

Induction of Pluripotent Stem Cells from Mouse Embryonic Fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds

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SUMMARY

Somatic cells can be induced into pluripotent stem cells (iPSCs) with a combination of four transcription factors, Oct4/Sox2/Klf4/c-Myc or Oct4/Sox2/Nanog/LIN28. This provides an enabling platform to obtain patient-specific cells for various therapeutic and research applications. However, several problems remain for this approach to be therapeutically relevant due to drawbacks associated with efficiency and viral genome integration. Recently, it was shown that neural progenitor cells (NPCs) transduced with Oct4/Klf4 can be reprogrammed into iPSCs. However, NPCs express Sox2 endogenously, possibly facilitating reprogramming in the absence of exogenous Sox2. In this study, we identified a small-molecule combination, BIX-01294 and BayK8644, that enables reprogramming of Oct4/Klf4-transduced mouse embryonic fibroblasts, which do not endogenously express the factors essential for reprogramming. This study demonstrates that small molecules identified through a phenotypic screen can compensate for viral transduction of critical factors, such as Sox2, and improve reprogramming efficiency.

INTRODUCTION

Previous studies demonstrated that stable genomic integration and high expression of four factors, Oct4/Sox2/Klf4/c-Myc or Oct4/Sox2/Nanog/LIN28, can reprogram fibroblast cells, B cells, and liver and stomach epithelial cells into induced pluripotent stem cells (iPSCs) (Hanna et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). It was later shown that iPSCs can be generated by viral integration of Oct4/Sox2/Klf4 without c-Myc (Aoi et al., 2008; Nakagawa et al., 2008). Such iPSCs exhibited reduced tumorigenicity in chimeras and progeny mice (Nakagawa et al., 2008). However, in the absence of c-Myc overexpression, the reprogramming process is much slower, and efficiency is substantially lower. These studies also suggested that the ectopic expression of these three transcription factors (TFs) is required for reprogramming, since combina-

tion of three factors excluding one of them (i.e., Oct4/Klf4/c-Myc, Oct4/Sox2/c-Myc, or Klf4/Sox2/c-Myc) was unsuccessful to reprogram general somatic cells into authentic iPSCs (Aoi et al., 2008; Nakagawa et al., 2008).

Since the viral integration of these TFs is not a viable therapeutic option, in this present study, we directed our efforts in finding small molecules that could replace the presence of virally transduced TFs in a quest to ultimately find a chemical cocktail that would allow reprogramming of somatic cells in chemically defined conditions. Recently, we and others have shown that if the target somatic cells are chosen carefully, it is possible to use an endogenously expressed reprogramming gene to help in generating iPSCs (Kim et al., 2008; Shi et al., 2008). In these studies, neural progenitor cells (NPCs), which endogenously express Sox2 (Bielloch et al., 2006), were transduced with Oct4 and Klf4 alone (OK) and were successfully reprogrammed to iPSCs. Furthermore, we found that this process was greatly enhanced by the presence of a G9a histone methyltransferase (G9a HMTase) inhibitor, BIX-01294 (BIX) (Kubicek et al., 2007; Shi et al., 2008). In addition, we observed that BIX could enable reprogramming of NPCs transduced with c-Myc, Klf4, and Sox2, in the absence of Oct4 ectopic expression. Therefore, in this particular system, BIX seemed to compensate for the lack of Oct4 overexpression.

This present study aimed to assess if a similar strategy could be used to find small molecules that can replace specific viral transduction to obtain iPSCs from a general cell lineage, in which none of the TFs deemed essential for reprogramming, Oct4, Sox2, and Klf4, are expressed. Hence, mouse embryonic fibroblasts (MEFs) were used. Finding a small molecule that could replace one of these TFs in the induction of MEF reprogramming might lead to the identification of general pathways involved in this process. Such chemical strategy might also be more amenable for therapeutic application. Consequently, we screened a collection of known drugs to identify small molecules that can enable the generation of iPSCs from MEFs transduced with Oct4 and Klf4 (OK) and thus could compensate for the lack of Sox2 overexpression. Through the different screens performed, we identified that a combination of BIX with BayK8644 (BayK), a L-channel calcium agonist (Schramm et al., 1983) was one of the most effective. BayK was of interest because it exerts its effect upstream in cell signaling pathways and does not directly cause epigenetic modifications. It is likely that this type of molecule can be exploited to induce reprogramming in a more specific manner

than molecules acting directly at the epigenetic level causing DNA or histone modification. Some of these epigenetic modifiers have already been shown to facilitate the reprogramming process, such as BIX (Shi et al., 2008), valproic acid (Huangfu et al., 2008), and 5' azacytidine (Mikkelsen et al., 2008).

This present study demonstrates that small molecules identified through a phenotypic screen can be used to effectively compensate for the viral transduction of a critical iPSC TF, Sox2. Moreover, it highlights the important contribution that small-molecule screens will eventually make to the discovery of new molecular targets and mechanisms involved in complicated biological processes such as reprogramming.

RESULTS

Phenotypic Screen Leads to the Discovery of Small Molecules that Enable MEF Reprogramming Transduced with Only Two TFs

Unmodified MEFs derived from E13–E14 embryos of the 129 mice were used for the initial screen. MEFs were plated on Matrigel at 3.5×10^4 cells/well of a 6-well plate and transduced with OK (retroviral vectors expressing Oct4 and Klf4) alone. Within 14–21 days, treated cells were assessed for the appearance of colonies that had the characteristic embryonic stem cell (ESC) colony morphology and were positive for the pluripotency marker alkaline phosphatase (ALP). Such OK-transduced cells generated only a few small noncompact colonies, which were weakly positive for ALP expression (Figures 1A and 1C). These colonies initially appeared within 21 days after viral transduction and were difficult to expand. Therefore, such assay system offered a clean background for the identification of small molecules having desirable reprogramming-inducing activity. Using this system, compounds from a library of around 2000 known small molecules (see [Experimental Procedures](#)) were screened and were identified as hits when they induced the appearance of ESC colonies that were strongly positive for ALP within 14–21 days after treatment. This image-based method provided a more accurate assessment of reprogramming as compared to homogenous reporter-based assay. Among several primary hits that induced formation of more than one to two compact ESC-like colonies with strong ALP staining, we focused on the small molecule with the strongest effect, BIX. We observed that when MEFs were treated with BIX after OK viral transduction, compact colonies with high levels of ALP expression could be readily detected within approximately 14–21 days (Figure 1A). These cells were also positive for Nanog, Oct4, and SSEA-1 expression (data not shown). This result, obtained with a more general cell type, which does not endogenously express any of the three essential reprogramming genes, further validates our previous observation that BIX has strong reprogramming-inducing activity and inhibition of the G9a HMTase can facilitate reprogramming (Shi et al., 2008). However, the reprogramming efficiency in MEFs transduced with OK and treated with BIX was still low in comparison to the four-factor-induced reprogramming of MEFs or the OK/BIX NPC reprogramming. Furthermore, this small molecule acts directly at the epigenetic level and appears to have a broad range of action. To identify additional compounds with reprogramming activity, we conducted a second screen using a similar

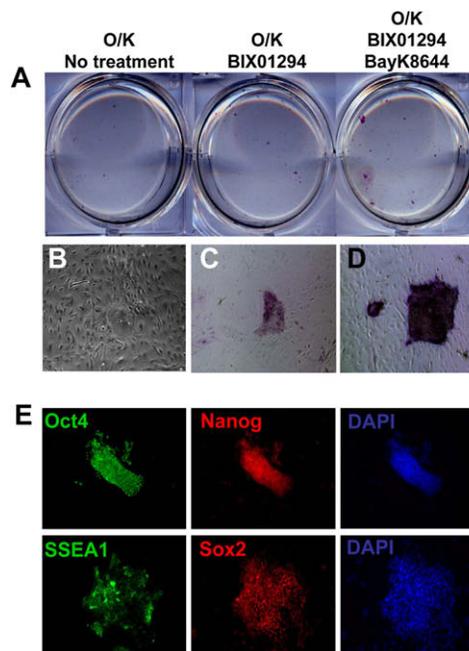


Figure 1. Screening for Small Molecules Inducing iPSCs from 129 MEFs after OK Viral Transduction

(A) Example of results obtained during screen confirmations. Shown here are photographs of 129 MEFs transduced with OK, untreated, treated with BIX alone, and treated with BIX/BayK combination after staining for ALP expression.

(B) Phase contrast image of 129 MEFs transduced with empty retrovirus (negative control); no colonies were observed.

(C) Image of 129 MEFs transduced with OK without small molecules; few small flattened colonies with weak ALP expression present.

(D) ESC-like iPSC colonies were observed 14–21 days after 129 MEFs were transduced with OK and treated with BIX/BayK; these ESC-like colonies exhibited strong ALP expression.

(E) iPSC colonies generated from 129 MEFs transduced with OK and treated with BIX/BayK expressed common ESC-specific markers; panels show iPSCs immunostained with Oct4, Nanog, Sox2, and SSEA1 antibodies. DAPI was used as nuclear staining.

protocol, but where BIX was added to the basal media after OK viral transduction. This provided a more permissive platform to identify new small molecules that could further improve reprogramming efficiency. More importantly, this second screen could facilitate discovery of small molecules that impact reprogramming in a more specific manner, for example, by acting on signal transduction pathways rather than on histone or DNA modifying enzymes. In this second screen, we again assayed a library of approximately 2000 known small molecules (see [Experimental Procedures](#)) and found two that were able to act with BIX to improve reprogramming, based on the criteria of the screen. One was RG108, a DNA methyltransferase (DNMT) inhibitor (Brueckner et al., 2005) that enhanced reprogramming of OK-transduced MEFs in the presence of BIX (Figure 2A). However, as this molecule, like BIX, is known to impact the cells at a general epigenetic level, and the DNA methyltransferase inhibitor 5-azacytidine has already been shown to enhance reprogramming (Mikkelsen et al., 2008), RG108 was not pursued further for this study. Instead, we focused our phenotypic and functional characterization on another small molecule that was identified in the

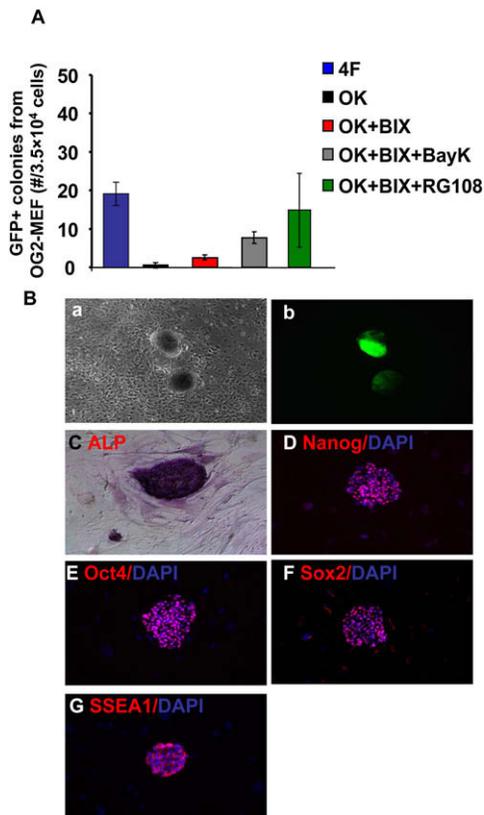


Figure 2. Generation of OK2B iPSCs from Primary OG2 MEFs

(A) Bar graph showing the average number of GFP⁺ colonies induced from OG2-MEFs in three independent experiments. This graph shows data for OG2 MEF cells transduced with four factors (Oct4, Klf4, Sox2, and cMyc; 4F); transduced with OK (OK); transduced with OK and treated with 1 μ M BIX (OK+BIX); transduced with OK and treated with 1 μ M BIX + 2 μ M BayK (OK+BIX+BayK); or transduced with OK and treated with 1 μ M BIX + 0.04 μ M RG108 (OK+BIX+RG108). $n = 3$; error bar represents standard deviation as calculated with Excel.

(B) Fourteen to twenty-one days after OK transduction and BIX/BayK treatment, GFP⁺ colonies were observed and are shown in a phase contrast (Ba) and a green fluorescence (Bb) image.

(C–G) Images of OK2B iPSC colonies showing ESC marker expression: ALP (C), Nanog (D), Oct4 (E), Sox2 (F), and SSEA1 (G). (D–G) Markers were revealed using an Alexa Fluor 555 secondary antibody. DAPI was used as nuclear staining.

second screen, BayK, an L-calcium channel agonist. This small molecule was studied further because it has no observable reprogramming activity on OK-transduced MEFs in the absence of BIX and is not known to impact the cells directly at the epigenetic level, but rather at the cell signal transduction level. Therefore, BayK might play a more specific role in the reprogramming process. When OK-transduced MEFs were treated with BIX in combination with BayK, a significant increase in the number and size of ALP⁺ colonies that closely resemble the mESC morphology could be observed (Figures 1A and 1D) as compared to OK-transduced MEFs treated with BIX alone. Further characterization of these primary iPSC colonies showed that they were positive for standard pluripotency markers such as Oct4, Sox2, Nanog, and SSEA1 (Figure 1E) as determined by immunofluorescence.

iPSCs Obtained from MEFs Transduced with OK and Treated with BIX/BayK Have Pluripotency Properties Characteristic of mESCs

To further confirm and characterize that OK transduction and BIX/BayK treatment can induce MEFs to become iPSCs, we used primary MEFs derived from OG2^{+/-}/ROSA26^{+/-} (OG2) transgenic mice, which contain an Oct4-GFP reporter (Do and Scholer, 2004). Once reprogrammed, these cells could then be used to conveniently assess chimera and germline competency. Similarly to 129 MEFs, OG2 MEFs transduced with OK could generate iPSCs when treated with a combination of BayK/BIX (OK2B iPSCs) (Figures 2A and 2B). GFP⁺ iPSC colonies could be first detected on day 14–21 after viral transduction and compound treatment. When OG2 MEFs were transduced with OK and not treated with any compounds, only a few small colonies appeared for an average of 0.5 ± 0.7 colony per 3.5×10^4 cells. These colonies were difficult to passage and therefore were not studied any further. Treatment of OK-transduced OG2 MEFs with BIX alone readily and reproducibly enabled reprogramming as compared to OK alone, with 2.5 ± 0.7 colonies per 3.5×10^4 cells (Figure 2A). There was a further significant improvement in the reprogramming efficiency when OG2 MEFs transduced with OK were treated with the combination of BIX (2 μ M) and BayK (2 μ M), where we observed 7.7 ± 1.5 colonies per 3.5×10^4 cells (Figure 2A). Treatment of OK-transduced OG2 MEFs with BayK alone, in the absence of BIX, did not increase reprogramming efficiency above OK-transduced MEF control (data not shown).

OK2B colonies were picked out and serially expanded on irradiated MEF feeder cells in the conventional mESC growth conditions in the absence of small molecules for more than 20 passages. Staining (Figures 2C–2G) and/or RT-PCR (Figure 3A) showed that these GFP⁺ OK2B iPSCs express typical pluripotency markers, including ALP, Nanog, Sox2, Oct4, SSEA1, c-Myc, eRas, Esg1, Ecat1, and Fgf4. RT-PCR assay also demonstrated that OK2B iPSCs expressed endogenous Oct4 and Klf4 (Figure 3A). Bisulphite genomic sequencing analyses of the Nanog promoter revealed that it is demethylated in OK2B iPSCs similarly to the mESC control (R1), while the MEFs' Nanog promoter was hypermethylated (Figure 3B). This result further suggests a reactivation of the stem cell transcription program in these OK2B iPSCs. In addition, transcriptome analysis showed that expression profile of OK2B iPSCs is greatly similar to the one of mESCs with a Pearson correlation value of 0.96, while significantly different to MEFs' profile with a Pearson correlation value of 0.84 as exemplified in the clustering analysis (see Figure S1 available online).

OK2B iPSCs Differentiate into Cells from All Three Germ Layers and Contribute to Germline Transmission

OK2B iPSCs could efficiently form embryoid bodies (EB) in suspension, which could differentiate into endodermal cells (Albumin and Pdx1), mesodermal cells/cardiac muscle cells (CT3), and ectodermal cells/neurons (β III-tubulin, Tuj1), derivatives of the three primary germ layers (Figure 4A). In addition, OK2B iPSCs could efficiently incorporate into the inner cell mass of a blastocysts (Figure 4Ba) following aggregation with an eight-cell embryo and lead to chimerism (Figure 4Bb) with germline contribution (Figure 4Bc) in vivo after the aggregated embryos

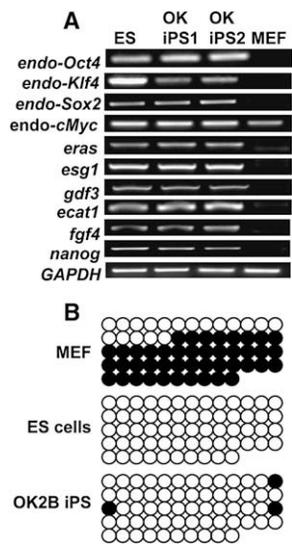


Figure 3. OK2B iPSCs Have a Transcriptional Profile Similar to R1 mESCs

(A) RT-PCR analysis of OK2B iPSCs indicated that they express genes specific to pluripotent mESCs. R1 mESCs were used as positive control, while OG2 MEFs were used as negative control. GAPDH was used as loading control.

(B) Bisulfite sequencing revealed that OK2B iPSCs' nanog promoter is demethylated, further suggesting a reactivation of endogenous genes specific to mESCs. Schematic depiction of the cytosine present in the region of the Nanog promoter that was amplified for this analysis. Open circle indicates demethylated cytosine, while filled/black circle indicates methylated cytosine.

were transplanted into pseudopregnant mice. Moreover, mating of one adult male progeny obtained from these blastocysts (Figure 4C) with a female CD1 wild-type mouse led to the production of LacZ⁺ progeny (Figure 4Da), three of which showed Oct4-GFP⁺ cells (Figure 4Db), further validating that these iPSCs could contribute to germline transmission (Figures 4B–4D). These in vitro and in vivo characterizations confirm retroviral transduction with only two genes, *Oct4* and *Klf4*, and in conjunction with BIX/BayK treatment are sufficient to reprogram MEFs into iPSCs, which are phenotypically and functionally similar to the classic mESCs.

DISCUSSION

The studies presented here provide a further demonstration that small molecules can be identified from rationally designed phenotypic screens to functionally replace viral transduction of certain TF(s), as well as improve reprogramming efficiency in generating iPSCs from a general cell type like MEFs. Such a chemical approach for the generation of iPSCs, which offers more precise and temporal control of the target/process, would be advantageous over the genetic manipulation with oncogenes that may also introduce harmful hard-to-detect insertional genomic alterations. Similar strategies are being used to find additional small molecules that may ultimately allow reprogramming of lineage-restricted cells to pluripotent or multipotent state in a completely chemically defined condition. BIX was originally identified and characterized as a specific inhibitor for G9a HMTase (Kubicek et al., 2007). It has been shown to reduce H3K9me2 levels at

G9a target genes (Feldman et al., 2006). Interestingly, histone H3K9 methylation, mediated by G9a, and heterochromatinization represent a highly specific mechanism for epigenetic silencing of embryonic genes such as Oct4 and Rex1 (Feldman et al., 2006). Furthermore, it was also demonstrated that knockdown of G9a can assist in fusion-based reprogramming of adult neuronal cells (Ma et al., 2008). It is therefore fitting that we previously observed that BIX can facilitate the generation of iPSCs from NPCs transduced with either OK or Klf4/Sox2/c-Myc (Shi et al., 2008), suggesting that it can compensate for the exogenous expression of Sox2 or Oct4. However, NPCs already express significant levels of Sox2, which might cause these cells to be more susceptible to reprogramming in the conditions mentioned above. This present study aimed at identifying small molecules that can enable reprogramming of MEFs, which do not express any of the TFs deemed necessary for reprogramming. It was fortuitous that we identified BIX in both the NPC and MEF screens, which further confirmed that this molecule has a role in enabling and improving the generation of iPSCs from somatic cells. Given BIX's characterized mechanism of action, our studies potentially identified a molecular target whose loss of function via pharmacological inhibition is sufficient to compensate for the gain of function of an essential iPSC reprogramming gene. It further mechanistically links a specific epigenetic process, inhibition of G9a-mediated H3K9me2, to iPSC generation. BIX may function to facilitate shifting of the epigenetic balance from a silenced state of pluripotency genes to an active transcription state for cell reprogramming. On the other hand, our observation that BayK, with a characterized activity as a specific L-type calcium channel agonist (Schramm et al., 1983), improves reprogramming efficiency is intriguing. L-type calcium channels are known to mediate intracellular processes in different tissues, such as blood pressure regulation, smooth-muscle contractility, insulin secretion, cardiac development, etc. (Tosti, 2006). Furthermore, activation of L-type calcium channels by different agonists, including BayK, has been shown to induce intracellular signaling through CREB activation, sarcoplasmic reticulum Ca²⁺ release, and change in cAMP activity. More importantly, some reports suggest that calcium might play a role in the control of mESC proliferation (Heo et al., 2006). However, in our hands, treatment of mESCs with 2 μM BayK alone or in combination with 1 μM BIX does not lead to a change in proliferation (Figure S2). Furthermore, treatment of OG2 MEF with 2 μM BayK alone or in combination with 1 μM BIX does not induce SOX2 expression (Figure S3). Needless to say, more work needs to be performed to dissect the precise mechanism by which BayK impacts the reprogramming process. However, it is interesting to find that a small molecule with activity in signaling pathways that have not been previously linked to reprogramming can significantly enhance its efficiency. So far, it is the first small molecule of its type to show an effect on reprogramming, as most of the other small molecules found up to date appear to directly modify the epigenetic status of the cell: i.e., BIX (Shi et al., 2008), valproic acid (Huangfu et al., 2008), and 5'azacytidine (Mikkelsen et al., 2008). Importantly, BayK seems to have several important characteristics that would be ultimately desirable for a molecule to be therapeutically relevant for in vivo reprogramming and/or regeneration. The fact that it does not act/reprogram on its own but needs the presence of BIX to exert its effects suggests that cells

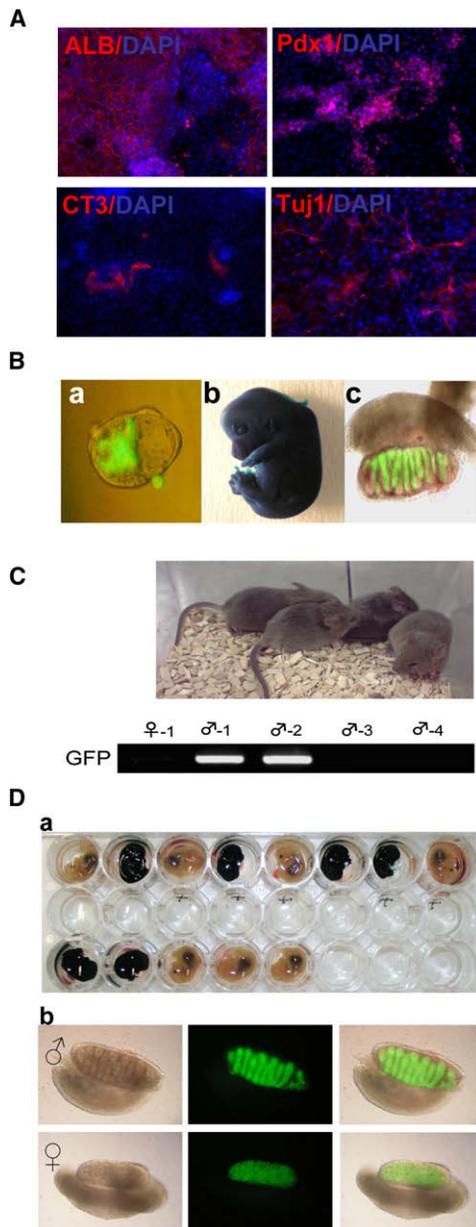


Figure 4. In Vitro and In Vivo Developmental Pluripotency of OK2B iPSCs

(A) OK2B iPSCs differentiate in vitro into cells from the three germ layers: endoderm (Albumin⁺ and Pdx1⁺), mesoderm (cardiac troponin T [CT3⁺]), and ectoderm (neurons, β III-tubulin [Tuj1⁺]).

(B) (Ba) iPSCs incorporate into the ICM of a blastocyst after aggregation with an eight-cell embryo. (Bb and Bc) Chimeric embryos (13.5 dpc) were obtained after transfer of the aggregated embryo into a pseudopregnant mouse (LacZ staining and Oct4 GFP⁺ cells show contribution from iPSCs that are OG2^{+/-}/ROSA26^{+/-}).

(C and D) OK2B iPSCs are germline competent. (C) The surrogate mother gave birth to a total of five pups (one female and four male). PCR on tail-tip tissues showed that two adult males were chimeras, since their genome contained the GFP cassette. PCR was performed because the eight-cell embryos used for aggregation were recovered from an agouti B6C3F1 mouse and MEFs for derivation of iPSCs were derived from OG2xROSA26 mice, from which F1 coat is mostly agouti color and seldom black; iPSCs may be derived from agouti color MEFs. These results clearly suggest that the OK2B iPSCs aggregated with the

that are already undergoing a form of reprogramming, perhaps caused by injury, might be more susceptible to its effect. This might allow us to ultimately reprogram the target cell in a more specific manner, without impacting healthy cells systemically, as direct epigenetic modifiers might.

In summary, we have identified a combination of two small molecules, BIX and BayK, that can enable MEF reprogramming into iPSCs in conjunction with the transduction of only two TFs: Oct4 and Klf4. This study further confirms the usefulness of a phenotypic screening approach in identifying small molecules that can effectively compensate for the viral transduction of a critical iPSC TF, such as Sox2. Ultimately, phenotypic small-molecule screens may lead to the identification of small molecules that will become powerful tools in providing us with new insights into the reprogramming process and may ultimately be useful to in vivo stem cell biology and therapy.

EXPERIMENTAL PROCEDURES

MEF Derivation

129S2/SvPasCrif or ROSA26^{+/-}/OG2^{+/-} MEFs were derived according to the protocol reported on the WiCell Research Institute website: "Introduction to human embryonic stem cell culture methods" (http://www.wicell.org/index.php?option=com_content&task=category&id=310&Itemid=149§ionid=16). Animal experiments were performed according to the Animal Protection Guidelines of the Max Planck Institute for Biomolecular Research, Germany.

Retrovirus Transduction and Compounds

pMX-based retroviral vectors for mouse Oct4, Klf4, c-Myc, and Sox2 were obtained from Addgene (Cambridge, MA). The viral production and transduction process was performed as described (Takahashi et al., 2007). The synthesis and full characterization of compound BIX-01294 was as previously described (Kubicek et al., 2007), and Bayk8644 was purchased from EMD/Calbiochem Biochemical (San Diego, CA).

Screen for iPSC Generation from MEFs

For the primary and secondary screens, a collection of known compounds was used. This collection was composed of roughly 2000 known bioactive molecules that are commercially available, including FDA-approved drugs, known inhibitors and activators of characterized enzymes (the LOPAC collection from Sigma-Aldrich [St. Louis, MO]), Known Bioactive Library from BIOMOL (Plymouth Meeting, PA), and nonoverlapping known compounds from EMD Calbiochem (San Diego, CA).

Primary 129S2/SvPasCrif (primary screen) or ROSA26^{+/-}/OG2^{+/-} (secondary screen) MEFs were plated onto Matrigel (1:50; BD Biosciences, Bedford, MA)-coated dishes at a density of 3.5×10^4 cells per well of a 6-well plate. Twenty-four hours later, these cells were transduced overnight with defined retroviruses at 37°C, 5% CO₂. Twelve to fourteen hours later, the media on the transduced cells were changed to mESC medium (knockout DMEM, 10% ES-qualified FBS, 10% knockout serum replacement, 1% Glutamax, 1% nonessential amino acids, penicillin/streptomycin, 0.1 mM β -mercaptoethanol, 1% EmbryoMax ESC Qualified Nucleosides [Millipore, Temecula, CA], and 10^3 U/ml LIF [Millipore]) (all products were from Invitrogen, Carlsbad, CA, except where mentioned). On that same day, individual small molecules from our known drug collection were added to the cells at a range between 0.5 and 2 μ M. Compound treatment was continued for 10–14 days; the cells were fixed and stained on day 14–21 using a standard ALP detection kit (Millipore). For the second screen, 1 μ M BIX was added to the mESC medium 1 day after transduction. Five days later, in addition to 1 μ M BIX, an individual small

eight-cell embryo contributed to adult chimeric mice. (Da) One male adult chimera was mated with a female CD1 wild-type mouse. X-gal staining indicated six germline transmitted F1 embryos, three of which exhibited Oct4-GFP⁺ germ cells, originating from OK2B iPSCs (Db). This result indicates that the OK2B iPSCs can contribute to germline transmission.

molecule from the known drug collection was added to each well, at a range between 0.5 and 2 μ M. Mouse ESC media with defined small molecules were refreshed every 3 days until colonies with a similar morphology to mESCs were observed, which was usually between 14 to 21 days after transduction. In addition to the confirmed compounds as indicated in the text, primary hits from the second synergist screen that were not further followed up also include PD173074, reversine, 5'azacytidine, pluripotin, and dexamethasone. Further characterization studies and repeats were carried out either on primary 129S2/SvPasCr1f or ROSA26^{+/+}/OG2^{+/+} MEFs. When ROSA26^{+/+}/OG2^{+/+} MEFs were used, the iPSC colonies could also be identified through GFP expression, as a marker of Oct4 expression. Once iPSC colonies were identified, they were picked for expansion on MEF feeder cells in mESC medium. Some colonies were expanded in the presence of the MEK inhibitor PD0325901 at concentration of 0.5–2 μ M to further confirm their pluripotentiality.

Immunocytochemistry and Immunofluorescence Assay

ALP staining was performed according to the manufacturer's instructions using the Alkaline Phosphatase Detection Kit (Millipore). For immunofluorescence assay, cells were fixed in 4% paraformaldehyde for 15 min at room temperature (RT) and washed with PBS. They were then incubated in blocking buffer (BB) (0.3% Triton X-100 [Sigma-Aldrich], 10% normal donkey serum [Jackson ImmunoResearch Laboratories Inc.] in PBS [Invitrogen]) 30 min at RT. They were then incubated with primary antibody overnight at 4°C in BB. Afterward, cells were washed with PBS and incubated with secondary antibody in BB for 45–60 min at RT. Primary antibodies were mouse anti-Oct4 (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-SSEA1 (1:200) (Santa Cruz Biotechnology Inc.), rabbit anti-Nanog (1:500) (Abcam Inc., Cambridge, MA), mouse anti-Sox2 (1:200) (Millipore), rabbit anti-Pdx1 (1:200) (a kind gift from Dr. C. Wright), mouse anti- β III-Tubulin (Tuj1) (1:500) (Covance Research Products Inc., Denver, PA), mouse anti-cardiac troponin T (CT3) (1:200) (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), and rabbit anti-albumin (DAKO). Secondary antibodies were Alexa Fluor 555 donkey anti-mouse or rabbit IgG (1:500) (Invitrogen). Nuclei were detected by DAPI (Sigma-Aldrich) staining. Images were captured using a Nikon Eclipse TE2000-U/X-cite 120 EXFO microscope with a photometric CoolSnap HQ² camera.

RT-PCR Assay

RNA was extracted from iPSCs and control cell lines using the RNeasy Plus Mini Kit in combination with QIAshredder. The RNA was converted to cDNA using iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Amplification of specific genes was done using primers previously published (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) and Platinum PCR SuperMix (Invitrogen) on a Mastercycler ep gradient PCR machine (Eppendorf).

Methylation Assay

DNA from R1 cells, OG2 MEFs, and OK iPSCs (passage 10) was isolated using the Non Organic DNA Isolation Kit (Millipore). The DNA was then treated for bisulfite sequencing with the EZ DNA Methylation-Gold Kit (Zymo Research Corp., Orange, CA). The treated DNA was then used to amplify sequences of interest. Primers used for promoter fragment amplification were as previously published (Blelloch et al., 2006). The resulting fragments were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen) and sequenced.

Aggregation of iPSCs with Zona-free Embryos

iPSCs were aggregated with denuded postcompacted eight-cell stage embryos to obtain aggregate chimeras. Eight-cell embryos (B6C3F1) were flushed from females at 2.5 dpc and cultured in microdrops of KSOM medium (10% FCS) under mineral oil. Clumps of iPSCs (10 to 20 cells) after short treatment of trypsin were chosen and transferred into microdrops containing zona-free eight-cell embryos. Eight-cell embryos aggregated with iPSCs were cultured overnight at 37°C, 5% CO₂. Aggregated blastocysts that developed from eight-cell stage were transferred into one uterine horn of a 2.5 dpc pseudopregnant recipient. One adult male chimera was mated with a female CD1 wild-type mouse. X-gal staining showed that six F1 embryos obtained from this natural mating of chimeric mouse and wild-type mouse were generated by germline transmission.

Statistical Analysis

Bar graphs and statistical analyses were performed using a standard Student's t test using Excel.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and three figures and can be found with this article online at [http://www.cellstemcell.com/supplemental/S1934-5909\(08\)00527-4](http://www.cellstemcell.com/supplemental/S1934-5909(08)00527-4).

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