSox treatment, cells were harvested at less than 60% confluence. RNA was amplified and labeled with biotin using the Illumina Total Prep RNA Amplification Kit from Ambion, hybridized to Illumina Whole-Genome Expression BeadChips (MouseRef-8), and analyzed by an Illumina Beadstation 500. All lines were analyzed in biological duplicate or triplicate. Data were processed using Resolver software.

Western Blots

Lysates were generated from cells treated with 25 μM RepSox for 48 hours in mES media. Cells were harvested using a cell scraper into lysis buffer containing protease inhibitors. For analysis of phospho-Smads, an anti-phospho-Smad2/3-specifc antibody (Epitomics) and an anti-phospho-Smad1/5/8-specific antibody (R&D) were used.

Cell Cyle Analysis

Cells were treated with 25 μM RepSox in mES media without feeders for 72 hours and subjected to cell cycle analysis by propidium iodide staining and flow cytometry. Cells were harvested with .25% trypsin and fixed with 70% ethanol overnight. Following at least one hour of incubation with propidium iodide staining solution (50 μg/ml propidium

iodide in PBS, .1% BSA, .1% Rnase A) in the dark, samples were analyzed on a BD LRSII Flow Cytometer (BD Biosciences).

RT-PCR

For experiments measuring *Nanog* induction, cells were treated with 25 μM RepSox in KSR mES media. RNA was harvested with Trizol (Invitrogen) and treated with Turbo-free (Ambion) to remove DNA contamination. RNA was reverse transcribed using random hexamer primers and superscript III reverse transcriptase (Invitrogen). Primer sequences for endogenous genes were the following: *Nanog* (5'-CAGGTGTTTGAGGGTAGCTC and 5'- CGGTTCATCATGGTACAGTC), *Sox2* (5'-TAGAGCTAGACTCCGGGCGATGA and 5'- TTGCCTTAAACAAGACCACGAAA), *Oct4* (5'- TCTTTCCACCAGGCCCCCGGCTC and 5'-TGCGGGCGGACATGGGGAGATCC), *Rex1* (5'-ACGAGTGGCAGTTTCTTCTTGGGA and 5'-TATGACTCACTTCCAGGGGGCACT). The reverse primer (5'-TTTCTACAAGAAAGCTGGGT) was used for all transgenes, plus the following forward primers: *Nanog* (5'- TTGGAATGCTGCTCCGCTCC), *Sox2* (5'-CTACAGCATGTCCTACTCGC), *Oct4* (5'- GCTATGGAAGCCCCCACTTC), and *Klf4* (5'- TGACTATGCAGGCTGTGGCA). QPCR was performed using these primers and SYBR green (Bio-Rad).

shRNA-mediated Knockdown of *Nanog* and *Sox2*

OKM 10 cells or MEFs transduced 4 days earlier with *Oct4*, *Klf4*, *cMyc*, and *Sox2* (OKMS-MEFs) were transduced with shRNA constructs in the lentiviral vector pLKO.1 that were specific to murine *Nanog* (5'-CCGGCCTGAGCTATAAGCAGGTTAACTCGAGTTAACCTGCTTATAGCTCAGGTTTTTG) or *Sox2* (5'-CCGGCGAGATAAACATGGCAATCAACTCGAGTTGATTGCCATGTTTATCTCGTTTTTG) (Open Biosystems). Lentiviruses were packaged by co-transfection of pLKO.1-shRNA plasmids with VSVG envelope and delta 8.9 plasmids into 293T cells using Fugene 6. Starting two days after infection, the population was enriched for transduced cells by selection with 4 μg/ml puromycin for three days. For OKM 10 cells, RepSox treatment (25 μM) was initiated after puromycin selection. RepSox treatment was performed in KSR mES media for 9 days before GFP+ colonies were scored.

Reprogramming of Stable Intermediate Cell Lines by Viral Transduction

Oct4::GFP-negative cell lines were transduced using the same methodology and reagents as MEFs were in the original screen. Cells were infected with three rounds of viral supernatant diluted 1:8 in MEF media in a 48-hour period on gelatin. Two days after the last viral supernatant was added, the cells were trypsinized and replated onto feeders. The media was changed to mES media containing knockout serum replacement (KSR) instead of FBS on the following day. *Oct4*::GFP+ colonies were counted at 9 days post-transduction.

Reprogramming of MEFs Using *Nanog*

MEFs were infected as described for the original screen, except that murine *Nanog* cDNA was cloned into the pMXs retroviral vector and used instead of pMXs-*Sox2*. Two days after the last viral supernatant was added, the cells were trypsinized and replated onto feeders. The media was changed to mES media containing knockout serum replacement (KSR) instead of FBS on the following day, and *Oct4*::GFP+ colonies were counted on day 9 post-transduction.

Figure S1. *Oct4*::GFP+ colony formation in *Oct4, Klf4, cMyc*-infected MEFs as a function of compound concentration. 2 mM VPA was used in all wells. **(A)** E-616452 (RepSox) **(B)** E-616451 **(C)** EI-275

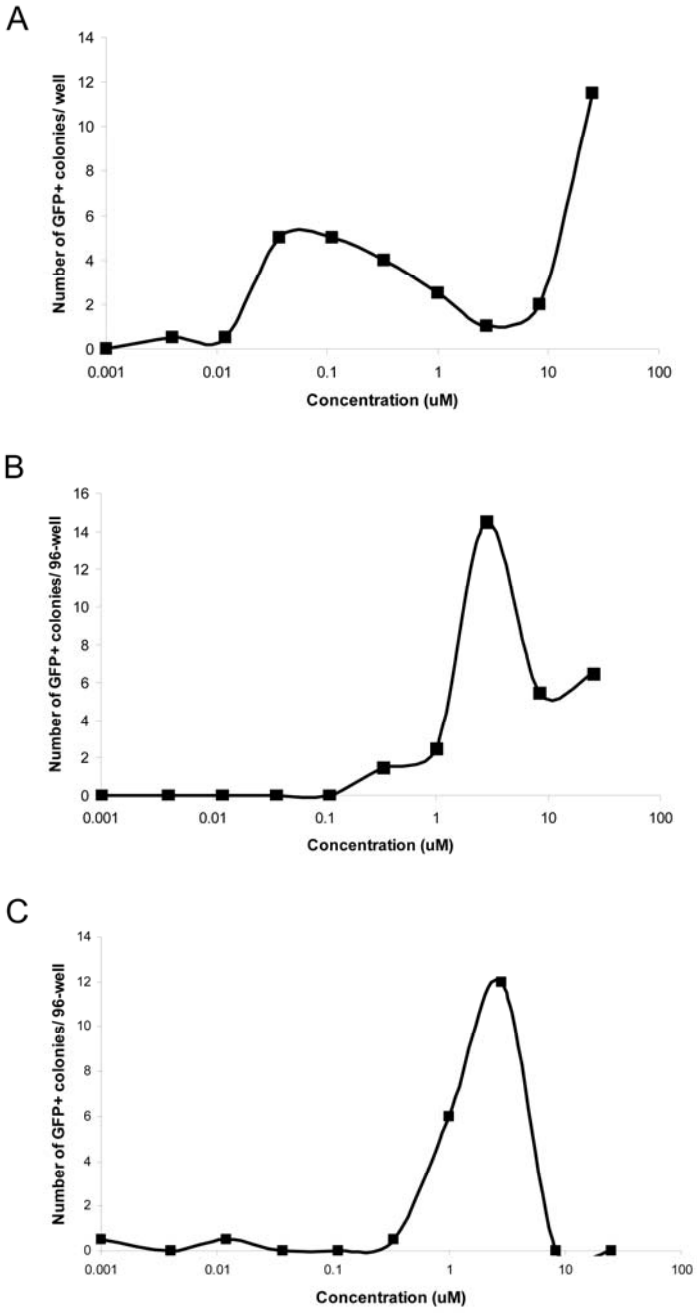


Figure S2. RepSox can replace *Sox2* in defined factor reprogramming of adult tail tip fibroblasts. (A) *Oct4*::GFP+ P0 colony derived from *Oct4, Klf4, cMyc*-infected tail tip fibroblasts treated with RepSox for 14 days. (B) Passage 5 *Oct4*::GFP+ cell line derived from a P0 colony. Scale Bars = (A) 200 μm (B) 500 μm

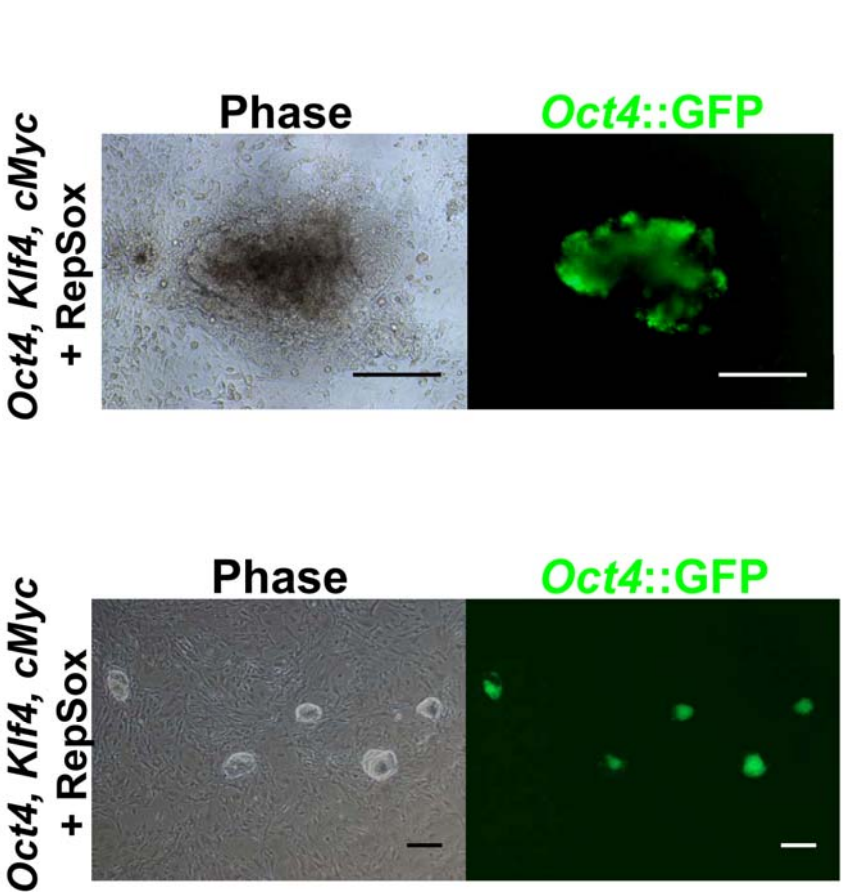


Figure S3. A RepSox-reprogrammed cell line contains transgenic *Oct4, Klf4,* and *cMyc*, but not *Sox2*, is karyotypically normal, uniformly expresses the embryonic stem cell marker alkaline phosphatase and has a global gene expression profile highly similar to that of an iPS line generated with *Oct4, Klf4, cMyc*, and *Sox2*. **(A)** PCR with primers specific for the transgenic versions of *Oct4, Klf4, cMyc* and *Sox2* (Takahashi and Yamanaka, 2006) was performed on genomic DNA isolated from a control iPS cell line generated with *Oct4, Klf4, cMyc*, and *Sox2* and a RepSox-reprogrammed cell line generated with *Oct4, Klf4*, and *cMyc* + RepSox. **(B)** Shown is the normal karyotype of a passage 8 cell from *Oct4, Klf4*, and *cMyc* + RepSox line 1. 20 cells were counted and 5 cells were karyotyped by GTL banding. All cells were karyotypically normal 40, XY. **(C)** Shown are passage 5 *Oct4, Klf4*, and *cMyc* + RepSox line 1 cells. Red color indicates alkaline phosphatase activity. Scale bar = 500 μm **(D)** A microarray scatter plot shows that the global gene expression profile of cells generated with *Oct4, Klf4*, and *cMyc* + RepSox (OKM + RepSox line 1) is highly similar to that of an iPS line generated with *Oct4, Klf4, cMyc*, and *Sox2* (OKMS-iPS).

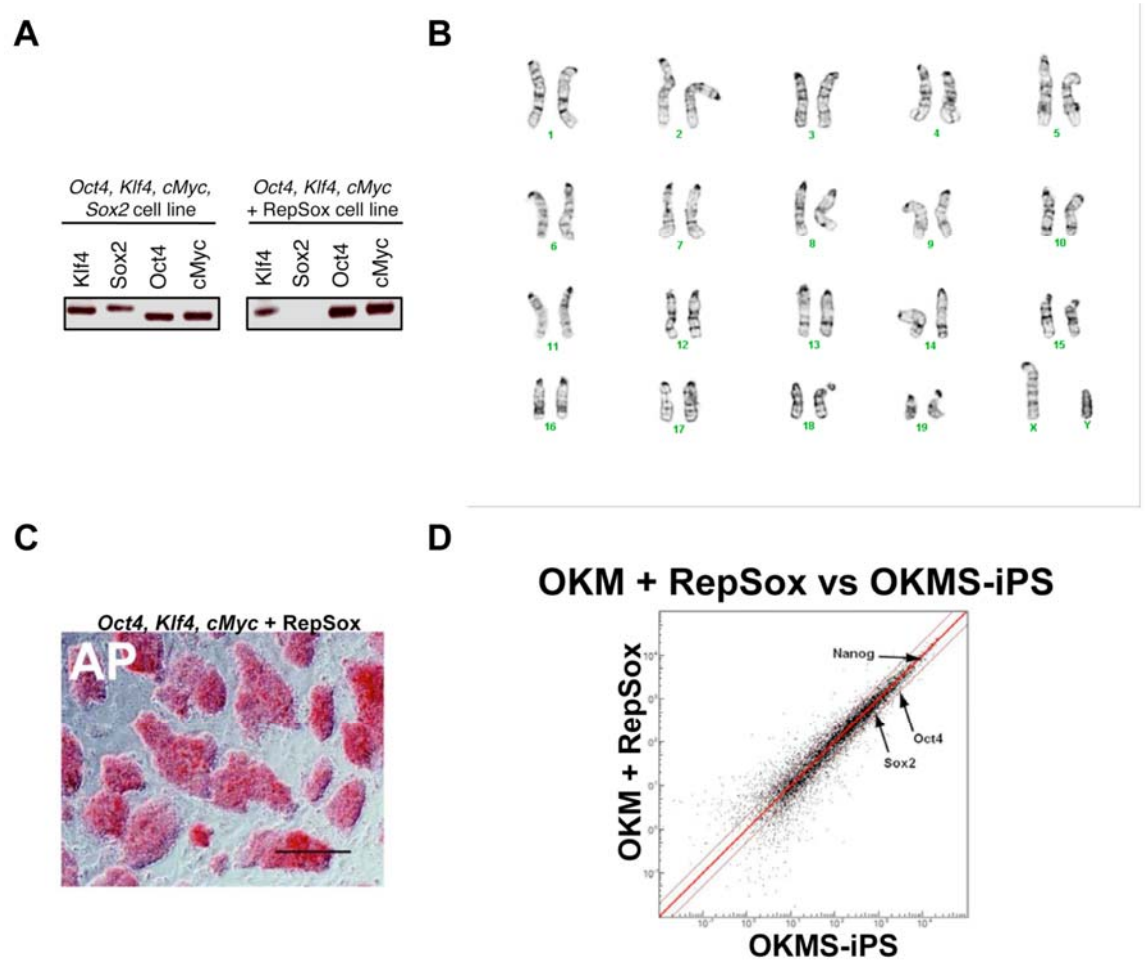


Figure S4. OKM + RepSox line 1 and OK + RepSox line 1 cells form embryoid bodies and differentiate into cells of all three germ layers *in vitro*.
(A) OKM + RepSox line 1 form embryoid bodies after 3 days in suspension culture **(B)** Spontaneously differentiated OKM + RepSox line 1 cells express TUJ1 (Beta III tubulin, ectoderm), MF20 (Myosin heavy chain, mesoderm), and AFP (Alpha fetoprotein, endoderm) **(C)** OK + RepSox line 1 form embryoid bodies after 3 days in suspension culture **(D)** Spontaneously differentiated OK + RepSox line 1 cells express TUJ1 (Beta III tubulin, ectoderm), MF20 (Myosin heavy chain, mesoderm), and AFP (Alpha fetoprotein, endoderm). Note that *Oct4*::GFP+ areas of the EBs are undifferentiated and do not overlap with the TUJ1+, MF20+, or AFP+ regions. Scale bars- TUJ1 and MF20 for OKM + RepSox line 1 = 100 μm, all others = 50 μm.

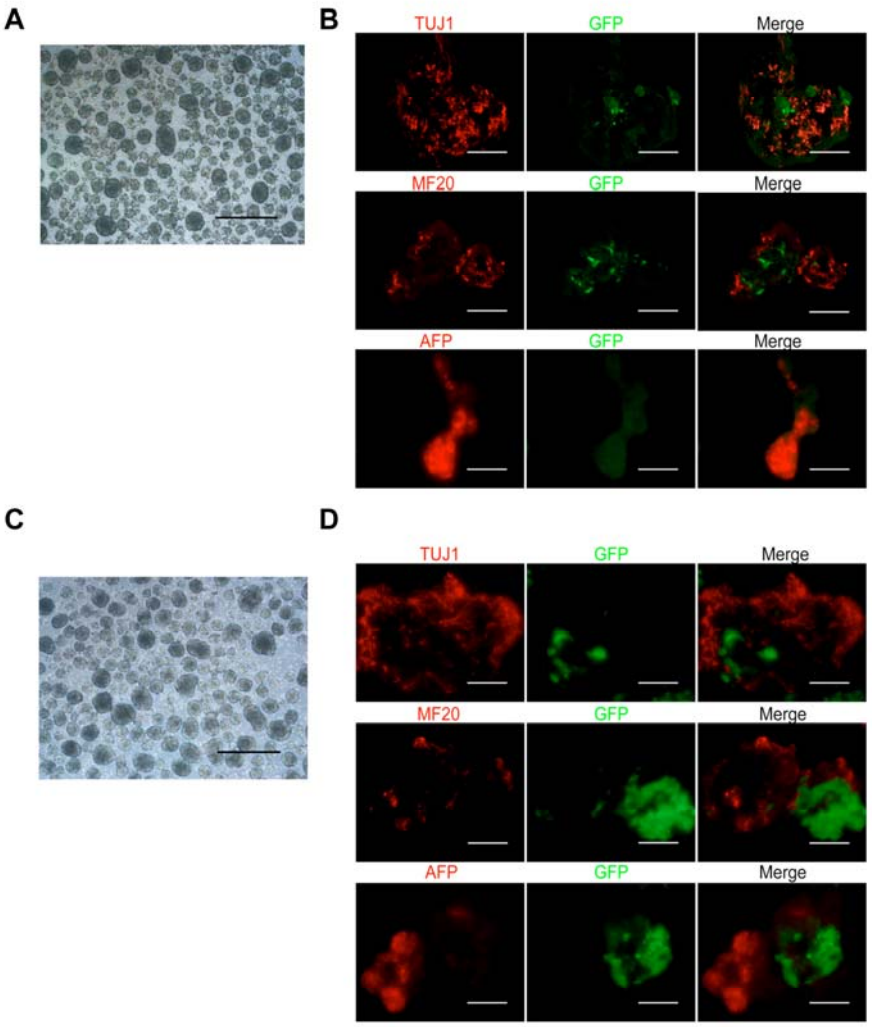
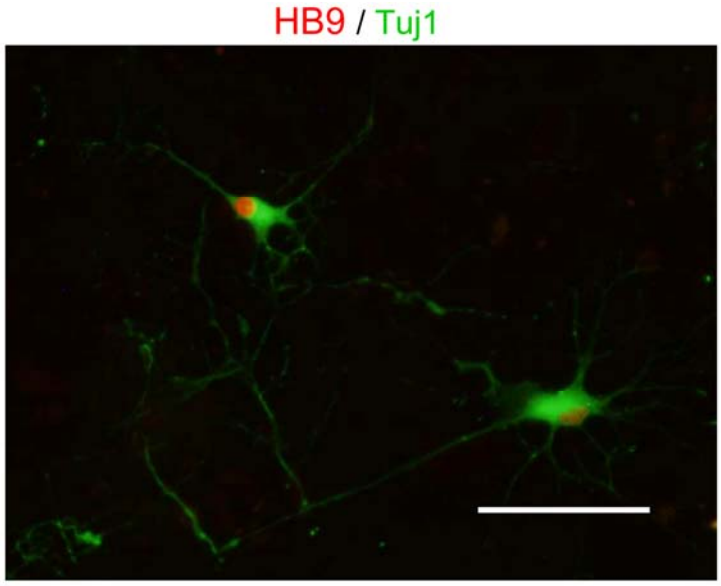


Figure S5. OKM + Rep Sox line 1 and OK + Rep Sox line 1 cells efficiently differentiate into HB9+ motor neurons *in vitro*. (A) Motor neurons differentiated *in vitro* from OK + RepSox line 1 (Scale bar = 100 μm) **(B)** Quantitation of motor neurons derived from RepSox-reprogrammed, four-factor iPS, and mES cell lines. The error bars denote the standard error derived from quantification of three separate wells (of cells).

A.



B.

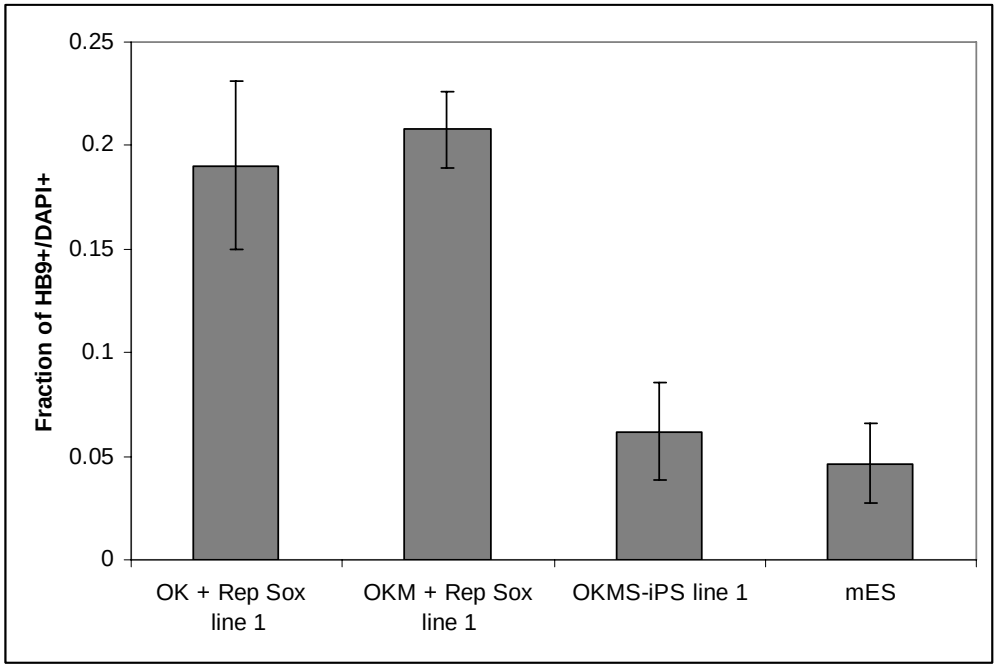
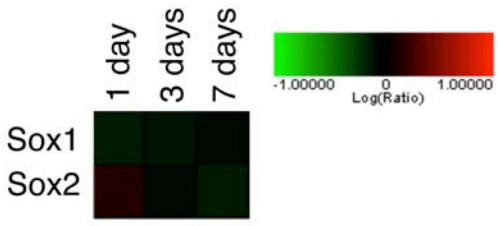
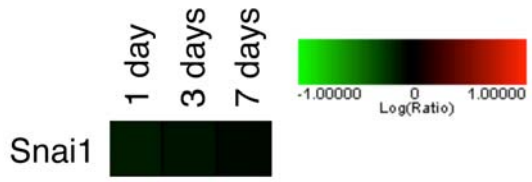


Figure S6. RepSox treatment in *Oct4, Klf4,* and *cMyc*-transduced MEFs does not induce the expression of *Sox*-family members or decrease the expression of fibroblast-specific genes, but it does increases *L-Myc* mRNA expression in MEFs. (A) *Sox*-family gene expression. Note- *Sox3* is absent from part A because its expression did not change enough to generate a low enough P value. Shown are changes relative to untreated controls. (B) Fibroblast-specific gene expression. (C) *L-Myc* expression analysis. Untransduced MEFs were treated with 25 μM RepSox for 7 days and mRNA expression was determined by microarray analysis. Fold-induction is relative to untreated control samples. The error bars denote the standard error derived from quantification of three separate wells (of cells).

A.



B.



C.

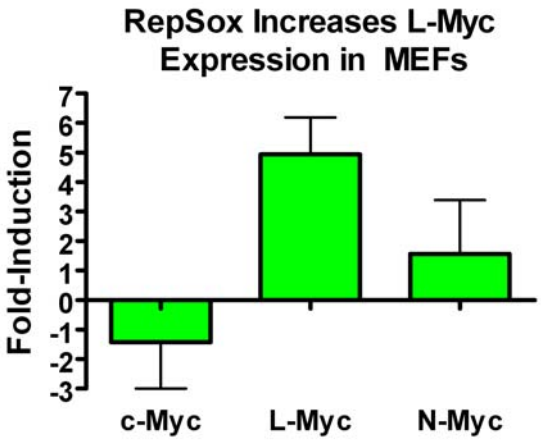


Figure S7. *Oct4*::GFP+ colonies appear at day 14 regardless of whether RepSox treatment is initiated at day 7 or day 10 post-transduction. The error bars denote the standard error derived from quantification of three separate wells (of cells).

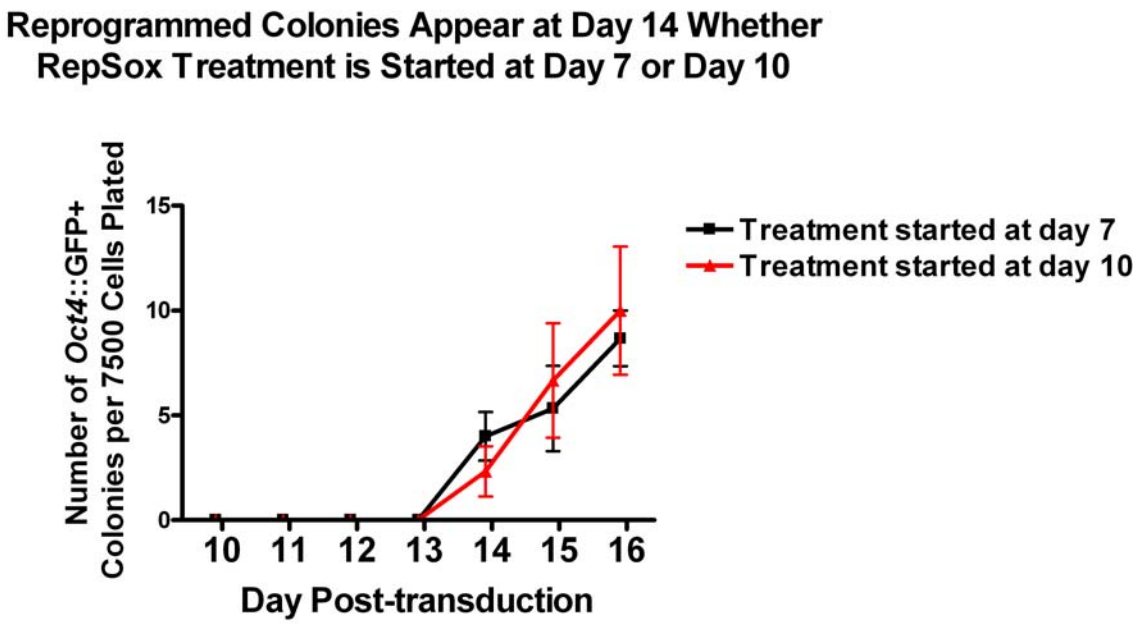


Figure S8. Stable *Oct4::*GFP-negative cell lines derived from *Oct4*::GFP negative colonies in *Oct4, Klf4, cMyc* and *Sox2*-infected MEF cultures can be reprogrammed by RepSox. *Oct4*::GFP-negative colonies (indicated by arrows) were picked at day 14 post-infection, propagated, treated with 25 μM RepSox for 48 hours at passage 4, and scored for *Oct4*::GFP+ colonies 12 days after RepSox

treatment. Scale bars in "P4 line + RepSox" panels = 500 μm, all other scale bars = 200 μm.

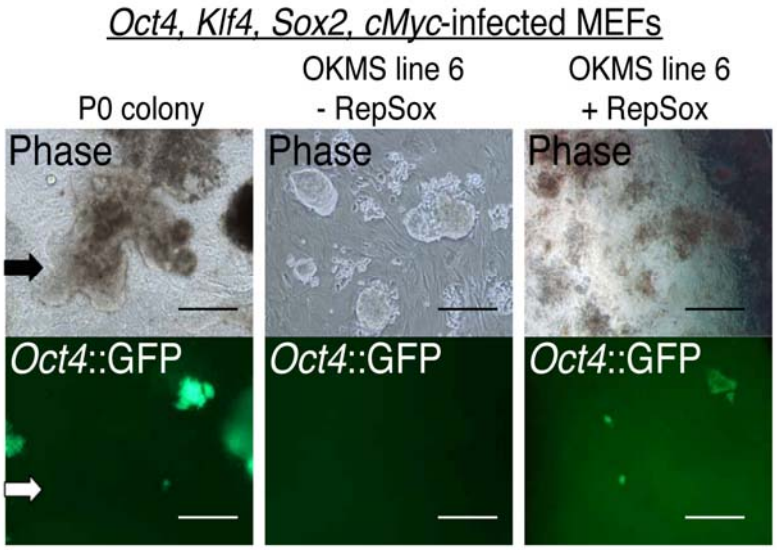


Figure S9. RepSox treatment of RepSox-responsive intermediate line OKMS 6 increases the expression of *Id1*, *Id2*, and *Id3*, genes that are repressed by Tgf-β signaling, but does not increase *Sox*-family gene expression, and shRNA-mediated knockdown of *Sox1* does not inhibit reprogramming with RepSox. (A) *Id* gene expression increases. Cells were treated with 0 or 25 μM RepSox for 48 hours before RNA was harvested and analyzed by microarray.

Shown are the fold-inductions of the genes with RepSox treatment versus without RepSox treatment. The error bars denote the standard error derived from quantification of three separate wells (of cells). **(B)** *Sox*-family gene expression does not increase. RepSox treatment for 10 hrs, 1 day, or 2 days is relative to untreated, time-matched controls. *Sox*-3, 4, 6, 8, 17, and 18 were included in the microarray but are not represented in the figure because they did not change significantly enough to generate a low enough P value. The error bars denote the standard error derived from quantification of three separate wells (of cells). **(C) .** *Oct4, Klf4, cMyc*-transduced MEFs were transduced once or twice with lentiviral particles encoding 5 different *Sox1*-specific shRNA constructs or an empty vector control and subjected to RepSox treatment (25 μM) in KSR mES media. KSOM MEFs = *Klf4, Sox2, Oct4,* and *cMyc*-transduced MEFs. The error bars denote the standard error derived from quantification of two separate wells (of cells).

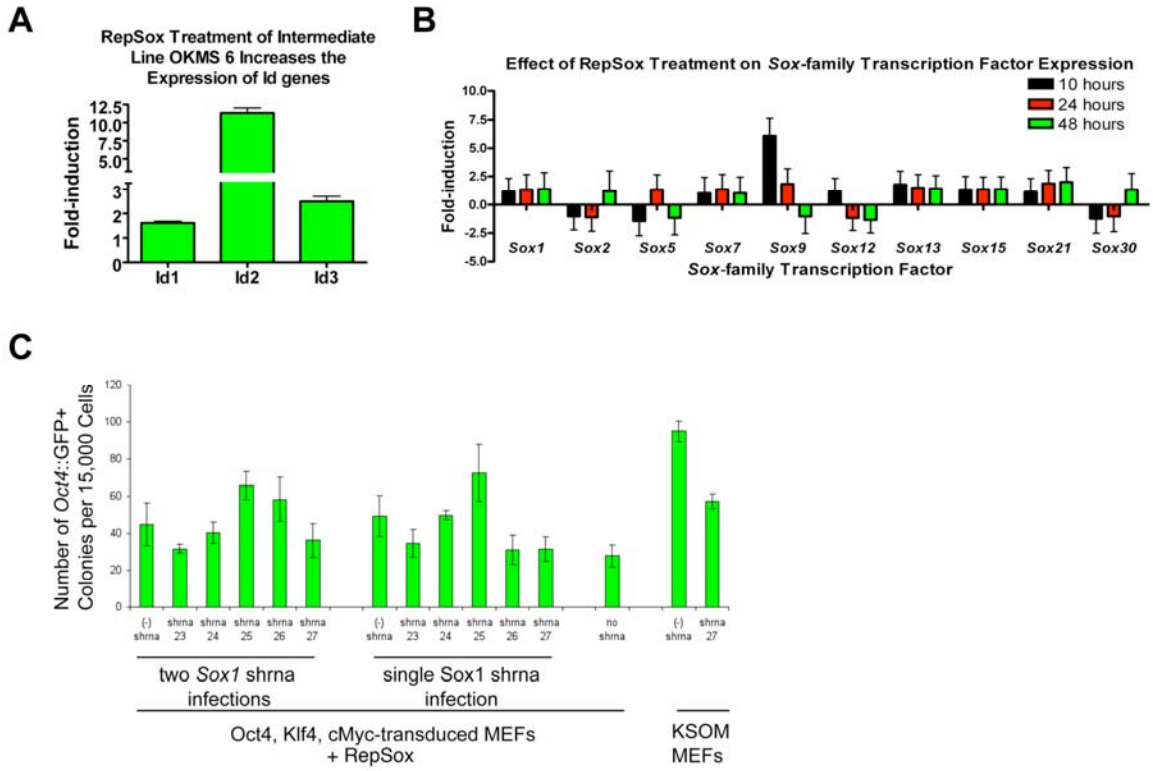


Figure S10. RepSox does not increase *Nanog* expression in non-RepSox-responsive intermediate lines OKMS 9 and OKM 9. Cells were treated with RepSox for 2 days in KSR mES media before RNA was harvested.

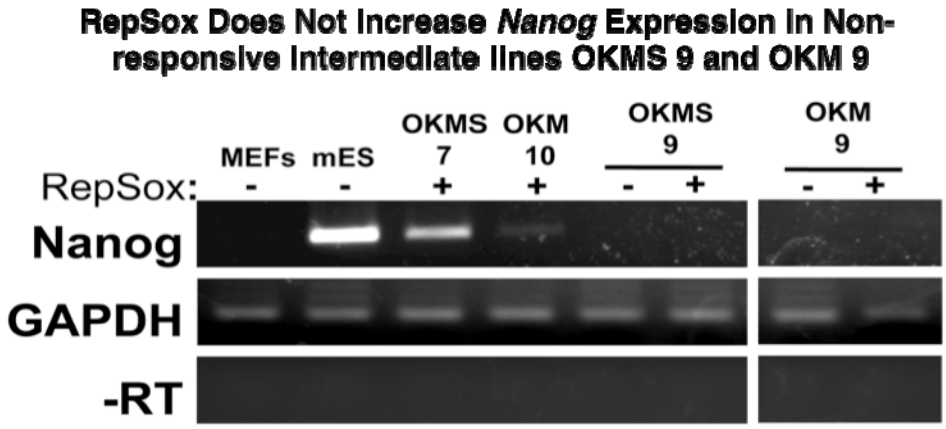
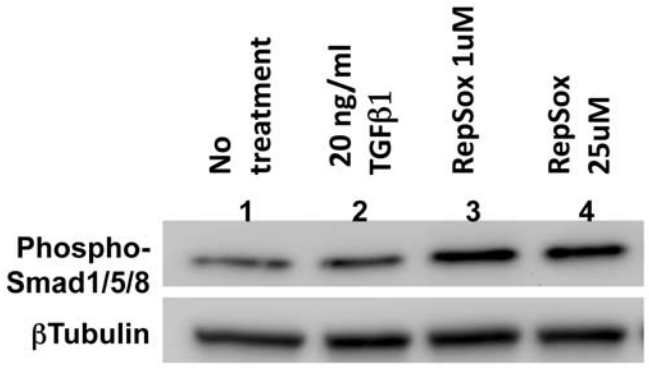


Figure S11. Bmp signaling increases in response to RepSox treatment. (A) Western blot for phospho-Smad1/5/8 shows an increase in the amount of the phosphorylated protein after a 48-hr RepSox treatment. (B) mRNA expression analysis shows that Bmp-3 levels increase upon RepSox treatment. Data are relative to untreated controls. The error bars denote the standard error derived from quantification of three separate wells (of cells).

A.



B.

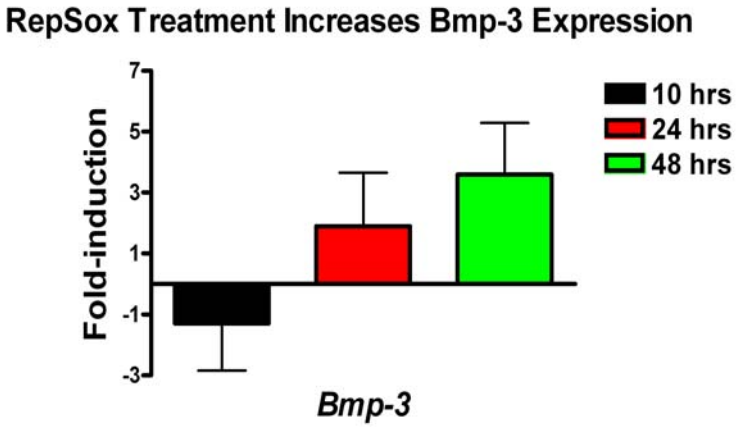
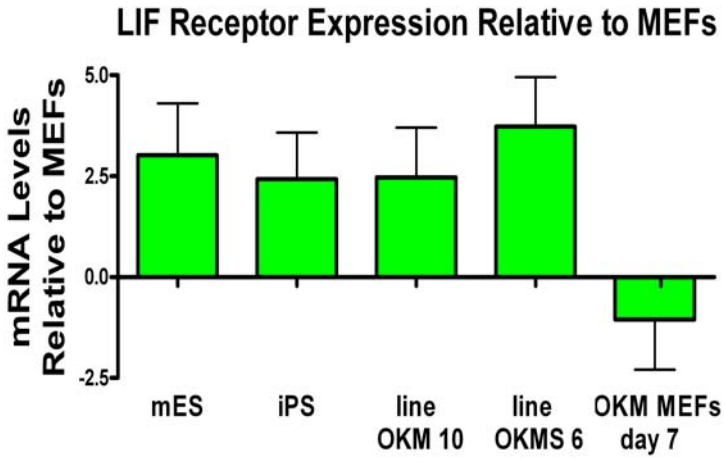


Figure S12. mRNA Expression analysis shows that non-pluripotent stable intermediate cell lines express the LIF receptor at the same level as mES cells, but freshly transduced MEFs do not, and MEFs do not upregulate *Nanog* significantly after RepSox treatment. (A) MEFs freshly infected with *Oct4, Klf4,* and *cMyc* (OKM MEFs day 7) express lower levels of the LIF receptor. The error bars denote the standard error derived from quantification of three separate wells (of cells). **(B)** *Nanog* mRNA levels in MEFs freshly transduced with *Oct4, Klf4,* and *cMyc* (within 7 days) do not increase upon RepSox treatment. The error bars denote the standard error derived from quantification of three separate wells (of cells).

A.



B.

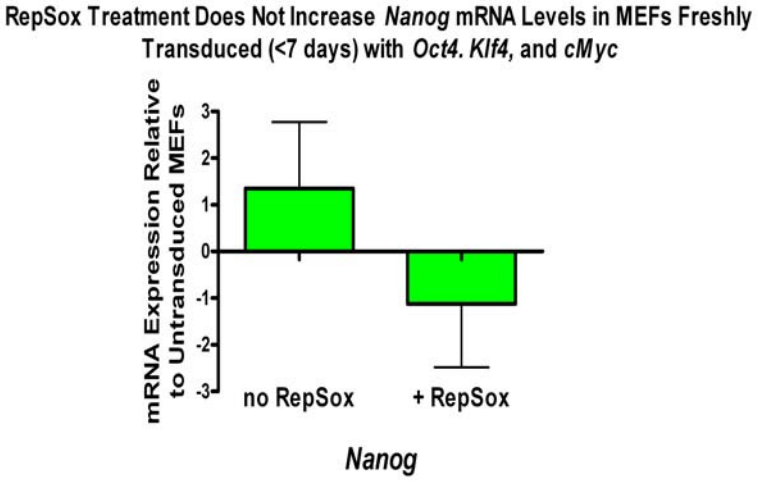


Figure S13. Cells from a line derived from MEFs transduced with *Oct4, Klf4, cMyc,* and *Nanog* (OKMN line 1) are iPS-like. **(A)** OKMN line 1 cells self-renew and remain *Oct4*::GFP+. Shown are cells at passage 5. Scale bars = 500 μm. **(B)** Immunocytochemistry showing that these cells strongly express *Sox2* from the endogenous allele. **(C)** QPCR analysis showing that these cells have activated expression of endogenous *Sox2, Oct4, Nanog,* and *Rex1*. The error bars denote the standard error derived from quantification of three separate wells (of cells). **(D)** QPCR analysis showing viral *Oct4, Klf4, and cMyc* are silenced but leaky expression from the *Nanog* transgene remains in OKMN line 1 cells. The error bars denote the standard error derived from quantification of three separate wells (of cells). **(E)** OKMN line 1 cells readily form embryoid bodies after 3 days in culture. Scale bar = 500 μM.

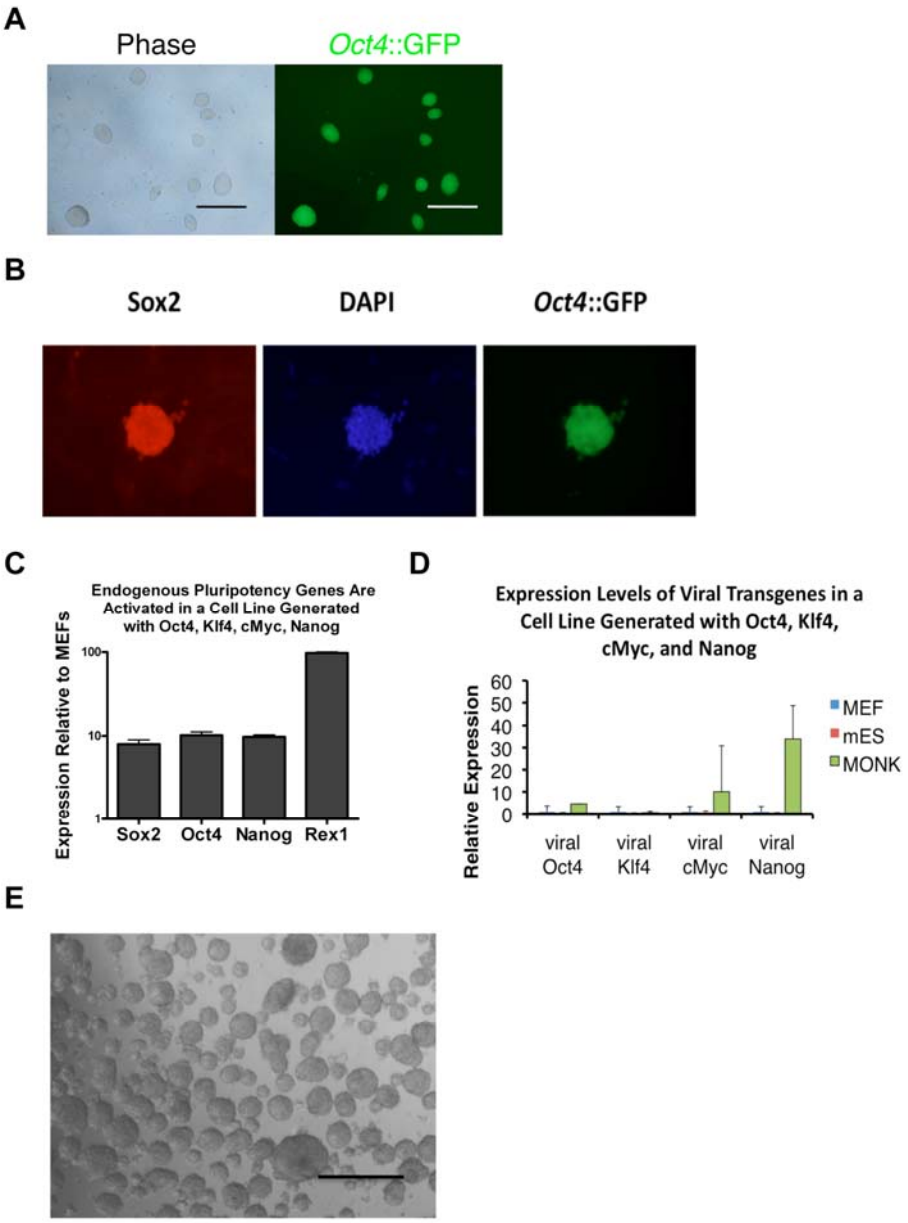


Table S1. Pearson correlation coefficients between mES cell lines, *Oct4, Klf4, cMyc,* and *Sox2* iPS line 1 (OKMS-iPS), *Oct4, Klf4,* and *cMyc* + RepSox iPS line 1 (OKM + RepSox), and *Oct4*::GFP MEFs (MEF).

Pearson (*R*)

	mES1 (R1)	mES2 (V6.5)	OKMS-iPS	OKM + RepSox	MEF
mES1 (R1)	1.00	0.96	0.98	0.96	0.80
mES2 (V6.5)		1.00	0.99	0.97	0.81
OKMS-iPS			1.00	0.97	0.82
OKM+RepSox				1.00	0.79
MEF					1.00

Table S2. *In vitro* assays of kinase inhibition activity show that RepSox does not inhibit the kinase targets of the 2i cocktail. Assays were performed in duplicate using the Z'-LYTE system (Invitrogen).

	Average % inhibition	Standard error
Mek1	3	1
Mek2	-4	1
Erk1	7	0
Erk2	-2	2
GSK-3β	1	0