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THE LEVELS OF REPROGRAMMING FACTORS INFLUENCE THE INDUCTION AND MAINTENANCE OF PLURIPOTENCY: THE CASE OF CD1 MOUSE STRAIN CELLS

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ABBREVIATIONS

AP, alkaline phosphatase

Dox, tetracycline analogue doxycycline

ESC, embryonic stem cell

iPSC, induced pluripotent stem cell

MEF, mouse embryonic fibroblast

MKOS, Myc, Klf4, Oct4, Sox2 polycistronic transgene

PGRN, pluripotency gene regulatory network

pPGK, Phosphoglycerate kinase 1 gene promoter

PSC, pluripotent stem cell

pTRE, tetracycline responsive promoter

rtTA, reverse Dox-dependent transactivator

2i medium, medium supplemented with MEK and GSK3β inhibitors
ABSTRACT

The amount of proteins of the regulatory pluripotency network can be determinant for somatic cell reprogramming into induced pluripotent stem cells (iPSCs) as well as for the maintenance of pluripotent stem cells (PSCs). Here we report a transposon-based reprogramming system (PB-Booster) that allowed high expression levels of a polycistronic transgene containing *Myc, Klf4, Oct4* and *Sox2* (*MKOS*) and showed increased reprogramming efficiency of fresh mouse embryonic fibroblasts (MEFs) into iPSCs under low but not under high *MKOS* expression levels. In contrast, MEFs after 2 passages derived into similar number of iPSC colonies than fresh MEFs at high MKOS dose but this number was reduced at low MKOS dose. Timing of reprogramming was not affected by *MKOS* expression levels but, importantly, exogenous *MKOS* expression in established PSCs caused a significant cell loss. At high but not at low *MKOS* expression levels, MEFs of the CD1 strain produced more initial cell clusters than iPSCs and, although reprogrammed at a similar efficiency as MEFs of the 129/Sv strain, iPSCs could not be maintained in the absence of exogenous *MKOS*. In CD1-iPSCs, *Oct4, Nanog, Rex1* and *Esrrb* expression levels were reduced when compared with the levels in PSCs derived from the 129/Sv strain. Culture of CD1-iPSCs in medium with MEK and GSK3β inhibitors allowed their self-renewal in the absence of exogenous *MKOS*, but the expression levels of *Oct4, Nanog, Rex1* and *Esrrb* were only partially increased. Despite the reduced levels of those pluripotency factors, CD1-iPSC kept high capacity for contribution to chimeric mouse embryos. Therefore, levels of regulatory pluripotency factors influence reprogramming initiation and PSC maintenance *in vitro* without affecting their differentiation potential *in vivo*. 
INTRODUCTION

In the last two decades, significant advances have occurred in the understanding of factors that regulate pluripotency. Recently, a significant number of those factors have been identified and their interactions defined in what is presently known as the pluripotency gene regulatory network (PGRN) (Li and Belmonte, 2018). This intrinsic regulatory network is influenced by a variety of extrinsic factors that maintain the PGRN in balance under particular environmental conditions (Papatsenko et al., 2018).

Although some components of the PGRN have shown to be essential for pluripotency maintenance, the precise stoichiometry among them has not been defined. Thus, it is possible that the PGRN could be disrupted due to very high or very low amount of its components. Interestingly, an apparently non-functional PGRN in embryonic stem cells (ESCs) derived from some species (e.g., rat; Buehr et al., 2008) or some mouse strains (Nichols et al., 2009; Ohtsuka and Niwa, 2015) under regular culture conditions can be overcome when a MEK and a GSK3β inhibitors are added to the medium (i.e., 2i medium).

Somatic cell reprogramming mediated by a combination of transcription factors has not only increased the number of cell sources for cell type- and patient-specific therapeutic purposes, but also revealed the significant plasticity of the genome of differentiated cells (Brumbaugh et al., 2019; Hochedlinger and Jaenisch, 2015). In particular, the induction of pluripotency by a combination of transcription factors (e.g., Oct4, Sox2, Klf4 and Myc) has shown that the genetic network of a differentiated cell can be disrupted and cause epigenetic modifications driven by both, the forced expression of exogenous genes encoding transcription factors and the culture
conditions (Hochedlinger and Jaenisch, 2015). The derivation of induced pluripotent stem cells (iPSC) using the latter procedure is highly reproducible, however, only a limited number of cells succeed in reaching a stable pluripotent state. Presently, although some barriers that prevent reprogramming have been identified (Vierbuchen and Wernig, 2012; Xu et al., 2016), including epigenetic modulators and cell cycle checkpoints, it is apparent that some can be overcome, at least partially, by increasing the expression of reprogramming factors (Carey et al., 2010; Polo et al., 2012; Sebban and Buganim, 2016; Stadtfeld et al., 2009; Wernig et al., 2008).

Here we report a reprogramming transposon vector with the ability to express high levels of an *MKOS* polycistronic transgene. Using this transposon, we explored the reprogramming capacity of mouse embryonic fibroblasts (MEFs) and the effect on the maintenance of PSCs from the CD1 outbreed strain. Our data show that iPSC derived from CD1 MEFs cannot retain pluripotency under standard culture conditions which relates to low expression levels of some genes of the PGRN.

**RESULTS**

**Induced rtTA expression allows high MKOS expression levels**

Aiming to get high expression levels of a polycistronic transgene encoding the Myc, Klf4, Oct4 and Sox2 reprogramming factors (MKOS), and based on the effectiveness of a retroviral vector for the production short-hairpin-RNAs (Zuber et al., 2011), we constructed the piggyBac transposon PB-*pTRE-pPGK-Venus-IRES-rtTA* (named PB-Booster/rtTA; Fig. 1A). This transposon expresses *Venus* (encoding the Venus
fluorescent protein) and rtTA (encoding the reverse Dox-dependent transactivator) from the Phosphoglycerate kinase 1 gene (PGK) promoter (pPGK) but, upon the addition of doxycycline (Dox), rtTA is expected now to be expressed from a tetracycline-responsive promoter (pTRE) (Fig. 1A); in either condition, the same polyA sequence is used and the rtTA is translated from an IRES element located between Venus and rtTA. When the PB-Booster/rtTA is combined with a transposon encoding MKOS under the control of a Dox-inducible promoter (i.e., PB-pTRE-MKOS-imO), high expression of MKOS is expected due to the ‘booster’ induction generated by the increase in rtTA production in the presence of Dox (Fig. 1A). In agreement with this positive feed-back regulatory circuit, MEFs electroporated with the PB-pTRE-MKOS-imO in combination with the PB-Booster/rtTA showed an evident expression induction of rtTA and of MKOS when treated for 24 h with as low as 0.05-0.10 µg/ml Dox, concentration at which no induction was detected when MEFs expressed rtTA from the pPGK constitutive promoter (i.e., encoded in the PB-pPGK-rtTA vector) (Fig. 1B). Furthermore, at least 5-fold ‘booster’ induction was noted when MKOS expression was compared between MEFs containing the PB-Booster/rtTA and those containing the PB-pPGK-rtTA treated with 0.15-1.5 µg/ml Dox for 24 h (approximately 10-fold vs. up to 2-fold induction; Fig. 1B). The return to near basal expression levels occurred about 24 h after Dox removal from the culture medium (data not shown). We also constructed an ‘all-included’ booster transposon which contains the polycistronic MKOS and allows rtTA booster expression (named PB-Booster/MKOS; Fig. 1C). In agreement with a booster expression induction of MKOS using this transposon, MKOS expression reached 10-fold induction in MEFs
electroporated with the PB-Booster/MKOS and treated with 0.15-1.5 μg/ml Dox (Fig. 1C).

**Influence of MKOS dose on reprogramming and PSC maintenance**

To test the reprogramming efficiency using the PB-Booster transposons, fresh MEFs from the 129/Sv mouse strain were electroporated with PB-pTRE-MKOS-imO in combination with either the PB-Booster/rtTA or the PB-pPGK-rtTA. The electroporated cells (i.e., up to 20% of total MEF population seeded; see Materials & Methods) were, then, induced to reprogram in the presence of 1.5 μg/ml Dox, which is within the range most frequently used for reprogramming, and the number of iPSC generated compared with that obtained at 10-fold lower Dox concentration (0.15 μg/ml; Fig. 2A). No difference in the efficiency of iPSC generation was noted between constitutive and booster rtTA expression when regular 1.5 μg/ml Dox was used, but this level of efficiency was reduced with constitutive but not with booster rtTA expression in the presence of 0.15 μg/ml Dox (Fig. 2B). Reprogramming timing was apparently not influenced by the level of MKOS expression such that emergence of iPSC-like colonies occurred nearly at the same time and almost all were positive for AP (Fig. 2C, right pictures).

The initiation of reprogramming can be identified by the formation of cell clusters (see typical cell clusters at 3 days of induction; Fig. 2C and Fig. 3C). Some early cell clusters might degenerate and do not succeed in generating iPSC, a phenomenon that can result from reprogramming conditions but also can be due to the mouse strain from which the MEFs are isolated. In our case, using highly proliferating 129/Sv MEFs, most cell clusters derived into iPSC colonies, independent of the expression system or Dox
concentration used for reprogramming (Fig. 2B). On the other hand, termination of reprogramming is generally determined by evaluating the ability of cells in primary iPSC-like colonies to form secondary iPSC colonies in the absence of reprogramming factors (i.e., without Dox; Fig. 2D). The comparison of number of secondary iPSC-like colonies generated in the presence or absence of Dox indicated that almost all primary iPSCs generated after 15 days of reprogramming with booster expression were committed to the iPSC phenotype, whereas an iPSC population fraction appeared Dox-dependent when the constitutive system was used in the presence of 0.15 \( \mu \text{g/ml} \) Dox (Fig. 2D). Therefore, when fresh MEFs are used, low MKOS expression levels are sufficient for reprogramming (i.e., using constitutive \( rtTA \) expression at 0.15 \( \mu \text{g/ml} \)) but reprogramming efficiency can still be increased by higher MKOS expression levels (i.e., using booster \( rtTA \) expression at 0.15 \( \mu \text{g/ml} \)) up to a limit that, apparently, cannot be overcome by increasing further MKOS expression levels (i.e., using booster \( rtTA \) expression at 1.5 \( \mu \text{g/ml} \)).

Reprogramming of 2-passage MEFs from the 129/Sv strain with the PB-Booster/MKOS generated iPSC with slightly lower efficiency than with fresh MEFs when 1.5 \( \mu \text{g/ml} \) Dox was used but, in contrast, a marked reduction in iPSC-like colony generation was noted in the presence of 0.15 \( \mu \text{g/ml} \) Dox (Fig. 3A, B). Interestingly, despite the lower efficiency in iPSC generation, most initial cell clusters derived into iPSC as when fresh 129/Sv MEFs were used. In contrast, although similar iPSC generation efficiency was obtained when 2-passage MEFs from the outbreed CD1 strain were used for reprogramming at 1.5 \( \mu \text{g/ml} \) and 0.15 \( \mu \text{g/ml} \) Dox, many more cell clusters than iPSC-like colonies were generated with 1.5 \( \mu \text{g/ml} \) Dox, whereas similar
number of cell clusters and iPSCs was determined in the presence of 0.15 µg/ml Dox (Fig. 3B). Again, timing for iPSC generation was not evidently changed by the expected different MKOS levels, though better formed iPSC-like colonies were detected at day 9 of reprogramming with 1.5 µg/ml Dox than with 0.15 µg/ml Dox, independent of the MEF strain source (Fig. 3C); as expected, nearly all colonies obtained were positive for AP (Fig. 3C, right pictures). These results support the above conclusion regarding the relationship between MKOS expression levels and reprogramming efficiency, though the sensibility to the MKOS dose appears reduced after MEFs have been passaged at least twice. In addition, it is apparent that higher MKOS expression levels within the CD1 genetic background, bring more cells to initiate reprogramming but only about a half succeed in deriving into iPSCs.

The booster system increased the total Oct4 expression levels above 10-fold the normal levels of the endogenous Oct4 gene (Fig. 4A), condition that could be deleterious for the maintenance of pluripotency (Niwa et al., 2000). Accordingly, ESCs carrying either the PB-pTRE-MKOS-imO plus the PB-Booster/rtTA or the PB-Booster/MKOS generated fewer and smaller colonies when grown in the presence of 0.15 µg/ml or 1.5 µg/ml for 3 days (Fig. 4B, C). Therefore, it is possible that the number of iPSC-like colonies determined after reprogramming is a sub-estimation of MEFs with reprogramming potential in the presence of MKOS factors, particularly when the booster rtTA system is used.

**MEFs of CD1 mouse strain efficiently reprogram into iPSCs that are unable to retain pluripotency**
Although reprogramming timing with CD1 MEFs was similar as with 129/Sv MEFs, cell density in putative CD1 iPSC was notably lower (Fig. 3C, opaque patches correspond to high cell density in BF pictures). Lower cell density in colonies could be due an increased propensity of CD1 iPSC to differentiate. Of note, however, was that reprogramming of 129/Sv MEFs for 25 days at low MKOS expression levels (i.e., in the presence of 0.15 µg/ml Dox), in contrast with reprogramming for the same days at high MKOS expression levels (i.e., in the presence of 1.5 µg/ml Dox), did not commit all reprogramming cells up to a Dox-independent stage (Fig. 5A, B). Remarkably, maintenance of putative CD1 iPSCs was Dox-dependent, independent of whether they were derived from reprogramming at low or high MKOS expression levels, such that most iPSCs were lost over two passages in the absence of Dox (Fig. 5A, B). Confirming the Dox-dependence for maintenance of putative CD1 iPSC, two independent CD1 iPSC clones isolated in the presence of Dox could not grow without Dox but could be rescued by adding Dox after one passage in the absence of Dox (Fig. 5C). Interestingly, CD1 iPSCs grown in the presence of Dox expressed about one-fifth the levels in 129/Sv iPSCs of Oct4, Nanog, Rex1 and Esrrb which, as expected, were further decreased when grown in the absence of Dox (Fig. 5D; see also below).

The failure of putative CD1 iPSCs to grow in the absence of Dox could be due to incomplete reprogramming (Silva et al., 2008) or to the inability of CD1 iPSCs to maintain pluripotency in the regular ESC culture medium, as it has been shown for rat ESCs (Buehr et al., 2008) and ESCs from some mouse strains (Nichols et al., 2009; Ohtsuka and Niwa, 2015). In agreement with this latter possibility, a CD1 iPSC population or a clone selected from them could grow in the absence of Dox when the
medium was supplemented with either MEK or GSK3β inhibitors or both (2i medium), conditions that allowed the maintenance of 129/Sv iPSCs and ESCs and favor the ground state of pluripotency (Ying et al., 2008; Fig. 6A, B). CD1 iPSCs retain growth characteristics of PSCs (e.g., continuous formation of well-defined compact refringent colonies) after several passages in this condition, but colonies degenerate when the MEK and GSK3β inhibitors are removed from the medium (data not shown). This latter observation contrast with that reported by Silva et al. (2008), which show that MEK and GSK3β inhibitors contribute to the completion of reprogramming from pre-iPSCs. The requirement of 2i medium for pluripotency maintenance is not a characteristic of CD1 iPSCs but, rather, is an intrinsic property of the CD1 strain since CD1 ESC could only be derived and maintained in the 2i medium (Fig. 6C). The inability of CD1 iPSCs to retain pluripotency appears related to the relatively low expression levels of at least some typical pluripotency genes (i.e., Oct4, Nanog, Rex1 and Esrrb), which were up-regulated in the 2i medium though remained about half the levels determined in 129/Sv iPSCs or in wild-type ESCs (Fig. 6C). Notably, Rex1 expression was only mildly up-regulated in the 2i medium and remained at much lower levels (between 5- to 10-fold) than those found in well-established pluripotent stem cells (Fig. 6D).

The capacity of cells under the CD1 genetic background to reprogram and to maintain pluripotency was also tested using MEFs from an E13.5 transgenic embryo carrying the PB-Booster/MKOS transposon (TgCD1/MKOS; Fig. 7A). iPSCs derived from these MEFs were similar to the ones described above and only could be expanded and passaged in the presence of 2i medium (Fig. 7B). One randomly selected individual CD1 iPSC clone (iPSC-TgCD1/MKOS-11) derived from these experiments showed a
high percentage of cells (around 70%) with a normal number of chromosomes and a high level of pluripotency as demonstrated by the capacity to form teratomas under the skin of immunodeficient mice (Fig. 7B) and by the ability to contribute to the inner cell mass and to embryonic tissues after injection into embryos at a morula stage (Fig. 7C and Table 1).

DISCUSSION

The transcription factor-mediated reprogramming into iPSCs initiates by disrupting the stable regulatory network that determines the identity of specific somatic cells due to the forced expression of pluripotency reprogramming factors. Later in time, the emergence of iPSC depends on the ability of these pluripotency factors to gradually establish the PGRN (Buganim et al., 2012; Buganim et al., 2013). Therefore, the number and amount of pluripotency factors should have a profound effect on reprogramming efficiency. Adding genes of the PGRN to the reprogramming transcription factors have shown a variable increase in reprogramming efficiency (Buganim et al., 2014; Sebban and Buganim, 2016) resulting, in few cases, a pattern according to deterministic reprogramming (Hernandez et al., 2018; Mor et al., 2018). Also, the dose of reprogramming factors, mainly MKOS, has shown to influence the reprogramming efficiency (Carey et al., 2010; Polo et al., 2012; Stadtfeld et al., 2009; Wernig et al., 2008), though it has also been shown that higher than required levels for iPSC derivation of specific reprogramming factors can deviate cells towards non-pluripotency fates (Shu et al., 2013; Velychko et al., 2019). Without considering altering the barriers that naturally block somatic cell reprogramming, in the present study we evaluated the
effect of expression levels of reprogramming factors in the iPSC derivation efficiency as well as the effect on the maintenance of the pluripotent state.

The MEFs compose a heterogeneous cell population that may include cells more or less susceptible to reprogram upon MKOS expression. Considering this fact, it is possible that the effect of Dox dose on reprogramming is targeting, though overlapping, different set of cells, such that cells resistant to reprogram at low MKOS expression levels can reprogram at a higher dose. This cell population could include those Thy1+ cells remaining at the early phase of reprogramming but that can derive into iPSC after increasing the levels of reprogramming factors (Polo et al., 2012). It has been proposed that these refractory-to-reprogram cells are unable to maintain the production of at least the Oct4 protein. Alternatively, since MEFs after few passages showed a marked reduction in reprogramming efficiency at the low Dox dose, cell proliferation capacity could be an additional barrier that MKOS expression levels may mitigate. Accordingly, we show here that the reduced number of iPSC after 2-3 passages in comparison with the number obtained from early passage MEFs was nearly recovered by increasing the Dox dose. At an early passage stage, it is unlikely that reduced proliferation of MEFs is the only barrier relieved by MKOS expression levels for reprogramming, since most cells hold a high proliferation capacity. Therefore, increasing levels of pluripotency factors can facilitate the conversion of MEFs into iPSCs by acting on distinct cell types with different reprogramming potential and/or different proliferation capacity.

It is apparent that the effect of MKOS expression levels on iPSC derivation occurs at an early stage of reprogramming since the number of initial cell clusters formed correlated with the number of iPSC colonies obtained. In agreement with this possibility,
the levels of MKOS did not markedly affected the reprogramming dynamics such that emergence of cell clusters and iPSC-like colonies occurred almost at the same reprogramming point disregarding the Dox dose. Interestingly, within the CD1 genetic background, high MKOS levels promote the formation of more cell clusters than iPSC colonies, whereas at low MKOS levels similar number of cell clusters than iPSC colonies were generated. Therefore, it is apparent that higher MKOS expression does not largely affect reprogramming kinetics and pluripotency establishment but, rather, facilitates the initiation of reprogramming in a subset of MEFs that cannot reprogram at low MKOS expression levels, as mentioned above.

Although high levels of MKOS expression improved reprogramming efficiency with the booster system, this condition was incompatible for the maintenance of pluripotency, such that most PSC overexpressing MKOS were prompt to differentiate. Considering the multiple feedback circuits working within the PGRN, it is not unexpected that exogenously increasing the amount of one of its components could disrupt the PGRN regulatory balance. Alternatively, high levels of Oct4 and/or Sox2 could alter the balance of the lineage specifier activity provided by these transcription factors, which has been proposed to be required to maintain pluripotency (Shu et al., 2013; Velychko et al., 2019).

Under standard culture conditions, low expression levels of one or several pluripotency factors appears also to be incompatible with the maintenance of PSCs. In particular, we show here that PSCs derived from the CD1 strain (ESCs and iPSCs) have low expression levels of pluripotency factors and are prompt to differentiate. In agreement with this interpretation, CD1 iPSCs can be rescued by re-activating the
expression of exogenous MKOS. PSC from CD1 could also be maintained in 2i medium, condition at which expression levels of Oct4, Nanog, Esrrb and Rex1 increased but still were below those detected in 129/Sv iPSCs or ESCs. Remarkably, Rex1 mRNA levels remained low under high MKOS expression levels or under 2i culture conditions, which was unexpected because it has been shown that high Rex1 expression levels in ESCs correlate with high Nanog expression levels and with the recruitment, within a 5' located super enhancer, of Nanog, Oct4 and Sox2 (Zhang et al., 2019). Low expression levels of Rex1 have been correlated with loss of developmental potential (Kalkan et al., 2017), interestingly however, at least one CD1 iPSC clone, expressing low Rex1 expression levels, showed very high contribution in chimeric mouse experiments. Therefore, it is apparent that CD1 iPSC captured by either MKOS expression or a medium supplemented with GSKβi and/or MEKi inhibitors occurs trough a mechanism that does not compensate for Rex1 expression and only partially influence the expression of some genes of the PGRN (e.g., Esrrb, Nanog and Oct4).

High expression levels of MKOS have shown to promote the derivation of a distinct class of PSCs known as F-class (Tonge et al., 2014). F-class PSCs were originally recognized by the fuzzy colonies they form in a regular culture medium, in contrast with the usual compact colonies derived from the best characterized PSC (i.e., ESC; C-class). In our studies, independent of the mouse strain used to obtain MEFs, we were unable to clearly observe any colony resembling the morphology of those colonies derived from F-class PSCs, despite the booster induction lead to much higher MKOS expression levels than those achieved with constitutive rtTA expression. Furthermore, in conditions not optimized for F-class PSC specific growth, induction of high expression
levels of \textit{MKOS} in ESCs caused cell differentiation and death rather than establishing F-class-like colonies. If derivation of F-class PSCs requires progressive increase in \textit{MKOS} expression, this would not occur in our experiments where high \textit{MKOS} expression level is obtained at an early reprogramming stage.

In conclusion, the data presented suggest that the dynamic range of \textit{MKOS} expression increased with the booster system used here. Depending on the cell type, high level of \textit{MKOS} expression could be required to disrupt the gene regulatory network that gives identity to a somatic cell, and could also contribute to surpass certain barriers that prevent reprogramming at the initiation stage. It is also worth mentioning that the PB-Booster/MKOS vector is self-contained such that no additional vector is required for getting gene expression induction by Dox; this particular setting is convenient for reprogramming human cells. On the other hand, it has been shown that different mouse strains have different ability to establish cultures of PSCs (i.e., derived from either preimplantation embryos or after reprogramming; (Hanna \textit{et al.}, 2009; Nichols \textit{et al.}, 2009; Ohtsuka and Niwa, 2015). As for the CD1 outbreed strain, in several instances, the difficulty to establish these PSC cultures can be accomplished by growth in culture media such as the 2i medium. However, this latter condition does not identify the deficiency that prevents the establishment of the PGRN under basic culture condition (M15 medium). Finding the origin of these deficiencies in different mouse strains or even in different mammalian species, could contribute to understand the diversity of mechanisms that regulate the PGRN and to develop strain- or specie-specific protocols for cellular reprogramming.
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MATERIALS & METHODS

Construction of piggyBac transposon plasmid vectors

To construct the piggyBac transposons used here, we initially transferred the TREtight and the PGK-Venus-IRES-rtTA3 DNA fragments from the TtRMPVIR retroviral plasmid vector (a gift from Dr. Scott Lowe; Addgene #27995) to a piggyBac transposon plasmid vector; this transposon was named PB-Booster/rtTA. The MKOS polycistronic sequence, derived from the PB-pTRE-MKOS-imO (a gift from Dr. Keisuke Kaji), was inserted as an EcoRI fragment between TREtight and the PGK-Venus-IRES-rtTA3 to generate the PB-Booster/MKOS. The PB-pPGK-rtTA, which expresses the rtTA constitutively, was generated by deleting the TREtight sequence from the PB-Booster/rtTA.

Cell Culture

The culture medium used was composed of Dulbecco’s Modified Eagle Medium (DMEM), nonessential amino acids (1X), sodium pyruvate (1X), Glutamax (1X), all from GIBCO, plus penicillin and streptomycin (30 μg/ml and 50 μg/ml, respectively; Sigma-
Aldrich). For MEFs, the medium (M10) was supplemented with 10% fetal bovine serum (FBS; Byproducts), whereas for ESCs, iPSCs and MEFs under reprogramming, the medium (M15) was supplemented with 15% FBS, 100 µM 2-mercaptoethanol (Sigma-Aldrich) and leukemia inhibitory factor (1000 u/ml LIF; StemR&D). ESCs and iPS cells were routinely seeded at a density of 2.5-5.0x10^4 cells/cm^2 on a MEF feeder layer, unless otherwise indicated; media was daily changed and passaged every other day. For self-renewal analysis, ESCs or iPSCs were seeded at a density of 1000 cells/cm^2 over a feeder layer in the presence or absence of Dox (1.5 µg/ml), MEKi (1 µM PD0325901; STEMGENT) or GSK3βi (3µM CHIR99021; STEMGENT); then, after five days in culture, colonies were fixed with 4% PFA and stained for alkaline phosphatase activity (see below).

Electroporation and reprogramming procedures

MEFs (5x10^5) from 12.5-13.5 dpc embryos at passage 1 or 3 were resuspended in 1X PBS and immediately centrifuged. The pellet was resuspended in an electroporation universal buffer (BTXpress electroporation solution; BTX) containing 5 µg of total DNA composed of 5:1 ratio of transposon:transposase plasmid vectors, the suspension was then transferred to a 2 mm electroporation cuvette (BTX), and electroporated by a single 20 milliseconds pulse under 170 V with the BTX ECM 830 electroporator (BTX). The electroporated suspension was centrifuged at 14,000 rpm for 30 seconds, the pellet resuspended in 100 µl of M10 media and incubated for 20 minutes at 37 °C. Cells were plated on a 6 cm dish and cultured in a medium supplemented with 20% FBS. The next day, media was changed for M10 medium, and 24 hours later the efficiency of electroporation was calculated by counting the proportion of Venus+ fluorescent cells in
images captured with the Axio Observer.Z1 microscope. Regularly, the electroporation efficiency was 18-20\%. Two days post-electroporation, 1x10$^3$ electroporated MEFs (Venus$^+$)/cm$^2$ were plated on a feeder layer-covered well of a 6-well plate and cultured in fresh M15 media. The next day, Dox was added (0.15-1.5 µg/ml) and media was changed every other day. Dox was added until day 15 when, usually, most iPSC-like colonies have emerged. In some cases, putative iPSC were revealed by staining for alkaline phosphatase (AP). In this latter case, plates were washed twice with 1X PBS and cells fixed with 4\% PFA, pH 7.4, 10 minutes at room temperature (RT). AP staining was performed using a commercial kit (86R-1KT; Sigma-Aldrich) according to manufacturer instructions. Briefly, 100 µl of sodium nitrite and 100 µl of FRV-alkaline solution were mixed and incubated for 2 minutes; the mixture was then added to 4.5 ml of distilled water with 100 µl of naphthol AS-BI-alkaline solution. Fixed cells were incubated in this solution for 15 minutes at RT and, afterwards, washed twice with distilled water. Cell were observed and photographed under a stereoscopic microscope (Leica).

**RNA extraction and RT-qPCR**

Cells were fed with fresh media two hours before RNA extraction. After washing with 1X PBS, 1 ml of RiboEX$^\text{TM}$ (GeneAll) was added for every 10 cm$^2$ surface area and incubated 5 minutes at RT. The solution was transferred to an Eppendorf tube and 200 µl of cold chloroform added for every 1 ml of RiboEX$^\text{TM}$ used. The mixture was vigorously shaken and centrifuged at 10,000 rpm at 4°C for 15 minutes, the aqueous phase was recovered and 500 µl of 100\% isopropanol added for every 1 ml of RiboEX$^\text{TM}$ used. This mixture was again vigorously shaken and centrifuged at 10,000
rpm at 4°C for 30 minutes, the supernatant was removed and 1 ml of 75% ethanol-DEPC added to the pellet. After centrifuging at 10,000 rpm for 5 minutes at 4°C, the pellet was left to dry for 15-20 minutes and dissolved in 20-50 µl of H2O-DEPC. When necessary, to eliminate contaminating genomic DNA, samples were treated with DNase I (Roche) according to manufacturer instructions. cDNA was generated from 1 µg of total RNA using the Hyperscript™ Reverse Transcriptase (GeneAll) according to manufacturer instructions. Gene expression was quantified using KAPA SYBR® FAST Universal qPCR kit (KAPA BIOSYSTEMS) in a Rotor-Gene Q (QIAGEN). Relative expression was calculated using the comparative C_T method using the constitutive RPLP0 or Cyclophilin expression level as a reference. The specific primers used were: for rtTA, forward: 5’-ACGACAAGGAAACTCGCTCA-3’, reverse: 5’-TCTTGCCATGACTCGCCTTC-3’; for total Oct4: forward: 5’-CACGAGTGGAAGCAACTCA-3’, reverse: 5’-AGATGGTGGTCTGGCTGAAC-3’; for endogenous Oct4, forward: 5’-TAGGTGAGCCGCTTTCCAC-3’, reverse: 5’-GCTTAGCCAGGTTGAGG-3’; for Rex1, forward: 5’-CAGCTCCTGCACACAGAAGA-3’, reverse: 5’-ACTGATCCGCAAACACCTG-3’; for Nanog, forward: 5’-CTTACAAGGGTCTGCTACTGAGATGC-3’, reverse: 5’-TGCTTCCTGGCAAAGGACCTT-3’; for Esrrb, forward: 5’-CATGAAATGCCTCAAAGTG-3’, reverse: 5’-AAATCGGCGAGTTGTTGAG-3’; for Oct4-Sox2 (MKOS transgene), forward (in Oct4 of MKOS transgene): 5’-CCTTTCCCTCTCTCCGTACGTA-3’, reverse (in Sox2 of MKOS transgene): 5’-CGGGGTTACTTTCAACTCATCAGGCCGAGGAA-3’, reverse: 5’-AGGGGGAGATGTTACGATG-3’.
for *Cyclophilin*, forward: 5’-GGCAATGCTGGACCAAACAC-3’, reverse: 5’-
TTCCTGGACCCAAAACGCTC-3’.

**Teratoma assay and generation of mouse chimeras**

To generate teratomas from CD1 iPSCs, 1x10^6 cells (clone iPSC-TgCD1/MKOS-11, p-2), previously grown in the presence of 1.5 µg/ml Dox, were subcutaneously injected into the flank of immunodeficient nude mice. Mice were examined for tumor formation twice a week and sacrificed 4 weeks after injection. Tumor was fixed in 4% PFA and 10 µm cryosections were stained with hematoxylin/eosin or safranin/fast green for analyses. Donors of 8-cell embryos for chimera generation were CD1 mice superovulated by injection of 5 IU PMSG followed by 5 IU hCG 48 hours later; these mice were immediately mated to stud males and checked for a vaginal plug the next morning. Eight-cell embryos were collected and injected with 5-6 iPSCs; injected embryos were cultured for 24 hours before being surgically transferred to the oviduct of pseudopregnant mice. Generally, pregnant mice were sacrificed at E13.5 gestation stage and chimeric embryos photographed with an inverted fluorescence microscope. To obtain the TgCD1/MKOS transgenic embryos, the PB-Booster/MKOS together with a transposase expressing vector at 10:1 ratio were injected into fertilized oocytes of the CD1 strain of mice; the injected zygotes were transplanted to the oviducts of pseudopregnant CD1 mice. At E13.5, one Venus+ embryo was selected for derivation of MEFs for reprogramming.

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Table 1. Contribution of iPSCs from clone TgCD1/MKOS-11 to the ICM of blastocysts or E13.5 embryos as estimated by the percentage of Venus+ cells in embryos (Fig. 7C).

| Experiment # | Embryos analyzed | Category 1 (without contribution to embryos) | Category 2 (2-4 iPSCs/ICM or <50% iPSC contribution to E13.5 embryos) | Category 3 (all iPSC-derived ICM or >50% iPSC contribution to E13.5 embryos) |
|--------------|------------------|---------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| 1 (blastocysts) | 29               | 1 (3.4%)                                   | 16 (55.1%)                                      | 12 (41.4%)                                      |
| 2 (blastocysts)  | 18               | 1 (5.5%)                                   | 9 (49.5%)                                       | 8 (44%)                                         |
| 3 (blastocysts)  | 20               | 0                                           | 12 (60%)                                        | 8 (40%)                                         |
| 4 (E13.5 embryos) | 1                | 0                                           | 0                                               | 1 (100%)                                        |
| 5 (E13.5 embryos) | 5                | 0                                           | 3 (60%)                                         | 2 (40%)                                         |
| 6 (E13.5 embryos) | 6                | 1 (16%)                                    | 2 (33%)                                         | 3 (50%)                                         |
| 7 (E13.5 embryos) | 4                | 0                                           | 1 (25%)                                         | 3 (75%)                                         |
| 10 (E13.5 embryos) | 8                | 3 (38%)                                    | 2 (25%)                                         | 3 (38%)                                         |
Fig. 1. PiggyBac transposon vectors for booster gene expression. (A) A schematic representation of the booster expression of a polycistronic MKOS transgene. In contrast with Dox-induced MKOS expression when rtTA is constitutively expressed from the Pgk
promoter (left), booster expression of MKOS is achieved when rtTA is expressed from both the pPGK promoter and the tetracycline-responsive promoter (pTRE, Dox-dependent; right); in this latter condition, Dox induces MKOS expression and also the production of more rtTA, forming a positive regulatory circuit. (B) Induced booster expression. MEFs were electroporated with the plasmid sets for constitutive (gray labels) or booster rtTA expression (black labels) shown in A (approximately 20% electroporation efficiency for both sets). Twenty-four hours after electroporation recovery, cells were induced with different Dox doses and expression determined by a specific RT-qPCR (an Oct4-Sox2 segment for the determination of mRNA levels of the polycistronic MKOS transgene; see Materials & Methods). Note that the booster vector combination increased the expression of both MKOS (left) and rtTA (right) at low Dox doses (0.05-0.10 µg/ml) and higher levels of MKOS expression were reached at higher Dox doses (0.15-1.50 µg/ml) than with constitutive rtTA expression. (C) The PB-Booster/MKOS transposon. A single transposon allows MKOS and rtTA booster expression. MEFs were electroporated with the plasmid containing this transposon and significant Dox induction of MKOS expression was observed, as determined by a specific RT-qPCR for Oct4 (constitutive Cyclophilin expression level was used as a reference).
Fig. 2. Reprogramming with booster MKOS expression. (A) Reprogramming protocol used with MEFs of the 129/Sv mouse strain. (B, C) Determination of cell clusters and iPSC-like colony formation with constitutive (gray labels) and booster rtTA expression (black labels); phase contrast images are shown (scale bars, 100 µm). Note
that at high Dox dose (1.5 µg/ml; left graph in B and top row of pictures in C), the time of emergence of cell clusters and iPSC colonies as well as the number of each were similar when either constitutive or booster rtTA expression was used. In contrast, at low dose (0.15 µg/ml; right graph in B and bottom row of pictures in C), although time of emergence of cell clusters and iPSC colonies (phase contrast images shown in C) was similar, the number obtained was significantly reduced when constitutive rtTA expression was used (*, P< 0.001; two-tailed unpaired t-test). In agreement with complete reprogramming after 15 days of Dox induction, nearly all colonies at this stage were positive for alkaline phosphatase (AP⁺; C, right pictures). (D) Dox-dependence of iPSCs derived with constitutive or booster rtTA expression. Fifteen days after Dox induction, iPSC colonies formed were dissociated and seeded in the presence or absence of Dox. iPSC formed were stained for AP (left pictures) and colonies counted (right graph). Notably, although most iPSCs derived at high Dox dose with either constitutive or booster rtTA expression had become Dox-independent at day 15 of reprogramming, at low dose, more Dox-independent iPSCs were obtained with booster rtTA expression at this time (**, P<0.005; two-tailed unpaired t-test).
Fig. 3. Comparison between reprogramming of CD1- and 129/Sv-derived MEFs with booster *MKOS* expression. (A) Reprogramming protocol used with MEFs derived from the 129/Sv or CD1 mouse strains after two passages. As in Fig. 2, Dox induction for reprogramming started 3 days after electroporation. (B, C) Determination of cell clusters and iPSC-like colony formation during reprogramming of either 129/Sv or CD1 MEFs with booster *rtTA* expression. Two independent experiments were performed (one graph for each shown). As expected, fewer cell clusters and iPSC-like colonies were obtained with 0.15 µg/ml Dox than with 1.5 µg/ml Dox, independent of the source of MEFs but, although higher number of cell clusters was observed at the beginning of reprogramming with CD1 MEFs in comparison with 129/Sv MEFs, the total number of iPSC-like colonies at day 15 of reprogramming was similar; nearly all colonies at this
stage were positive for alkaline phosphatase (AP⁺; C, right pictures). Note the evident increased growth of cell clusters and iPSC-like colonies in phase contrast (PC; scale bars, 100 µm) images when 1.5 µg/ml Dox was used. In addition, as estimated from the opaque areas in bright field (BF) images at 15 days of reprogramming, iPSC from 129/Sv MEFs showed higher cell density than those derived from CD1 MEFs.
Fig. 4. Effect of high MKOS expression level on ESC maintenance. (A) MKOS overexpression in ESC. ESCs from the line W9.5 were electroporated with the PB-Booster/MKOS transposon. Total Oct4 expression levels were determined by RT-qPCR (constitutive RPLP0 expression level was used as a reference) for samples obtained...
from ESCs containing the PB-Booster/MKOS cultured in the presence of 1.5 µ/ml Dox for 24 h and from same cultures but 24 h after Dox removal; MEFs and wild-type W9.5 ESCs were used as controls. Oct4 expression in ESC-PB-Booster/MKOS in the presence of Dox increased near 30-fold in comparison with the same cells without Dox or with the levels detected in wild-type ESCs. (B, C) Maintenance of ESCs under high MKOS expression level. Clones of ESCs containing either PB-MKOS-imO/PB-Booster/rtTA (1 clone) or PB-Booster/MKOS (2 clones) were seeded in the absence or presence of different Dox dose, and the capacity to form colonies positive for alkaline phosphatase (AP⁺; scale bars, 100 µm) evaluated 3 days later. Note the reduction in number and size of colonies due to the treatment with two Dox doses (0.15 and 1.5 µg/ml).
Fig. 5. Dependence on *MKOS* transgene expression for growth of iPSCs from MEFs of the 129/Sv or CD1 strains. (A, B) MKOS-dependence for maintenance of a pool of iPSCs from MEFs of 129/Sv or CD1 strains. Fifteen days after reprogramming, generated iPSCs were subcultured for additional 10 days in the presence of Dox (0.15 or 1.5 µg/ml); then, the pool of iPSCs were subcultured in the presence or absence of Dox for 2 passages. Inlets in A show a higher magnification of AP-stained iPSC colonies (scale bars, 100 µm). Note that, a high Dox dose for 25 days produced a moderate higher number of 129/Sv Dox-independent iPSCs than a low Dox dose (*, P<0.01). In contrast, Dox-independent iPSCs could not be established with CD1 MEFs
at either high or low Dox dose for 25 days (compare +Dox vs. –Dox in derivatives of CD1 iPSCs; **, p<0.001). (C) MKOS-dependence and rescue by MKOS expression of two CD1 iPSC clones. Selected clones were cultured in presence of Dox (1.5 µg/ml) for several passages (41 days after reprogramming) and then cultured for 2 passages in the presence or absence of Dox; at the second passage, Dox was added to iPSCs grown in the absence of Dox. These experiments indicate that iPSC loss of pluripotency is not a sudden event but rather a slow process occurring throughout several days, such that pluripotency can be recovered by re-expressing the MKOS transgene. (D) Expression of pluripotency markers in CD1 and 129/Sv iPSC. Expression of the genes indicated was determined by RT-qPCR (constitutive RPLP0 expression level was used as a reference) in a pool of iPSC derived from the corresponding strain. 129/Sv iPSCs were grown in the absence of Dox, whereas for CD1 iPSC, determination was done in the presence or absence (for 3 days) of Dox. Note the marked lower expression levels of pluripotency genes in CD1 iPSC in the presence of Dox in comparison with levels determined in 129/Sv iPSC (more than 5-fold reduction), which were further reduced after removing Dox from the culture medium.
Fig. 6. Pluripotency maintenance of CD1 iPSC in culture in the presence of MEK and/or GSK3β inhibitors. (A, B) Growth of 129/Sv and CD1 iPSC in 2i medium. Established iPSC (in the presence of Dox for CD1 iPSCs) were grown in M15 medium supplemented with MEKi, GSK3βi or both (2i medium) and compared with the growth observed in regular M15 medium without Dox (DMSO added). Inlets in A show a higher magnification of AP-stained iPSC colonies (scale bars, 1 mm). 129/Sv iPSC (a pool or a clone) generated similar number of colonies in all culture media tested comparable with the growth of cells from an established ESC line (R1B5). In contrast, CD1 iPSC (a pool,
from TgCD1/MKOS MEFs [see Fig. 7, or a clone] started to lose pluripotency since 24 h after Dox removal, and by 72 h could grow only when MEKi and/or GSK3βi were added to the medium. (C) Derivation of CD1 ESCs. CD1 blastocyst (without zona pellucida) were cultured in M15 medium with or without 2i; then, the grown inner cell mass (arrows in left images; scale bars, 100 µm) was dissociated and cells cultured in the presence or absence of 2i (middle images; scale bars, 1 mm). To test the requirements for pluripotency maintenance, the pool of putative ESCs generated in M15 medium with 2i (M15+2i; arrowheads) were subcultured in M15 medium with or without 2i (right images; scale bars, 100 µm). Note that generation and maintenance of ESC-like colonies could only occur in M15 medium with 2i. (D) Expression of pluripotency markers in a CD1 iPSC clone. A CD1 iPSC clone was cultured in M15 medium with Dox or without Dox in the presence of 2i and expression of the pluripotency markers indicated determined by RT-qPCR and compared against the expression levels in wild type ESCs (R1B5) (constitutive RPLP0 expression level was used as a reference). Expression levels of all genes tested were between 5- to 10-fold lower in CD1 iPSC than those determined in ESCs. Adding 2i to the medium in the absence of Dox, increased the expression levels of all genes tested but remained in a much lower range than levels in ESCs; particularly, a limited increase was observed for Rex1. An independent experiment showed the same pattern though comparable values between genes were different (data not shown).
Fig. 7. Derivation of teratomas and chimeric mice from a CD1 iPSC clone. (A)

Derivation of PB-Booster/MKOS transgenic embryos. Transgenic embryos were generated by pronuclear injection of the PB-Booster/MKOS transposon in the presence of transposase. iPSCs were derived from MEFs of an E13.5 transgenic mouse embryo carrying the PB-Booster/MKOS transposon (TgCD1/MKOS). A single clone was isolated (iPSC-TgCD1/MKOS-11) and pluripotency tested by the capacity to form teratomas and to contribute to embryos after injection into a 8-cell morula. (B) Teratoma formation. A teratoma formed (green arrow) contained tissues belonging to the three germ layers (hematoxylin/eosin staining for ectoderm and endoderm, and safranin fast green staining for mesoderm). (C) Chimeric embryos. Using Venus as a reporter, high frequency of iPSC incorporation into the inner cell mass was observed (left pictures),
many of which developed to produce chimeric embryos with high iPSC contribution to, apparently, all tissues (see also Table 1).
