

Reprogramming Factor Stoichiometry Influences the Epigenetic State and Biological Properties of Induced Pluripotent Stem Cells

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SUMMARY

We compared two genetically highly defined transgenic systems to identify parameters affecting reprogramming of somatic cells to a pluripotent state. Our results demonstrate that the level and stoichiometry of reprogramming factors during the reprogramming process strongly influence the resulting pluripotency of iPS cells. High expression of Oct4 and Klf4 combined with lower expression of c-Myc and Sox2 produced iPS cells that efficiently generated “all-iPSC mice” by tetraploid (4n) complementation, maintained normal imprinting at the *Dlk1-Dio3* locus, and did not create mice with tumors. Loss of imprinting (LOI) at the *Dlk1-Dio3* locus did not strictly correlate with reduced pluripotency though the efficiency of generating “all-iPSC mice” was diminished. Our data indicate that stoichiometry of reprogramming factors can influence epigenetic and biological properties of iPS cells. This concept complicates efforts to define a “generic” epigenetic state of iPSCs and ESCs and should be considered when comparing different iPS and ES cell lines.

INTRODUCTION

Direct reprogramming generates induced pluripotent stem cells (iPSCs) with the molecular profile and developmental potential of embryonic stem cells (ESCs); however, several studies have suggested that subtle differences in gene expression or chromatin modifications yield iPS cell lines with reduced pluripotency when compared to ESCs. Tissue-specific iPSCs have been reported to harbor “epigenetic memory,” which correlates with the ability to give rise to somatic cells of their tissue-of-origin more efficiently than other lineages (Kim et al., 2010; Polo et al., 2010; Ohi et al., 2011; Bar-Nur et al., 2011). Loss of imprinting (LOI) at the *Dlk1-Dio3* locus has been associated with lower pluripotency as seen by poor chimera formation and

loss of potential to generate “all-iPSC mice” in 4n complementation assays, the most stringent assay for pluripotency (Liu et al., 2010; Stadtfeld et al., 2010a). These data argued that incomplete reprogramming is a common outcome of factor-mediated reprogramming.

While the successful generation of all-iPSC mice suggests that at least some iPSC lines can pass the most stringent test of pluripotency (Boland et al., 2009; Kang et al., 2009; Zhao et al., 2009), it remains unclear how technical or biological conditions used to generate iPS cells influence the epigenetic and biological properties of iPSCs. Comparisons between ESCs and iPSCs often lack proper ESC controls and to date it has been difficult to simultaneously control variables in reprogramming experiments such as complete reactivation of endogenous pluripotency genes, basal reprogramming vector expression (Soldner et al., 2009), genetic background of donor cells, unique reprogramming factor cocktails (Yu et al., 2007), and distinct proviral integrations (Wernig et al., 2007).

In this study we used two transgenic systems that eliminate most of these variables in the generation of iPSCs and demonstrate that expression and stoichiometry of reprogramming factors influence the biological properties of iPS cells. Our data suggest the epigenetic state and pluripotency of iPSCs are influenced by unique and difficult-to-control parameters during direct reprogramming.

RESULTS

Derivation of Tissue-Specific iPSCs

To generate iPSCs under highly defined conditions from multiple somatic tissues of origin, we isolated cells from a previously published mouse model of factor-mediated reprogramming (Carey et al., 2010). These mice carry a single polycistronic vector carrying Oct4, Sox2, Klf4, and c-Myc (Carey et al., 2009) flanked by LoxP sites (in the following termed “Col1a1-OSKM”) under the control of a doxycycline-inducible promoter that was inserted into the collagen type 1 α locus (*Col1a1*) by gene targeting in ESCs expressing the reverse tetracycline inducible M2rtTA (rtTA) from the *ROSA26* promoter. Cells derived from four different somatic tissues including liver (Liv), CD19+ proB cells

(pB), whole Brain tissue (B), and tail-tip fibroblasts (TTF) were isolated and induced with DOX under optimized conditions for iPSC derivation as described previously (Figure 1A) (Carey et al., 2010). All iPSC lines stained positive for the pluripotency markers alkaline phosphatase (AP), SSEA-1, and Nanog (Figure 1B) and expressed pluripotency genes at levels similar to control ES cell lines as measured by qRT-PCR (Figure 1C). iPSC lines from each somatic lineage generated differentiated tissues of all three germ layers in teratoma assays (Figure S1A, available online). In addition, in vitro differentiation to mature neurons was performed on four iPSC lines and an ESC control, all of which generated Tuj1- and MAP2-2-positive neurons (Figure S1B). Factor-free iPSCs were isolated upon exposure to Cre recombinase with precise excision of the Col1a1-OSKM transgene confirmed by Southern blot (Figure 1D) and PCR analyses (Figure 1E). In agreement with previous studies all-iPSCs derived with the Col1a1-OSKM transgene generated teratomas and adult chimeras after blastocyst injection, most of which were shown to contribute to the germline (Carey et al., 2010) (Table S1).

The Majority of iPSC Cells Can Generate “All-iPSC” Mice

To determine whether Col1a1-OSKM iPSC lines could generate full-term “iPSC mice” we performed tetraploid (4n) complementation assays. Two ESC lines of genetically identical background to the iPSCs were used as control (Col1a1-OSKM ES cell lines #1B and #1C) and gave rise to live-born mice with efficiencies ranging from ~1.67% to 3%, respectively (Figure 1F). We injected all nine factor-free iPSCs that had been independently derived from different donor tissues into 4n blastocysts and obtained normal-appearing breathing E19.5 pups from seven out of nine lines with efficiencies ranging from 0.5% to 7.5% (avg. 2.2%) (Figure 1F), which is similar to ES and SCNT-ES cells as well as other reports with embryonic or adult fibroblast derived iPSC mice (Boland et al., 2009; Eakin et al., 2005; Eggan and Jaenisch, 2003; Kang et al., 2009; Stadtfeld et al., 2010a; Zhao et al., 2009). Most newborns were sacrificed after breathing was established while some gave rise to healthy pups that survived to adulthood (BC2 Ad.5), all of which were male and had a uniformly agouti coat color (Figure S1C). It has been shown previously that the presence of vectors carrying reprogramming factors affects the gene expression profile of iPS cells (Soldner et al., 2009); therefore, to determine whether the presence of the Col1a1-OSKM vector interfered with the potential to generate all-iPSC mice, we injected vector-containing parental iPSCs into 4n-blastocysts. As shown in Table S2, five out of the nine parental-vector-containing Col1a1-OSKM lines were able to produce all-iPSC mice (avg. 2%) (Figure S1D).

These data indicate that the majority of iPSCs isolated from different tissues were able to generate all-iPSC mice after injection into 4n blastocysts with efficiency similar to that of ES cells (2.5% and 2.1%, respectively) (Figure 1F and Table S3). Finally, aging of OSKM iPSC mice did not reveal elevated mortality due to tumors over a 16 month period of observation (Figure 1G) in contrast to “Col1a1-OKSM” transgenic mice (Stadtfeld et al., 2010b), which exhibit elevated mortality. The high tumor incidence in the latter strain may be due to IRES-mediated read-through from the Col1a1 promoter into the Col1a1-OKSM transgene leading to occasional c-Myc activation.

Silencing of the *Dlk1-Dio3* Locus Is Not an Absolute Marker of Reduced Pluripotency

Two recent reports found that LOI at the *Dlk1-Dio3* locus decreased the efficiency of iPS cell lines to produce high-grade chimeras and precluded the generation of all-iPSC mice by 4n complementation (Liu et al., 2010; Stadtfeld et al., 2010a). To determine whether this could faithfully predict the failure of iPSCs from our system to produce all-iPSC mice, we tested two maternally expressed imprinted RNAs, the noncoding RNA *Gtl2* (also known as *Meg3*) and the small nucleolar RNA *Rian*. Both genes were expressed at levels similar to ES cell controls (seven different ESC lines tested, data not shown) in a majority of lines (6/9), while a minority (3/9) exhibited reduced expression (Figure 2A). High-contribution chimeras were obtained from all lines exhibiting reduced *Gtl2* expression (Figure 2B). Col1a1-OSKM iPS cell lines were analyzed by Southern analysis with two methylation-sensitive restriction enzymes (HhaI and HpaII) to assess the methylation status of a control element for the imprinted *Dlk1-Dio3* locus, the intergenic germline-derived differentially methylated region (IG-DMR) (Lin et al., 2003; Zhou et al., 2010). Figure 2C demonstrates that two ES cell clones and three iPSC lines exhibiting ESC-like levels of *Gtl2* and *Rian* have both methylated and unmethylated IG-DMR alleles. In contrast, only methylated IG-DMR alleles were detected in two iPSC lines (Livp2E2, pBL4) with reduced levels of *Gtl2* and *Rian* expression, while the third line with reduced expression (BE1), did not show a gain in methylation at the CpGs tested by Southern. Thus, the majority of iPSC lines with repressed transcripts within the *Dlk1-Dio3* locus showed a gain of DNA methylation at CpGs within the IG-DMR locus, whereas ESCs and iPSCs with ESC-like expression did not.

To assess whether loss of imprinting (LOI) at the *Dlk1-Dio3* locus affected the developmental potential of the Col1a1-OSKM iPSCs, we performed 2n blastocyst injections to generate adult chimeras from all nine lines to analyze chimerism based on agouti coat color (n = 44), dividing the chimeras into two groups based on *Gtl2* expression (*Gtl2*-ON or *Gtl2*-LOW). All lines, independent of the status of *Dlk1-Dio3*, had variable contributions with no significant difference between the two groups (Figures 2D and 2E).

Our results differ from previous studies that demonstrated the predictive value of the imprinting state of the *Dlk1-Dio3* locus in determining the potential of iPSCs to produce all-iPSC mice (Figure 2F). Factor-free iPSC line pBL4 Ad.1 gave rise to all-iPSC mice (Table S1) and late-stage embryos with Southern blot analysis of the IG-DMR locus, confirming that these mice carried only methylated alleles (Figure 2G). We conclude that reduced expression of genes within the *Dlk1-Dio3* locus does not strictly correlate with reduced pluripotency in iPSCs.

Stoichiometry of Reprogramming Factors Influences the Genetic and Epigenetic State of the *Dlk1-Dio3* Locus

The Col1a1-OSKM reprogrammable mouse strain is based upon a genetically highly defined and almost identical transgenic system described previously (Stadtfeld et al., 2010a, 2010b). Both strains use a DOX-inducible polycistronic vector inserted into the *Col1a1* locus that was activated by the M2rtTA inserted into the *ROSA26* locus. Figure 3A shows the three differences in design of the two vectors inserted into the *Col1a1* locus in the

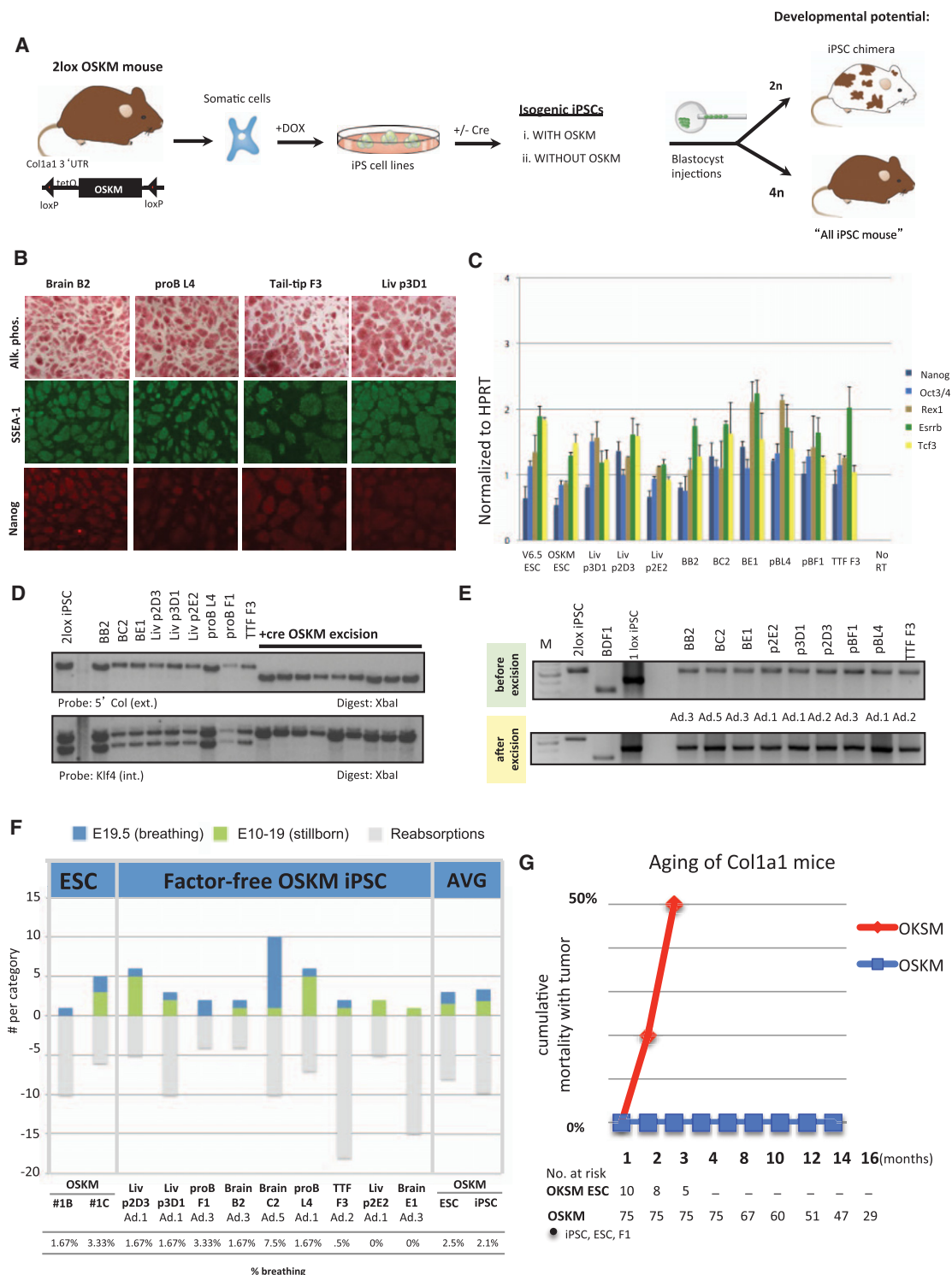


Figure 1. The Majority of iPS Cells Can Generate "All-iPSC" Mice

(A) Experimental scheme describing the Col1a1 2lox OSKM mouse strain for deriving iPSCs.

(B) Immunostaining for alkaline phosphatase (Alk. Phos.), SSEA1, and Nanog on iPS cell lines derived from four adult tissues.

(C) qRT-PCR of pluripotency genes in Col1a1 2lox OSKM iPSCs and control ES cell lines. Relative expression levels normalized to the housekeeping gene HPRT. Error bars represent standard deviation of triplicate reactions.

(D) Southern analysis of iPSCs obtained from sorted (GFP+) single iPSCs after transduction with adenovirus carrying Cre-recombinase. DNA was digested with XbaI and probed for correct targeting using a 5' Col1a1 probe and lack of signal with an internal probe using Klf4. Same indicated iPSC lines are loaded in +Cre OSKM excision (right).

OSKM and OKSM mice: (1) OKSM instead of OSKM; (2) F2A instead P2A; and (3) IRES instead T2A.

To investigate the basis for the different quality of iPSCs derived from each system we first compared the basal and DOX-induced level of factor expression. Single-copy transgenic MEFs from Col1a1-OSKM or Col1a1-OKSM mice were cultured in the presence or absence of DOX with transgene RNA levels analyzed by qRT-PCR using primers for Oct4 and Sox2 cDNA. Neither system showed significant differences in transgene RNA levels prior to or upon induction (Figure S2A). In addition, we observed that the number of copies of either the Col1a1-OSKM or the ROSA26 M2rtTA transgene did not affect the *Dlk1-Dio3* locus gene expression state (“ON” versus “LOW”) in resulting iPSCs, the majority of which show ESC-like expression and no difference between lines derived from mice heterozygous or homozygous at either locus (Figure S2B).

We next tested protein levels in Col1a1-OSKM and Col1a1-OKSM MEFs induced with DOX and both derived from the same gene-targeted parental V6.5 ES cell lines. Western analysis in Figure 3A shows that reprogramming factor protein levels were significantly different between the two strains. We observed higher protein levels of Oct4 and Klf4 in Col1a1-OSKM MEFs and lower levels of Sox2 and c-Myc than in those of similarly induced Col1a1-OKSM MEFs. Quantification of band intensities after normalization to the loading control indicated ~5- and 15-fold-higher protein levels of Oct4 and Klf4 and about 2-fold-lower protein levels of Sox2 and c-Myc in Col1a1-OSKM as compared to Col1a1-OKSM MEFs (Figure S2C). The specific concomitant accumulation of unprocessed high-molecular-weight polypeptides was consistent with inefficient processing to mature functional Oct4 and Klf4 proteins in Col1a1-OKSM cells (* in Figure 3A), an effect attributable to the F2A or P2A peptide variants used in the two vectors (Figure S2D). These results demonstrate that subtle differences in vector construction have a significant impact on protein expression.

To examine whether an increased expression of reprogramming factor protein levels could affect the epigenetic state of the iPSCs, we analyzed IG-DMR methylation in iPSCs created by induction of the Col1a1-OSKM transgene alone or by additional ectopic expression of Oct4 or Klf4 or of both factors using DOX-inducible lentiviruses. For this we derived tertiary MEFs from a single Gtl2-ON secondary iPSC line “BC2” by injection into 2n blastocysts and initiated reprogramming to generate stable DOX-independent iPSCs. Upon isolation of DOX-independent iPSCs DNA was isolated and analyzed by Southern blot to determine the extent of aberrant DNA methylation at the *Dlk1-Dio3* IG-DMR. Aberrant methylation occurred in about 43% of OSKM, OSKM+O, and OSKM+K MEFs. However, combined ectopic expression of Oct4 and Klf4 significantly reduced

aberrant methylation to ~15% of the tested iPSC lines (OSKM+O+K; $p = 0.0441$) (Figure 3B, Figure S3A), suggesting that the level of Oct4 and Klf4 expression during reprogramming can influence the resulting epigenetic conformation at the IG-DMR locus.

We also tested whether a change in factor level(s) could rescue the consistent aberrant silencing of the *Dlk1-Dio3* locus in the Col1a1-OKSM MEFs, which have been reported to consistently give rise to Gtl2-LOW iPSCs (Stadtfield et al., 2010a). ESC-derived secondary Col1a1-OKSM MEFs were infected with different combinations of DOX-inducible lentiviruses carrying the pluripotency factors Oct4 (O), Klf4 (K), and Nanog (N) with robust protein expression being observed by western blot (Figure 3C). Eight to ten DOX-independent iPSC lines were isolated from each condition after 2 to 3 weeks and expression of *Gtl2* was analyzed by RT-PCR. Of 57 clones analyzed from five separate conditions, four clones transduced with Oct4 and Klf4 showed ESC-like expression (O+K #12, 2.14, 2.29, 2.31) and normal methylation of the IG-DMR locus (Figure 3D; Figure S3B) while all other conditions gave rise to clones with reduced *Gtl2* between 0.5- and 0.1-fold normalized expression to ESCs (Figure S3C). Thus 22% (4/18) of the OKSM O+K clones had preserved normal imprinting of the *Dlk1-Dio3* locus and expressed *Gtl2* at levels similar to OSKM iPSCs.

In agreement with *Gtl2* expression being a predictor of 4n competence, the Col1a1-OKSM O+K #12 iPSC clone, carrying one and two proviral integrations of *Oct4* and *Klf4*, respectively (data not shown), gave rise to adult chimeras and all-iPSC mice when injected into 2n and 4n blastocyst embryos (Figures 3E and 3F), with PCR genotyping verifying the origin of the Col1a1-OKSM O+K #12 all-iPSC mouse (Figure S3D). These data confirm that ectopic expression of Oct4 and Klf4 in secondary MEFs can generate high-quality iPSCs even on a genetic background of OKSM, which generates mostly iPSCs with LOI and decreased pluripotency (Stadtfield et al., 2010a).

Isolation of Gtl2-ON and Fully Pluripotent Cells following Stoichiometric Correction

Our data predict that preselection for optimal stoichiometry should further increase normal imprinting at the *Dlk1-Dio3* locus in iPSCs. To this end we generated a “tertiary” Col1a1-OKSM transgenic system carrying identical *Oct4* and *Klf4* proviral integrations as a parental secondary cell line by injecting iPSC clone #12 into 2n blastocysts to generate tertiary Col1a1-OKSM O+K #12 MEFs (Figure 4A). DOX addition induced robust expression of proviral Oct4 and Klf4 as detected by western blot (Figure 4B).

Ectopic Oct4 and Klf4 expression in the tertiary system greatly altered the growth and proliferative characteristics of reprogramming MEFs typically observed using the Col1a1-OKSM strain (Figure 4C). Colonies were picked from multiple independent

(E) PCR genotyping of Col1a1 2lox OSKM iPSCs pre- and postexcision of reprogramming factor cassette. Homozygous targeted Col1a1 allele give rise to a band size ~550 bp whereas untargeted allele is ~300 bp. 1lox-Col1a1 after excision shows a slight higher band ~350–400 bp. Ad, adenovirus.

(F) Summary of 4n blastocyst injections for Col1a1-OKSM ESCs and factor-free Col1a1-OKSM iPSCs. Each cell line is indicated below along with relative efficiency (% breathing is relative to total blastocysts transferred, see Table S2). All data were collected at 19.5 d.p.c. Blue bars: pups that established breathing; green bars: development E10–19, stillborn; gray bars: reabsorbed. For summary see Table S2.

(G) Aging of Col1a1 mice. y axis is % mortality of mouse cohort due to tumors (relative to total at 1 month) for each timepoint. x axis shows time (in months). “No. at risk” below x axis indicates total mice analyzed at each time point. To control for different ages all mice are normalized by time from birth. Few mortalities in the OSKM strain were due to extreme dermatitis or hernia (not shown). Blue line indicates mice from Col1a1-OSKM strain including iPSC chimeras (both factor-containing and factor-free) in addition to OSKM iPSC F1 mice ($n = 10$) and ESC chimeras ($n = 4$). Red line indicates ESC-derived chimeric mice from Col1a1-OKSM strain.

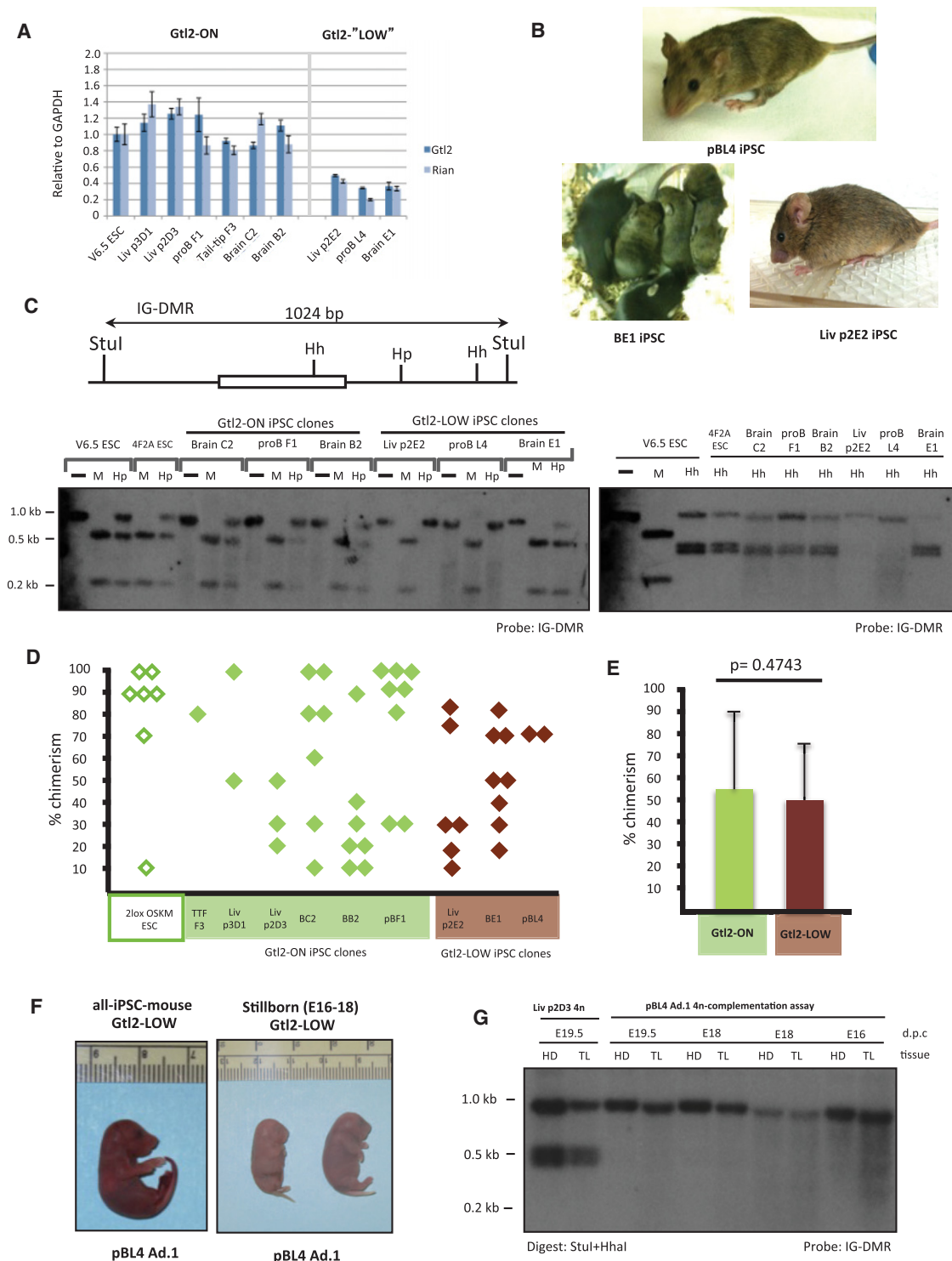


Figure 2. Silencing of the *Dlk1-Dio3* Locus Is Not an Absolute Marker of Reduced Pluripotency

(A) qRT-PCR of nine Col1a1 2lox OSKM iPSCs and one ES cell control (V6.5) of *Gtl2* and *Rian*. Expression levels are relative to GAPDH. "Gtl2-LOW" is represented in iPS lines with < 50% expression of both genes (right). Error bars indicated standard error of the mean of three biological replicates (SEM). Expression in additional iPSCs are shown in Figure S2B.

(B) Bright-field pictures of Col1a1 2lox OSKM chimeras from Gtl2-LOW iPSCs.

(C) Methylation analysis of IG-DMR in Col1a1 2lox OSKM iPSC lines. All DNAs were digested with *StuI* in combination with either methylation-insensitive *MspI* or methylation-sensitive *HpaII* or *HhaI*. The digested DNAs were analyzed by Southern hybridization with a probe to IG-DMR. Restriction enzymes: M, *MspI*; Hp, *HpaII*; Hh, *HhaI*.

wells on day 8 and expanded to isolate DOX-independent cell lines, which stained positive for pluripotency markers Nanog and SSEA1 (Figure 4D). To determine the silencing at the *Dlk1-Dio3* locus, we isolated RNA and tested for expression of *Gtl2* by RT-PCR. Out of 41 iPSC lines analyzed from two independent experiments, 23 (~56%) exhibited *Gtl2* expression similar to the parental Col1a1-OKSM O+K #12 secondary iPSC line, with the remaining samples ranging from 0.5- to 0.1-fold relative to GAPDH, similar to Col1a1-OKSM iPSCs (Figure 4E and Figure S4A). To determine the epigenetic state of the *Dlk1-Dio3* locus, 20 randomly selected iPSC lines were analyzed for DNA methylation at the IG-DMR by Southern blot using two methylation-sensitive digests (HpaII and HhaI). ESC-like expression of *Gtl2* correlated with normal DNA methylation whereas reduced expression showed aberrant DNA methylation at the IG-DMR (Figure 4F and data not shown). The proportion of O+K #12 tertiary iPSCs exhibiting normal DNA methylation (50%) is consistent with the proportion exhibiting normal *Gtl2* expression (55% of total). We verified the pluripotency of early passage (p3) OKSM O+K #12 tertiary iPSCs in 4n complementation assays by generating all-iPSC mice from *Gtl2*-ON clones #12.21 and 12.1.2 (~2.8%–5.2%) (Figure 4H and Figure S4B). PCR genotyping of tissues from all-iPSC mice confirmed the origin from tertiary Col1a1-OKSM O+K #12 iPSCs (Figure S4C).

To test whether differentiated somatic cells of blood origin could also be rescued by ectopic expression of Oct4 and Klf4 we isolated CD11b+ macrophages from spleen of an adult Col1a1-OKSM O+K #12 chimera. In contrast to fibroblasts, blood cells show very low levels of *Dlk1-Dio3* transcripts (Stadtfeld et al., 2010a). After isolation of tertiary blood-iPSCs we found that, similar to MEF-derived iPSCs, 6/12 exhibited ESC-like expression of *Gtl2* (Figure 4G). These observations are consistent with the notion that reprogramming of Col1a1-OKSM somatic cells transduced with Oct4 and Klf4 generates a similar fraction of iPSCs that maintain normal *Dlk1-Dio3* locus expression as iPSCs derived from Col1a1-OSKM somatic cells.

As a control we characterized another Col1a1-OKSM tertiary reprogramming system carrying only ectopic *Klf4* (K #1) (Figure S4D) and found that six tertiary MEF-derived iPSC clones exhibited reduced *Gtl2* expression (Figure S4E). We also sought to determine whether additional epigenetic changes correlated with the *Gtl2* expression status or 4n competence in Col1a1-OKSM and Col1a1-OSKM iPSCs by comparing the methylation of regions associated with epigenetic memory (Kim et al., 2010). Bisulfite sequencing was used to determine the methylation of four loci (*Gcnt2*, *Kcnrg*, *Cd37*, and *Slc32a1*) in five fibroblast- and four blood-derived iPSCs. Figure S4F shows that no consistent DNA methylation differences were found between *Gtl2*-ON and *Gtl2*-LOW lines derived from either Col1a1-OKSM

or -OSKM mice. One Col1a1-OKSM O+K #12 tertiary MEF iPSC line (#12.1.2) had acquired some aberrant DNA methylation within the *Kcnrg* and *Gcnt2* regions analyzed but this did not preclude the ability to give rise to “all-iPSC” mice (Figure S4B). Thus, the methylation state of loci that are differentially modified in iPSCs derived from different donor cell types did not correlate with *Dlk1-Dio3*-LOI or 4n competence.

DISCUSSION

The main finding of our study is that the expression level and stoichiometry of reprogramming factors used to generate iPSCs play an important role in determining the epigenetic and pluripotent state of iPS cells (Table S4). This conclusion is based on the comparison of two genetically highly defined transgenic systems, both of which used an identical strategy to activate reprogramming factor expression, thus eliminating the variables inherent in conventional vector-mediated iPSC generation. Importantly, while factor transcription was equivalent in both systems, subtle differences in vector design (choice of different 2A peptides and the use of an IRES instead of a 2A sequence; Figure 3A) caused a significant difference in the stoichiometry and total factor protein level. High Oct4 and Klf4 combined with lower Sox2 and c-Myc expression in the Col1a1-OSKM mice was more optimal for generating high-quality iPS cells than in Col1a1-OKSM mice. In further support of this conclusion we found that the number of copies of the Col1a1-OSKM transgene or M2rTA did not correlate with *Dlk1-Dio3* LOI in Col1a1-OSKM iPSCs (Figure S2B), which is consistent with the notion that factor stoichiometry, rather than absolute factor expression levels, may be important.

The distinct factor expression levels of OSKM and OKSM reprogrammable mice strains emphasize that different factor stoichiometries can produce iPSCs but that their quality may vary. The more favorable vector design in OSKM mice significantly reduced the proportion of iPSC lines exhibiting LOI at the imprinted *Dlk1-Dio3* locus and robustly increased the proportion of iPSC lines with ESC-like pluripotency (e.g., 4n competence). Furthermore, aberrant DNA methylation within the *Dlk1-Dio3* locus did not, in contrast to previous observations, preclude the generation of all-iPSC mice by tetraploid complementation although the efficiency of generating mice by 4n complementation decreased with aberrant methylation at this locus. Nonetheless, our data are consistent with LOI at the *Dlk1-Dio3* locus being a criterion for low-quality iPS cells.

All somatic cells have the potential to generate iPS cells but reprogramming is an inefficient and stochastic process with long latency (Hanna et al., 2009). While factor expression in OSKM mice was more favorable for high-quality iPS cell

(D) Quantification of chimeras generated from Col1a1 2lox OSKM strain using whole-body agouti coat color. Open green diamonds, chimerism of adult mice from parental V6.5 Col1a1 2lox OSKM ESCs; closed green diamonds, iPSCs with ESC-like expression of *Gtl2* and *Rian* (*Gtl2*-ON); brown closed diamonds, iPSCs with repressed expression of *Gtl2* and *Rian* (*Gtl2*-LOW).

(E) Average chimerism for 44 mice analyzed from Col1a1 2lox OSKM iPSCs. *Gtl2*-ON (57%); *Gtl2*-LOW (50%). Error bars indicate standard deviations. p value, Student's t test = 0.4743.

(F) Bright-field image of live-born (E19.5) all-iPSC mouse from the *Gtl2*-LOW iPSC line pBL4 Ad.1. For summary see Table S3.

(G) Methylation of IG-DMR (*Stul*+*HhaI*) in 4n mice and embryos using Southern blot protocol described above. Only unmethylated bands were observed from embryos and mice from iPSC line pBL4 Ad.1. As a control DNA of a live-born pup generated from the *Livp2D3* iPSC line shows both methylated and unmethylated banding patterns. HD, head; TL, tail and limbs.

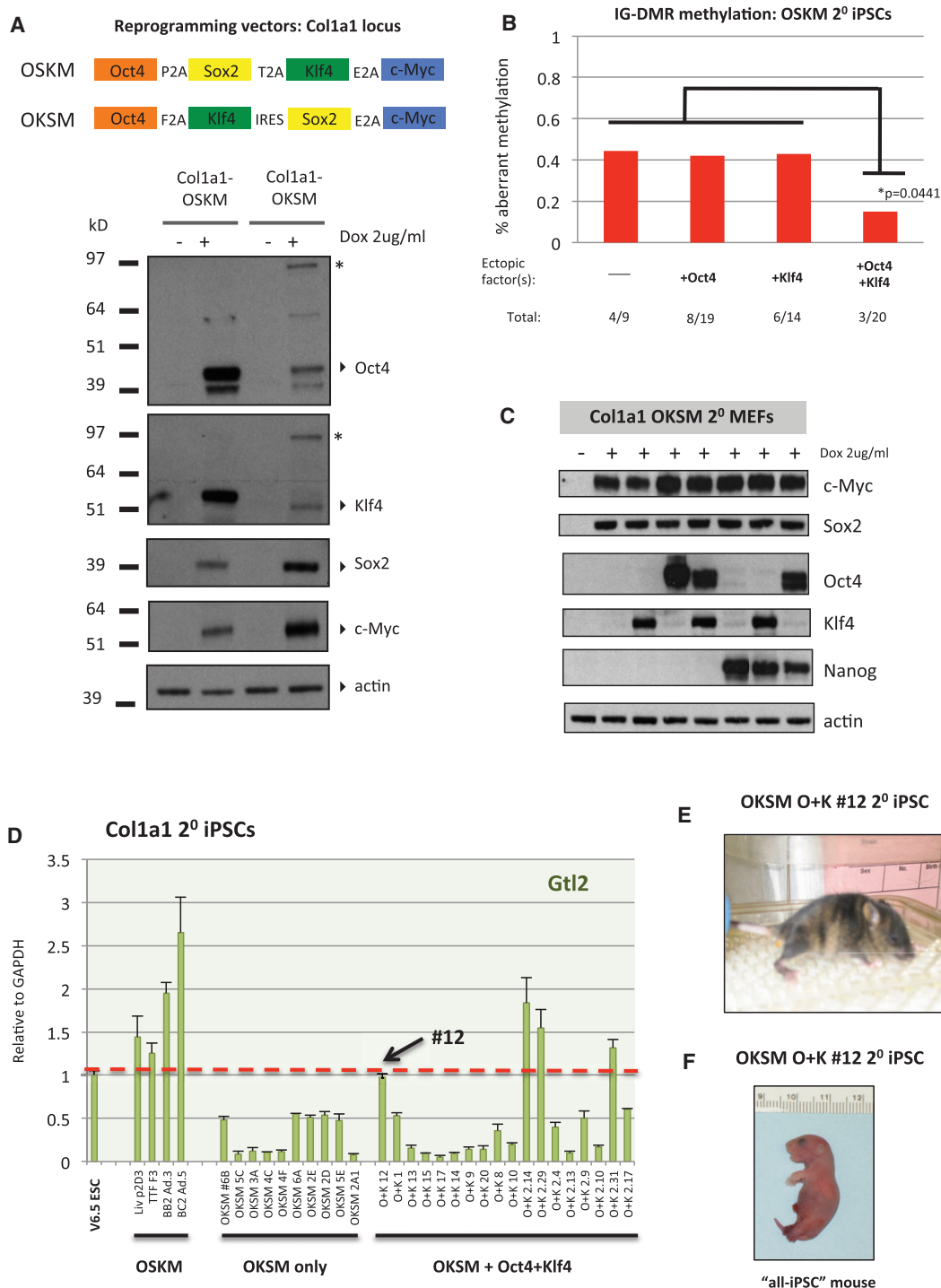


Figure 3. Stoichiometry during Reprogramming Influences the Genetic and Epigenetic State of the *Dlk1-Dio3* Locus

(A) (Top) Scheme of the two polycistronic vectors inserted into the *Col1a1* gene in the OKSM and the OKSM mouse strains, respectively. Note the unique 2A-peptides (F2A versus P2A), the IRES sequence present in OKSM, and the position of Sox2 and Klf4 in each cassette, respectively. (Below) Western blot analysis of reprogramming factor proteins upon induction with DOX in Col1a1 mouse embryonic fibroblasts. * indicates high molecular unprocessed precursor proteins for Oct4 and Klf4.

(B) Relative fraction of iPSCs recovered with aberrant DNA methylation as tested by Southern analysis at the IG-DMR region (StuI+HpaII digest). Secondary OSKM MEFs were reprogrammed with no additional factor (-), additional Oct4, Klf4, or both (+Oct4+Klf4). Total number of iPSCs recovered and examined in each condition indicated below. p value = fisher's exact t test.

(C) Western blot analysis of vector-mediated induction of additional pluripotency factors in Col1a1-OKSM secondary MEFs. Samples were harvested 48 hr after addition of DOX. Prolonged exposure shows Oct4 and Klf4 from the OKSM transgene (data not shown).

generation than in Col1a1-OKSM mice, it is probably not optimal as reflected in the higher incidence of normal imprinting at the *Dlk1-Dio3* locus when the Oct4 and Klf4 levels were further increased. In addition, the change of factor stoichiometry in Col1a1-OKSM mice by a vector-mediated increase of Oct4 and Klf4 partially rescued LOI at the *Dlk1-Dio3* locus (22% of the iPSCs had normal imprinting instead of none on the Col1a1-OKSM background) and resulted in 4n-competent iPSCs. When tertiary MEFs and adult blood cells were isolated from such a “rescued” Col1a1-OKSM iPSC clone and reprogrammed by DOX, ~50%–56% of the resulting tertiary iPS cells had normal imprinting of the *Dlk1-Dio3* locus, which supports the conclusion that a more optimal factor stoichiometry results in a reliably and consistently higher recovery of fully pluripotent iPS cells independent of their differentiation stage or tissue-of-origin.

The observation that Col1a1-OKSM iPSC mice did not develop tumors argues against the hypothesis that reprogramming is inherently tumorigenic. Recent analysis by Quinlan et al. utilizing whole-genome DNA sequencing on several viral-transduced iPSC lines displaying full developmental potential (e.g., 4n competence) found only a few (one or two) de novo genomic structural variants and did not find evidence for activation of endogenous retroelements during the acquisition of pluripotency (Quinlan et al., 2011). Whether 4n-competent iPSCs from the Col1a1-OKSM reprogrammable mouse strain show similar genomic stability will be an interesting area for future investigation.

A much-debated issue in the iPSC cell field is the question of how similar or different iPS cells are as compared to ES cells derived from naturally fertilized embryos or following somatic cell nuclear transfer (SCNT). While the transcriptional and epigenetic state of iPSCs is highly similar to ES cells (Bock et al., 2011; Guenther et al., 2010; Mikkelsen et al., 2008; Stadtfeld et al., 2010a), a number of recent studies have identified at least some genetic, epigenetic, and biological differences between iPS and ES cells though the functional consequences have not been clarified (Gore et al., 2011; Hussein et al., 2011; Lister et al., 2011). The results presented in this study argue that subtle and temporary differences in the level and stoichiometry of the reprogramming factors during early stages of iPSC formation can have irreversible consequences for the epigenetic state and the pluripotent potential of iPS cells. This is consistent with the notion that incomplete or imperfect reprogramming is not a fundamental biological problem of factor-induced iPSC generation but rather due to technical and often not well-controlled variables. Thus, comparisons between ES and iPS cells may often be influenced by idiosyncratic and difficult to control differences that occur during the generation of individual pluripotent cells, qualifying conclusions about generic differences between ES and iPS cells.

EXPERIMENTAL PROCEDURES

Somatic Cell Isolation and Culture

Organs were isolated from 6-week-old Col1a1 2lox OKSM transgenic male mice heterozygous for the *ROSA26* M2rtTA and homozygous at the *Col1a1*

locus harboring the 2lox OKSM cassette. The following isolation procedures were applied: brain was removed and plated immediately into ES cell medium with doxycycline (2 µg/ml); whole marrow and isolation of liver cells were performed as described previously (Carey et al., 2010); CD19+ pro-B cells or CD11b+ macrophages were isolated by MACS cell separation (Miltenyibiotec Cat# 130-052-201) following manufacturer's instructions; purified B cell subsets were resuspended in IMDM with 15% FCS as well as IL-4, IL-7, SCF (10 ng/ml each, Peprotech), and doxycycline (2 µg/ml) and plated on OP9 bone marrow stromal cells (ATCC). Three days later the medium was changed to ESC medium plus DOX.

Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) or RNasy kit (QIAGEN). Five micrograms of total RNA was treated with DNase I to remove potential contamination of genomic DNA using a DNA Free RNA kit (Zymo Research). One microgram of DNase I-treated RNA was reverse transcribed using a First Strand Synthesis kit (Invitrogen) and ultimately resuspended in 100 µl of water. Quantitative PCR analysis was performed in triplicate using 1/50 of the reverse transcription reaction in an ABI Prism 7000 (Applied Biosystems) with Platinum SYBR green qPCR SuperMix-UDG with ROX (Invitrogen). Equal loading was achieved by amplifying GAPDH mRNA and all reactions were performed in triplicate. Primers used for amplification were as follows: *Oct4* F, 5'-ACATCGCCAAATCAGCTTGG-3' R, 5'-AGAACCATACTCGAACCACATCC-3'; *Sox2* F, 5'-ACAGATGCAACCGATGCACC-3' R, 5'-TGGAGTTGACTGCAGGGCG-3'; *Nanog* F, 5'-AAGATGCGGACTGTGTCTC-3' R, 5'-CGCTTGCACTTCATCCTTTG-3'; *Zfp42 (Rex1)* F, 5'-CGAGTGGCAGTTTCTTCTTG-3' R, 5'-CTTCTTGAACAATGCCTATGACTCACTTCC-3'; *Esrrb* F, 5'-TTTCTGGAACCATGGAGAG-3' R, 5'-AGCCAGCACCTCTCTACA-3'; *Tcf3* F, 5'-TCTCAAGCCGTTCCACAC-3' R, 5'-TTTCCGGCAAGCTCATAGTATTT-3'; *OSKM (E2A-cMyc)* F, 5'-GGCTGGAGATGTTGAGAGCAA-3' R, 5'-AAAGGAATCCAGTGGCGC; *GAPDH* F, 5'-TTCACCACCATGGAGAAGGC-3' R, 5'-CCCTTTTGCTCCACCCT-3'; *HPRT* F, 5'-GCAGTACAGCCCAAAATGG-3' R, 5'-GGTCTTTTACCAGCAAGCT-3'; *Gli2* F, 5'-TTGCACATTTCTGTGGGAC-3' R, 5'-AAGCACCATGAGCCACTAGG-3'; *Rian* F, 5'-TCGAGACACAAAGGACTGC-3' R, 5'-ATTGGAAGTCTGAGCCATGG-3'; *Mirg* F, 5'-TTGACTCCAGAAGATGCTCC-3' R, 5'-CCTCAGTTCTTAAGCAAGG-3'.

Southern Blotting

For analysis of OSKM excision in iPSC lines 10 µg of XbaI digested genomic DNA was separated on a 0.7% agarose gel, transferred to a nylon membrane (Amersham). The membrane was prehybridized with hybridization buffer (1% bovine serum albumin in 7% SDS and 0.5M sodium phosphate, pH 7.5) at 65°C and hybridized with ³²P random primer (Stratagene) labeled probes for 5' Col1a1 probe (external) (Carey et al., 2010) and mouse Klf4 (full-length Klf4 cDNA). For analysis of IG-DMR, DNA was digested with StuI plus methylation-insensitive MspI or methylation-sensitive HpaII or HhaI. The blot was hybridized with probe M4 (IG-DMR) as previously described (Takada et al., 2002).

Western Blotting

Cell pellets were lysed on ice in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) for 30 min in presence of protease inhibitors (Roche Diagnostics), boiled for 5–7 min at 100°C, and subjected to western blot analysis. Primary antibodies: rabbit anti-Oct4 (1:1000, scH-134, Santa Cruz), rabbit anti-Sox2 (1:1000, #2748, Cell Signaling), goat anti-Klf4 (1:1000, AF3158, R&D Biosystems), rabbit anti-c-Myc (1:1000, #9402, Cell Signaling), and rabbit anti-actin (1:2000, A2066, Sigma), rabbit anti-Nanog (1:200, ab80892, Abcam), α-tubulin (1:10,000, # T-9026, Sigma). Blots were probed with anti-mouse, anti-goat, or anti-rabbit IgG-HRP secondary antibody (1:10,000) and visualized using ECL detection kit (GE Healthcare).

(D) Quantitative RT-PCR of Gli2 in secondary Col1a1 OKSM iPSCs generated without additional factors (OKSM only) or by additional vector-mediated transduction of Oct4 and Klf4 (OKSM + FUW-TetO Oct4 + Klf4). Four clones (#12, 2.14, 2.29, and 2.31) show ESC-like expression of Gli2. Error bars indicate standard deviation of triplicate reactions. Expression following other factor combinations is shown in Figure S3C.

(E) Adult chimera generated from secondary clone OKSM O+K #12 iPSC.

(F) 4n complementation assay using OKSM O+K #12 iPSC generated a live-born “all-iPSC” mouse.

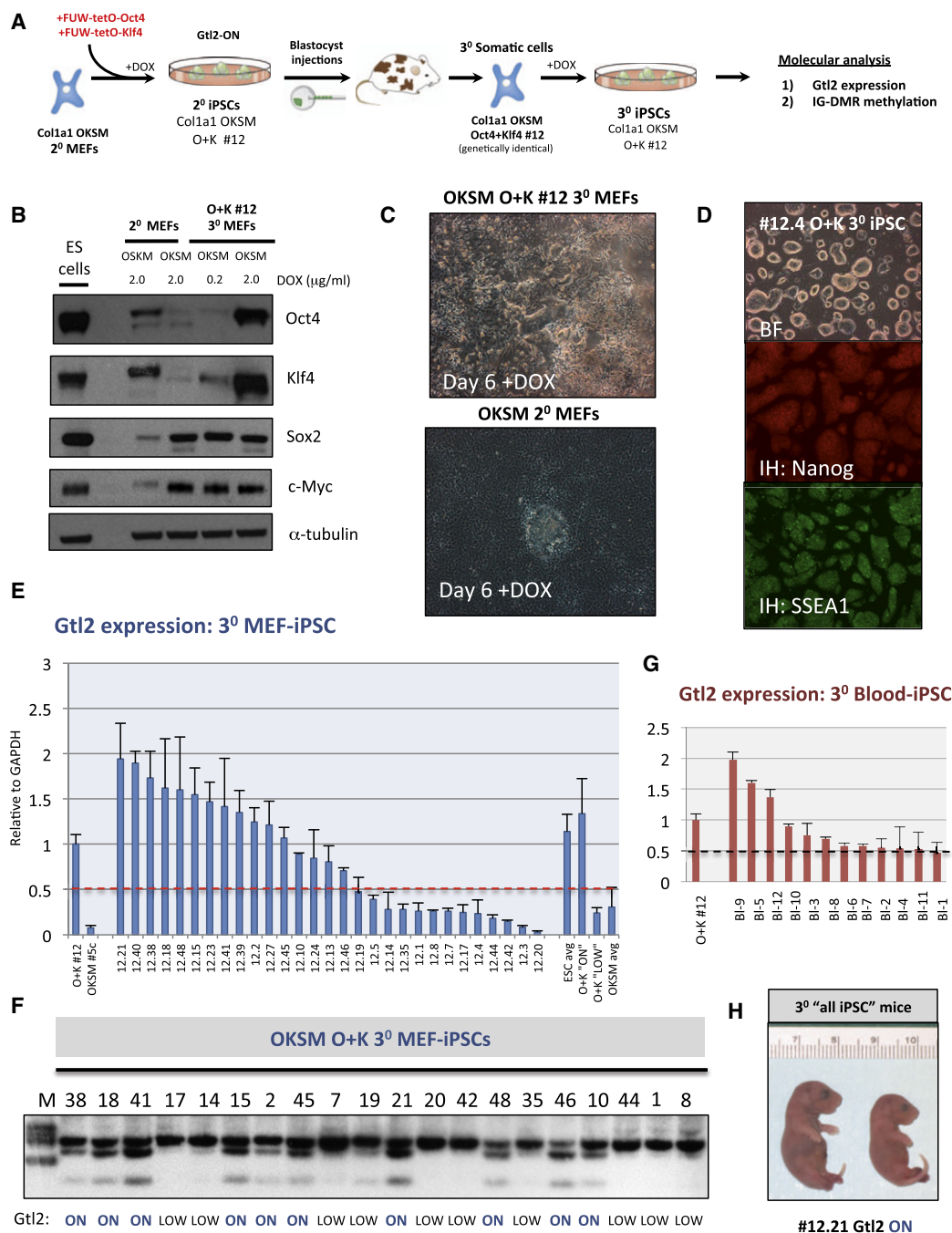


Figure 4. Isolation of Gtl2-ON and Fully Pluripotent iPSC Cells following Stoichiometric Correction

(A) Schematic for the generation of tertiary Col1a1 OKSM O+K system. Secondary OKSM O+K #12 iPSCs with normal Dlk1-Dio3 imprinting were injected into 2n blastocysts to obtain genetically identical "tertiary" OKSM O+K #12 MEFs. Addition of DOX reactivated both the Col1a1 OKSM transgene as well as the DOX-inducible Oct4 and Klf4 proviruses to obtain tertiary iPSCs, which were analyzed for Gtl2 expression and IG-DMR DNA methylation.

(B) Western blot analysis of ESCs as well as secondary and tertiary Col1a1 reprogramming systems from identical genetic backgrounds. Tertiary OKSM O+K #12 MEFs show robust reactivation of Oct4 and Klf4 proviruses upon addition of DOX media.

(C) Bright-field images of reprogramming tertiary OKSM O+K #12 MEFs after 6 days of DOX.

(D) Bright-field and immunofluorescence (IF) of pluripotency markers in tertiary OKSM O+K #12 iPSCs. Nanog (red) and SSEA1 (green).

(E) Quantitative RT-PCR of Gtl2 expression in 29 OKSM O+K #12 MEF-derived tertiary iPSCs. Sixteen out of twenty-nine show expression levels similar to parental OKSM O+K #12 secondary iPSCs. Dotted red line represents Gtl2-LOW cutoff. Error bars represent standard deviation of triplicate reactions (SD). Additional iPSCs are shown in Figure S4A.

(F) Representative Southern blot analysis of IG-DMR (Stul+HpaII digest) from tertiary OKSM O+K #12 iPSCs generated from MEFs. All clones with ESC-like expression show normal DNA methylation at the IG-DMR whereas clones with reduced expression show aberrant DNA methylation.

Mouse Blastocyst Injections and Teratoma Formation

All animal procedures were performed according to NIH guidelines and were approved by the Committee on Animal Care at MIT. All 2n and 4n injections were performed using B6D2F2 embryos as Col1a1 2lox OSKM iPSCs were derived from an agouti mouse and could be identified by coat color as adults. Diploid or tetraploid blastocysts (94–98 hr 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 μ m 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 ten injected blastocysts were transferred to each uterine horn of 2.5-day-postcoitum pseudopregnant B6D2F1 female. Pups were recovered at day 19.5 and fostered to lactating B6D2F1 mothers when necessary. Teratoma formation was performed by depositing 2×10^6 cells under the flanks of recipient SCID $^{-/-}$ mice. Tumors were isolated 3–6 weeks later for histological analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.stem.2011.11.003](https://doi.org/10.1016/j.stem.2011.11.003).

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(G) Quantitative RT-PCR of Gtl2 expression from 12 blood-derived (CD11b+) OKSM O+K #12 iPSCs. Dotted black line represents Gtl2-LOW cutoff. Error bars represent SD of triplicate reactions.

(H) 4n complementation assay on tertiary OKSM O+K #12.21 iPSC with normal ESC-like expression of Gtl2. Bright-field image shows 19.5 d.p.c live-born pups. An additional “all-iPSC” pup is shown in Figure S4B.

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