A Small-Molecule Inhibitor of Tgf-β Signaling Replaces Sox2 in Reprogramming by Inducing Nanog

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SUMMARY

The combined activity of three transcription factors can reprogram adult cells into induced pluripotent stem cells (iPSCs). However, the transgenic methods used for delivering reprogramming factors have raised concerns regarding the future utility of the resulting stem cells. These uncertainties could be overcome if each transgenic factor were replaced with a small molecule that either directly activated its expression from the somatic genome or in some way compensated for its activity. To this end, we have used high-content chemical screening to identify small molecules that can replace Sox2 in reprogramming. We show that one of these molecules functions in reprogramming by inhibiting Tgf-β signaling in a stable and trapped intermediate cell type that forms during the process. We find that this inhibition promotes the completion of reprogramming through induction of the transcription factor Nanog.

INTRODUCTION

Retroviral transduction with three genes, Sox2, Oct4, and Klf4, can directly reprogram somatic cells to a pluripotent stem cell state (Okita et al., 2007; Takahashi et al., 2007b). Unfortunately, the resulting induced pluripotent stem cells (iPSCs) are suboptimal for applications in transplantation medicine and disease modeling because both the viral vectors used for gene transfer and the reprogramming factors they encode are oncogenic (Hacein-Bey-Abina et al., 2003; Nakagawa et al., 2008; Thrasher and Gaspar, 2007).

One potential solution is to identify small molecules that can efficiently reprogram cells and produce unmodified iPSC lines better suited for downstream applications as a result. Identification of such compounds would allow reprogramming that would not be impeded by the laborious nature of protein transduction or the safety concerns surrounding transgenic approaches (Kaji et al., 2009; Kim et al., 2009; Okita et al., 2008).

Several small molecules that catalyze reprogramming have already been described. Compounds that alter chromatin structure, including the DNA methyltransferase inhibitor 5-aza-cytidine (AZA) and the histone deacetylase (HDAC) inhibitor valproic acid (VPA), can increase reprogramming efficiency and even reduce the number of factors required for reprogramming (Huangfu et al., 2008a; Huangfu et al., 2008b; Mikkelsen et al., 2008; Shi et al., 2008b). Treatment with these inhibitors presumably lowers the barrier to activation of endogenous pluripotency-associated genes. However, Oct4 and Sox2 not only activate genes required for pluripotency, they also function to repress genes promoting differentiation. It is therefore unlikely that this class of small molecules would be sufficient to completely replace the transgenic factors. As a result, there remains a need to identify novel small molecules that can function in reprogramming.

Here, we report the discovery of compounds that can replace the central reprogramming factor Sox2. We demonstrate that one of these chemicals specifically acts by inhibiting Tgf-β signaling. Interestingly, this compound does not act by inducing Sox2 expression in the target fibroblasts. Instead, we show that it enables reprogramming through the induction of Nanog transcription in a stable, partially reprogrammed cell type that accumulates in the absence of Sox2.

RESULTS

A Screen for Chemical Mediators of Reprogramming

To identify small molecules that function in reprogramming, we transduced fibroblasts with viral vectors encoding Oct4, Klf4, and cMyc and then screened for compounds that allowed for reprogramming in the absence of Sox2. We favored this approach because it was unbiased with respect to the mechanism by which a given chemical could function and would not only deliver chemical compounds with translational utility but also provide novel insights into the mechanisms controlling reprogramming.

Activation of an Oct4::GFP reporter gene in colonies with an embryonic stem cell (ESC) morphology has been shown to be a stringent assay for reprogramming (Meissner et al., 2007). In
mouse embryonic stem cell (mESC) culture medium supplemented with VPA, retroviral transduction of 7500 Oct4::GFP transgenic mouse embryonic fibroblasts (MEFs) with Oct4, Klf4, cMyc, and Sox2 (Boilani et al., 2004) routinely generated 100–200 GFP+ colonies (Figure 1A). In contrast, we observed no GFP+ colonies when Sox2 was omitted (Figure 1A). We used this robust difference to identify small molecules that can replace Sox2.

To facilitate the identification of cellular targets and signaling pathways affected by any compounds we discovered, we utilized a library of molecules with known pharmacological targets. We transduced Oct4::GFP MEFs with Oct4, Klf4, and cMyc and

Figure 1. Identification of Small Molecules That Replace of Sox2
(A) Oct4::GFP+ colonies form readily in Oct4, Klf4, cMyc, and Sox2-infected MEF cultures and do not form in Oct4-, Klf4-, and cMyc-infected MEF cultures. Scale bars represent 500 μm.
(B) Overview of chemical screen for replacement of Sox2.
(C) A P0 colony from Oct4-, Klf4-, and cMyc-infected MEFs + RepSox that displays a mESC-like morphology and is Oct4::GFP+. Scale bars represent 200 μm.
(D) Number of Oct4::GFP+ colonies detected for each hit in the primary screen after transduction of Oct4, Klf4, and cMyc and VPA treatment.
(E) Chemical structures of E-616452, E-616451, and EI-275, with the optimal concentrations for reprogramming listed.
(F) Quantification of small-molecule replacement of Sox2 in Oct4-, Klf4-, and cMyc-infected MEFs with and without VPA treatment. The error bars denote the standard error derived from quantification of three separate wells of cells.
(G) Sox2 replacement by RepSox is not dependent on cMyc (no VPA treatment). The error bars denote the standard error derived from quantification of three separate wells of cells.
then plated 2000 cells per well in a 96-well format. To each well, we added one of 800 distinct compounds for 7–11 days and also treated each well with 2 mM VPA for the first 7 days (Figure 1B). It was our hope that this approach would allow us to identify both compounds that required chromatin remodeling to induce reprogramming (Huangfu et al., 2008a) and compounds that did not. After 16 days, we scored each well for the presence of GFP+ colonies with a mESC-like morphology (Figure 1C) and identified three independent hit compounds (Figure 1D). Two of these compounds were distinct transforming growth factor-β receptor 1 (Tgfr1) kinase inhibitors (E-616452 and E-616451 [Figure 1E] [Gellibert et al., 2004]), whereas the third was a Src-family kinase inhibitor (EI-275 [Figure 1E] [Hanke et al., 1996]).

Efficient Small-Molecule Replacement of Sox2

Next, we optimized the effective concentration for each hit molecule (Figure S1 available online) and quantified the efficiency at which it synergized with VPA to replace Sox2. When 1500 MEFs were transduced with only Oct4, Klf4, and cMyc and then treated with VPA, we did not observe GFP+ colonies (Figure 1F). However, the addition of E-616452 (25 μM), E-616451 (3 μM), or EI-275 (3 μM) led to the formation of GFP+ colonies with an ESC morphology at a rate that was comparable to transduction with Sox2 (Figure 1F).

Given that the three compounds were identified in the presence of VPA, we next determined whether these molecules were dependent on this HDAC inhibitor for their reprogramming activities. We found that E-616451 and EI-275 could not induce the appearance of GFP+ colonies in the absence of VPA (Figure 1F), whereas E-616452 could do so and at a rate that was similar to a positive control transduced with the Sox2 retrovirus (Figure 1F).

Although cMyc does increase the efficiency of reprogramming, it is not required for the generation of iPSCs (Nakagawa et al., 2008). Because the elimination of cMyc is an important step toward reducing the risk of tumor formation, we tested whether E-616452 could function in the absence of this oncogene. When added to MEFs transduced with only Oct4 and Klf4, E-616452 induced the formation of GFP+ colonies with an efficiency similar to viral Sox2 (Figure 1G).

Previous reports on small molecules that affect reprogramming have focused on MEFS or neural stem cells (NSCs). These cells may be reprogrammed more easily because of either their proliferative capacity or their expression of iPSC factors (Huangfu et al., 2008a; Shi et al., 2008a; Shi et al., 2008b). However, it may be that chemical modulation of gene expression is cell-type specific, and we therefore determined whether the reprogramming compound we identified functioned in a more patient-relevant cell type. When we infected adult tail tip fibroblasts with Oct4, Klf4, and cMyc alone, we did not observe Oct4::GFP+ colonies. However, when we added E-616452, we readily observed reprogramming (Figure S2A). The resulting Oct4::GFP+ colonies could be expanded into cell lines that maintained homogeneous Oct4::GFP expression and self-renewed similarly to mESCs and 4-factor control iPSC lines (Figure S2B).

Because it could efficiently replace transgenic Sox2 in the absence of VPA and cMyc, as well as in both embryonic and adult fibroblasts, we chose to further characterize E-616452 and named it RepSox, for Replacement of Sox2.

RepSox-Reprogrammed Cells Are iPSCs

Investigation of self-renewal capacity (Figure 2A), gene expression program, and pluripotency demonstrated that Oct4::GFP+ cells induced by the RepSox replacement of Sox2 were bona fide iPSCs. PCR with primers specific to the Oct4, Klf4, cMyc, and Sox2 transgenes confirmed that this cell line did not harbor transgenic Sox2 (Figure S3A). Chromosomal analysis indicated it was karyotypically normal (Figure S3B).

The Oct4::GFP+ cells coexpressed alkaline phosphatase (Figure S3C) and the endogenous alleles of the Nanog and Sox2 genes, suggesting pluripotency had been established (Figure 2B). The global transcriptional profile of cells reprogrammed with RepSox was similar to that of an iPSC line produced with all four transgenes and as similar to those of mESCs (Pearson correlation coefficient = 0.95–0.97) as two distinct mESC lines profiles were to each other (Pearson correlation coefficient = 0.96) (Figure 2C, Figure S3D, and Table S1). The profile differed significantly from that of the somatic MEFs (Figure 2C).

Cells produced with RepSox could readily form both embryoid bodies and teratomas that contained differentiated cell types of the three distinct embryonic germ layers (Figure 2E and Figure S4A). In addition, we observed that these cells could respond to directed differentiation signals in vitro and robustly differentiate into Hb9+/Tuj1+ motor neurons (Figure 2D and Figure S5).

In order to more definitively confirm the pluripotency of cells reprogrammed with RepSox, we tested their ability to contribute to chimeric embryos in vivo. We labeled cells with a lentiviral transgene encoding the dTomato red fluorescent protein and injected them into blastocysts. Both embryos and adult mice with significant contribution from the iPSCs were obtained (Figures 2F and 2G). Although adult mice with high contribution from the iPSCs were observed, we found it difficult to assess the contribution of these cells to the germline because the majority of animals developed tumors at or before the time of sexual maturity. However, we did observe that the reprogrammed cells could contribute Oct4::GFP+ cells to the genital ridges of embryonic chimeras, demonstrating contribution of these pluripotent cells to the germline (Figure 2H). Together, these results demonstrate that the RepSox-reprogrammed cells are indeed iPSCs.

RepSox Can Replace Sox2 and c-Myc by Inhibiting Tgf-β1 Signaling

Previous studies with RepSox suggest that it can act as an inhibitor of the Tgfr1 kinase (Gellibert et al., 2004). Therefore, we investigated whether the mechanism by which RepSox functions to replace Sox2 is through the inhibition of Tgf-β1 signaling. If Tgfr1 is the functional target of RepSox, then a structurally unrelated inhibitor of Tgf-β1 signaling or depletion of Tgf-β1 ligands from the culture medium might also replace Sox2. The small molecule SB431542 (Figure 3A) is known to inhibit Tgfr1 kinase and is structurally distinct from RepSox (Inman et al., 2002). When we treated fibroblasts transduced with Oct4, Klf4, and cMyc with 25 μM SB431542, we observed ~10 GFP+ colonies per 7500 cells plated (Figure 3B). Likewise, when we transduced fibroblasts in the presence of either an antibody that neutralized a variety of Tgf-β1 ligands (R&D Systems, AB-100-NA) or an antibody specific to Tgf-β1 II (R&D Systems, AB-12-NA), Oct4::GFP+ colonies were generated (Figure 3B). In contrast, we observed no GFP+ colonies in transductions without these Tgf-β1 inhibitors.
These results are consistent with the notion that at least part of the mechanism by which RepSox replaces Sox2 in reprogramming is through the inhibition of Tgf-β signaling.

Our goal was to identify molecules that specifically replace Sox2 instead of generally increasing reprogramming efficiency. If RepSox acts specifically to replace Sox2, then we would not expect it to stimulate reprogramming in the presence of transgenic Sox2. When RepSox- or Tgf-β antibody-treated MEFs were transduced with Oct4, Klf4, cMyc, and Sox2, we observed less than a 2-fold increase in the number of GFP+ colonies over the untreated controls (Figures 3C and 3D). The magnitude by which RepSox stimulated reprogramming in this context was significantly less than the 10-fold increase that we observed after treatment with VPA, a compound thought to increase reprogramming efficiency (Figure 1F).

In order to further investigate the specificity of Sox2 replacement by RepSox, we tested the ability of this molecule to individually replace Oct4, Klf4, and cMyc in reprogramming. We found...
that RepSox could not induce GFP+ colonies in the absence of either Oct4 or Klf4, even in the presence of VPA (Figure 3E). In contrast, we found that RepSox did increase the number of Oct4::GFP+ colonies by 20-fold in the absence of cMyc, thereby fully replacing it in reprogramming (Figure 3F). In addition, the structurally distinct Tgf-β inhibitor SB431542 and a Tgf-β-specific neutralizing antibody both increased reprogramming efficiency in the absence of cMyc (Figure 3G). From these experiments, we conclude that RepSox enables the replacement of the reprogramming activities provided by both transgenic Sox2 and cMyc. In both cases, these complementing activities seem to be mediated through the inhibition of Tgf-β signaling.

**RepSox Replaces Sox2 by Acting on Intermediates Formed During the Reprogramming Process**

The development of cocktails of small molecules that can effectively reprogram somatic cells may require a detailed knowledge of the mechanism and kinetics by which each compound acts. Therefore, we determined the optimal duration of time by which inhibition of Tgf-β signaling with RepSox can help induce reprogramming.

Initially, we pretreated MEFs with RepSox, applying the chemical for 3 days, and then removed it at the time of transduction with Oct4, Klf4, and cMyc. In these experiments, no Oct4::GFP+ colonies were formed (Figure 4A), suggesting that RepSox does not act on the initial somatic cells to replace Sox2. Consistent with this result, we did not detect a significant increase in the expression of endogenous Sox2 or closely related Sox family members upon RepSox treatment (Figure S6A). In addition, RepSox treatment did not decrease the expression of the mesenchymal gene Snai1 (Figure S6B), which is downregulated 5- to 40-fold by transduction of the four reprogramming factors (Mikkelsen et al., 2008). Thus, RepSox does not destabilize the pre-existing MEF transcriptional program.

In contrast, we found that RepSox did increase by 5-fold the expression of L-Myc, a close homolog of cMyc that can functionally replace it in reprogramming (Nakagawa et al., 2008) (Figure S6C). Together, these data suggest that although RepSox...
probably functions at the level of the initial somatic cell population to replace cMyc, it does not act on the starting MEF population to replace Sox2.

Because RepSox did not seem to act directly on the fibroblasts to replace Sox2, we investigated whether it functioned on intermediates that arose during reprogramming. To address this question, we varied both the duration and timing of RepSox treatment in order to determine when it was most effective. First, we transduced 7500 MEFs with Oct4, Klf4, and cMyc, and Sox2, and subsequently expanded ten iPSC-like, GFP-negative colonies (Figure 5A). These cell lines continued to proliferate for at least four passages and often maintained an iPSC-like morphology (Figure 5A), but never further activated expression of Oct4::GFP. However, when we treated these cell lines with a 48 hr pulse of RepSox, 5%–10% of the colonies in two of the ten lines became Oct4::GFP+ (Figures 5A and 5B). These results demonstrate that partially reprogrammed cells can accumulate in the absence of Sox2 expression, we reasoned that it might also be possible to culture these responsive intermediates for prolonged periods of time. Instead, if RepSox acts during a critical window on very transient intermediates, this might not be possible. To distinguish between these models, we transduced Oct4::GFP MEFs with Oct4, Klf4, and cMyc, waited 10-14 days, and subsequently clonally expanded ten iPSC-like, GFP-negative colonies (Figure 5A).

Next, we varied the timing at which we initiated RepSox treatment, by administering the compound beginning at day 4, 7, 10, 13, or 16 after transduction. We found that delaying the start of RepSox treatment increased its reprogramming potency, with optimal treatment beginning at 10 days posttransduction (Figure 4A). Together, these results suggest that RepSox treatment is most effective between days 7–12 posttransduction.

To more precisely define the optimal treatment window, we determined the minimal duration of treatment required to induce reprogramming. We found that a treatment as short as only 1 day was sufficient for inducing detectable reprogramming (Figure 4B). Delaying this short treatment yielded more reprogrammed colonies, with a sharp increase at day 11 (Figure 4B). These results indicate that RepSox is most effective at replacing Sox2 during days 10–11 after transduction and that therefore cultures of Oct4, Klf4, and cMyc-transduced MEFs give rise to intermediates capable of responding to RepSox treatment. These intermediates appear at day 4 posttransduction and peak at days 10–11.

Interestingly, when we tracked the timing of the initial appearance of reprogrammed colonies as a function of the timing of RepSox administration, we found that regardless of whether we began treatment at day 7 or day 10 posttransduction, Oct4::GFP colonies first appeared at day 14 (Figure S7). This suggests that RepSox may not always be the rate-limiting step in this reprogramming process and that other, RepSox-independent events take place during the formation of the RepSox-responsive intermediates.

**RepSox-Responsive Cell Lines**

Our finding that a 24 hr pulse of RepSox can replace Sox2 (Figure 4B) differs strikingly from the 5–10 day period of transgene expression normally required (Sridharan et al., 2009; Wernig et al., 2007) and suggests that RepSox could trigger a switch activating reprogramming. If RepSox acts to flip a switch in semistable intermediate cell types that accumulate in the absence of retroviral Sox2 expression, we reasoned that it might also be possible to culture these responsive intermediates for prolonged periods of time. In contrast, if RepSox acts during a critical window on very transient intermediates, this might not be possible. To distinguish between these models, we transduced Oct4::GFP MEFs with Oct4, Klf4, and cMyc, waited 10-14 days, and subsequently clonally expanded ten iPSC-like, GFP-negative colonies (Figure 5A). These cell lines continued to proliferate for at least four passages and often maintained an iPSC-like morphology (Figure 5A), but never further activated expression of Oct4::GFP. However, when we treated these cell lines with a 48 hr pulse of RepSox, 5%–10% of the colonies in two of the ten lines became Oct4::GFP+ (Figures 5A and 5B). These results demonstrate that partially reprogrammed cells can accumulate in the absence of Sox2 and that some, but not all, of these cells can be clonally expanded and cultured for prolonged periods while maintaining responsiveness to RepSox.

As we had shown that this particular reprogramming molecule seems to replace Sox2 through the inhibition of Tgf-β signaling, we sought to determine whether RepSox treatment affected Tgf-β signal transduction pathways in these responsive cell lines. To this end, we determined the levels of phosphorylated Smad3 by western blotting in cell line OKM 10 both with and without
RepSox treatment. Without RepSox treatment, we detected relatively high levels of phosphorylated Smad3, suggesting that Tgf-β signaling was active (Figure 5C). In contrast, treatment with 25 μM RepSox almost completely eliminated Smad3 phosphorylation (Figure 5C), indicating that RepSox strongly inhibited Tgf-β signaling in these cells.

Because an increase in cell proliferation can also increase reprogramming efficiency (Hong et al., 2009) and possibly contribute to the replacement of transgenic Sox2, we measured the proliferation rate of partially reprogrammed OKM 10 cells both with and without RepSox. Treatment with RepSox decreased the proportion of cells in G2/M phase of the cell cycle (Figure 5D), indicating it does not increase the proliferation rate of these partially reprogrammed cells.

Cells that Respond to RepSox Treatment Are Distinct from Previously Described Intermediates

It has been shown that certain nonpluripotent, partially reprogrammed cell lines derived from MEFs transduced with Oct4, Klf4, cMyc, and Sox2 can be fully reprogrammed with AZA or a combination of chemical inhibitors of glycogen synthase kinase 3β (GSK-3β) and the Mek signaling pathway (2i conditions) (Mikkelsen et al., 2008; Silva et al., 2008). If the RepSox-responsive cell lines generated by overexpression of Oct4, Klf4, and cMyc are similar to these four-factor cell lines, then they should also be reprogrammed by AZA or 2i. However, when we treated the ten stable intermediate lines with either AZA or 2i for 48 hr, we found that none became reprogrammed (Figure 5B), indicating the presence of two different types of stable intermediates in the reprogramming cultures.
Figure 6. RepSox Replaces Sox2 by Inducing Nanog Expression

(A) RepSox treatment of RepSox-responsive line OKMS 6 strongly increases Nanog mRNA levels. Data were generated by microarray analysis and are relative to untreated controls. Nanog is induced more quickly and more significantly than Sox2, indicating it is upregulated before fully reprogrammed cells form. The error bars denote the standard error derived from quantification of three separate wells of cells.

(B) RT-PCR analysis showing that inhibition of Tgf-β signaling increases Nanog expression in the RepSox-responsive intermediate line OKMS 7.

(C) A 48 hr pulse of RepSox induces a persistent increase in Nanog expression in the RepSox-responsive intermediate line OKM 10. OKM 10 cells were treated with 25 μM RepSox for 48 hr and RNA samples were taken at 0, 48, and 96 hr (48 hr after removal of RepSox) and analyzed by RT-PCR.

(D) shRNA-mediated knockdown of Nanog in OKM 10 cells inhibits replacement of Sox2 by RepSox. The error bars denote the standard error derived from quantification of two separate wells of cells.

(E) Pictures of reprogrammed Oct4::GFP+ colonies induced by Sox2 (A) or Nanog (B) transduction of line OKM 10. Scale bars represent 200 μm.

(F) Nanog transduction can reprogram line OKM 10 at an efficiency similar to that of Sox2 transduction. The error bars denote the standard error derived from quantification of three separate wells of cells.
that like the stable intermediate cells generated with only Oct4, Klf4, and cMyc, certain incompletely reprogrammed cells generated by Oct4, Klf4, cMyc, and Sox2 transduction can also be reprogrammed by RepSox.

Next, in order to determine whether these RepSox-responsive intermediate cell lines derived after Oct4, Klf4, cMyc, and Sox2 transduction were similar to or distinct from previously described partially reprogrammed cell lines (Mikkelsen et al., 2008), we applied AZA to all nine lines. After 48 hr of AZA treatment and 12 subsequent days in culture, none of the RepSox-responsive cell lines expressed Oct4::GFP (Figure 5F). However, one of the lines that had been refractory to RepSox treatment did express Oct4::GFP after AZA treatment, indicating that it had undergone complete reprogramming (Figure 5F). Together, these results show that there are a variety of intermediates that can form after retroviral transduction and that they vary in their responsiveness to reprogramming molecules.

**RepSox Replaces Sox2 by Inducing Nanog Expression**

The causal molecular events that drive reprogramming are difficult to detect because of the low efficiency at which somatic cells are successfully reprogrammed (Amabile and Meissner, 2009). However, when we administered RepSox to cell lines that had been partially reprogrammed by retroviral transduction, Oct4::GFP expression was induced in up to 33% of the resulting colonies (Figure 5F). We used this more efficient reprogramming system to identify the changes in gene expression induced by RepSox that enable it to bypass the requirement for transgenic Sox2 expression.

We treated an Oct4::GFP-negative, partially reprogrammed cell line (OKMS 6) with RepSox and performed global gene expression analysis at 10, 24, and 48 hr after the initiation of treatment. To confirm that RepSox was inhibiting Tgf-β signaling in this intermediate cell line, we investigated expression changes in known Tgf-β-responsive genes after RepSox treatment. The *Inhibition of Differentiation* genes Id1, Id2, and Id3 are repressed by Tgf-β signaling in mESCs (Ying et al., 2003). After treating the RepSox-responsive intermediate cell line OKM 10 with RepSox for 24 hr, we observed increased expression of Id1, Id2, and Id3 (Figure S9A).

One way that RepSox could function to replace transgenic Sox2 would be to induce the expression of endogenous Sox2 or a Sox family member, such as Sox1 or Sox3, that can substitute for it in reprogramming (Nakagawa et al., 2008). However, we again did not observe a significant increase in the expression of Sox1, Sox2, Sox3, or any of the remaining Sox family transcription factors within the first 48 hr of RepSox treatment (Figure S9B). Additionally, shRNA-mediated depletion of Sox1, the most potent Sox family member other than Sox2 itself (Nakagawa et al., 2008), did not affect the rate of reprogramming in the presence of RepSox (Figure S9C). These results show that RepSox does not replace Sox2 by directly activating endogenous Sox2 or other closely related genes.

Next, we more broadly investigated changes in transcription factor expression after chemical treatment. We did not observe an increase in endogenous Oct4 or Klf4 expression at early time points after RepSox treatment. However, we found that the expression of the homeodomain factor Nanog was among the most increased after RepSox treatment. Relative to untreated controls, Nanog transcription increased 4-fold within 24 hr and 10-fold after 48 hr of RepSox treatment (Figure 6A). In contrast, we did not observe a rapid increase in Nanog expression in two Oct4::GFP-negative intermediate cell lines that could not be fully reprogrammed with RepSox (Figure S10). Therefore, we hypothesized that RepSox might replace Sox2 by inducing Nanog expression.

Because we had determined that inhibition of Tgf-β signaling by several different small molecules and antibodies can replace Sox2, we reasoned that if the increase in Nanog expression was critical for Sox2 replacement, the alternative inhibitors of Tgf-β signaling should also upregulate Nanog. To test this hypothesis, we treated the RepSox-responsive cell lines with RepSox, SB431542, or neutralizing antibodies and analyzed Nanog expression after 48 hr. In all cases, Nanog expression was strongly induced within 48–96 hr (Figure 6B).

If RepSox functions by increasing Nanog expression, then a short pulse of RepSox should induce a persistent increase in Nanog expression. To test this, we treated the RepSox-responsive intermediate cell line OKM 10 with RepSox for 48 hr, withdrew RepSox, and analyzed Nanog expression 48 hr later. A control time point taken just before RepSox withdrawal showed a significant increase in Nanog transcription (Figure 6C). Forty-eight hours after RepSox removal (96 hr after the initiation of treatment), Nanog expression continued to increase (Figure 6C).

If RepSox replaces Sox2 by increasing Nanog expression, then a forced reduction of Nanog expression should inhibit or even prevent reprogramming by RepSox. To test this hypothesis, we transduced the RepSox-responsive cell line with a lentivirus encoding a short-hairpin RNA specific for Nanog. The Nanog knockdown cells reprogrammed at a frequency that was 50-fold lower than cells transduced with Oct4, Klf4, cMyc, Sox2, and the Nanog shRNA construct only suffered a 50% loss in reprogramming efficiency (Figure 6D). These results demonstrate that increased Nanog expression in this context was only necessary for the replacement of Sox2 by RepSox.

Previous reports have shown that chemical inhibition of Tgf-β signaling by SB431542 increases bone morphogenetic protein (Bmp) signaling in embryonic stem cells (Xu et al., 2008). It has separately been shown that Bmp signaling in the presence of Stat3 induces Nanog expression in mESCs (Suzuki et al., 2006). The crossstalk between the Tgf-β and Bmp signaling pathways may be the result of a common requirement for Smad 4, which mediates transcriptional events in the nucleus (Attisano and Wrana, 2002). Similarly, we observed an increase in the levels of phosphorylated Smad1 protein and Bmp-3 mRNA in incompletely reprogrammed intermediates after RepSox.

(G) Nanog can substitute for Sox2 in defined-factor reprogramming of somatic fibroblasts. The error bars denote the standard error derived from quantification of three separate wells of cells.

(h) Picture of a reprogrammed Oct4::GFP+ colony induced by Oct4, Klf4, cMyc, and Nanog transduction of MEFs. Scale bars represent 100 μm.
treatment (Figure S11). Furthermore, the stable, partially reprogrammed cells that responded to RepSox expressed the LIF receptor at levels equivalent to those found in mESCs (Figure S12A). Expression of this receptor suggests that its downstream signal transduction pathway could be active in these cells, thereby resulting in the presence of activated Stat3, which is known to induce Nanog expression in conjunction with Bmp signaling.

Because RepSox does not act on the initial population of fibroblasts to replace Sox2, we would not expect Nanog to be upregulated in RepSox-treated MEFs. Indeed, within 7 days of transduction of MEFs with Oct4, Klf4, and cMyc, we did not observe an increase in Nanog expression upon RepSox treatment (Figure S12B). This may be explained in part by the observation that the LIF receptor, and thus activated Stat3, was not highly expressed in these cells (Figure S12A). Because Nanog plays a key role in maintaining ESCs in an undifferentiated state (Chambers et al., 2003) and has been shown to enhance the efficiency of reprogramming (Silva et al., 2006; Silva et al., 2009; Yu et al., 2007), we decided to test whether Nanog could directly replace Sox2 in reprogramming.

If RepSox replaces Sox2 by inducing Nanog expression, then retroviral transduction of RepSox-responsive intermediate cells (line OKM 10, Figures 5A and 5B) with Nanog should reprogram them. When we transduced line OKM 10 with Sox2 as a control, 0.2% of the colonies expressed Oct4::GFP after 10 days, indicating that reprogramming could be induced in this cell line by Sox2 (Figures 6E and 6F). When we transduced the same stable intermediate cell line with Nanog, it could also be reprogrammed, with 0.3% of the colonies expressing Oct4::GFP after 10 days (Figures 6E and 6F). In contrast, transductions with Oct4 or Klf4 resulted in only 0.04% and 0% reprogramming efficiencies (Figure 6F). These results suggest that Nanog can indeed functionally replace Sox2 and induce reprogramming in these stable intermediates formed from Oct4-, Klf4-, and cMyc-transduced MEFs.

If Nanog can compensate for the omission of Sox2 in defined-factor reprogramming, then MEFs transduced with Oct4, Klf4, cMyc, and Nanog might be as efficiently reprogrammed as MEFs transduced with Oct4, Klf4, cMyc, and Sox2. When we transduced MEFs with Oct4, Klf4, cMyc, and Sox2 and scored cultures 9 days later, an average of seven Oct4::GFP+ colonies appeared for every 7500 cells plated (Figure 6G). A control transduction with only Oct4, Klf4, and cMyc yielded no Oct4::GFP+ colonies (Figure 6G). Similar to the positive control transduction, MEFs transduced with Oct4, Klf4, cMyc, and Nanog gave rise to an average of five Oct4::GFP+ colonies for every 7500 cells plated (Figures 6G, H). These colonies could be picked and expanded and remained Oct4::GFP+ for at least five passages (Figure S13A). Immunocytochemistry indicated that these cells strongly activated Sox2 expression from the endogenous allele (Figure S13B). Importantly, QPCR analysis demonstrated that they also transcribed endogenous Oct4, Klf4, Nanog, and Rex1 (Figure S13C), indicating that a pluripotent gene expression program had been established. Furthermore, transgene-specific QPCR analysis showed that these cells had silenced the retroviral Oct4, Klf4, and cMyc transgenes (Figure S13D). Additionally, Oct4-, Klf4-, cMyc-, and Nanog-reprogrammed cells could readily form embryoid bodies in vitro (Figure S13E). However, we found that leaky expression of transgenic Nanog, which is a potent inhibitor of ESC differentiation (Chambers et al., 2003; Chambers et al., 2007), reduced the amount of differentiation in vitro (Figure S13D). We anticipate that efficient differentiation of cells created with Oct4, Klf4, cMyc, and Nanog will eventually require the use of an excisable transgenic Nanog cassette to completely remove ectopic Nanog expression. Although definitive proof of the pluripotency of these cells will be required to conclude that Nanog expression is sufficient for replacing Sox2 in defined factor reprogramming, our results suggest that this may be the case. Taken together, our results demonstrate that RepSox inhibition of Tgf-β signaling bypasses the need for Sox2 in defined-factor reprogramming through the induction of Nanog.

DISCUSSION

We have used a phenotypic chemical screen to identify compounds that can replace the reprogramming transcription factor Sox2 and have confirmed the mechanism by which the most potent compound acts: RepSox replaces Sox2 by inhibiting the broadly expressed Tgf-β signaling pathway (Attisano and Wrana, 2002) in cultures containing stable intermediate cells that are trapped in a partially reprogrammed state. This inhibition in turn leads to sustained transcription of Nanog, through which reprogramming is achieved in the absence of Sox2. These results demonstrate the feasibility of replacing the central reprogramming transgenes with small molecules that modulate discrete cellular pathways or processes rather than by globally altering chromatin structure. Furthermore, they show that the mechanisms by which these molecules act in reprogramming can be distinct from those of the factor(s) that they replace.

Importantly, and unlike many other studies (Mikkelsen et al., 2008; Shi et al., 2008a; Shi et al., 2008b; Utikal et al., 2009), the approach that we report here for replacing Sox2 did not rely on procurement of a highly specialized or rare cell type that already expresses Sox2. Furthermore, treatment with RepSox allowed the generation of iPSCs from both adult and embryonic fibroblasts with a frequency comparable to that of transduction with Sox2. Thus, reprogramming efficiency does not need to be compromised by small-molecule replacement of transgenic factors.

We observed that instead of working on the initial fibroblast population to replace Sox2, RepSox acts on cellular intermediates formed by overexpression of Oct4, Klf4, and cMyc. Without RepSox treatment, these intermediates are trapped in an unproductive state. Unlike previously described partially reprogrammed cells (Mikkelsen et al., 2008; Silva et al., 2009), the RepSox-responsive intermediates could not be reprogrammed with AZA or 2i treatment, suggesting that they are distinct. In addition, we found that RepSox does not target any of the kinases inhibited by the 2i cocktail, indicating that it works through a different mechanism. Furthermore, four-factor intermediates that reprogram with RepSox treatment are not responsive to AZA, indicating that they also are distinct.

These findings demonstrate that reprogramming can proceed in a stepwise fashion through different intermediates. Just as in a geographical setting in which there are multiple routes to travel from point A to point B, there exist different intermediate states
or “way stations” that somatic cells can transit through on the way to complete reprogramming. Interestingly, although our results indicate that defined-factor reprogramming with Oct4, Klf4, cMyc, and Sox2 can occur in the absence of Nanog, its induction is required for chemical reprogramming of both our RepSox-responsive intermediates and the recently described 2i-responsive intermediates made from Oct4, Klf4, and cMyc transduction of cells that express Sox2 endogenously (Silva et al., 2009). This indicates that commonalities can exist in the reprogramming routes used by some sets of distinct intermediates.

Originally, we found it surprising that Nanog was not included in the initial set of defined reprogramming factors (Takahashi and Yamanaka, 2006) given its critical role in maintaining pluripotency in ESCs (Boyer et al., 2005; Chambers et al., 2003) and its ability to stimulate reprogramming by cell fusion (Silva et al., 2006). However, Takahashi and Yamanaka reported that a combination of nine factors that included Oct4, Klf4, cMyc, and Nanog, but not Sox2, generated iPSC colonies at a detectable rate (Takahashi and Yamanaka, 2006). This combination of factors included other genes that may have inadvertently lowered the rate of reprogramming, thereby causing the combination of Oct4, Klf4, cMyc, and Nanog to be overlooked. Consistent with these data, work by Niwa and coworkers with inducible Sox2 null ESCs demonstrated that Sox2 is dispensable for modulation of the Oct-Sox enhancers that regulate pluripotent-specific gene expression and instead mainly governs pluripotency in ESCs by regulating the expression of Oct4 through other factors (Masui et al., 2007). Therefore, it is possible that Nanog may alleviate the requirement for Sox2 in reprogramming by stimulating or maintaining Oct4 expression. Indeed, Nanog is capable of maintaining Oct4 expression in mESCs (Chambers et al., 2003). Thompson and coworkers also reported that Nanog expression enhanced the reprogramming of human fibroblasts but that it was not able to replace Sox2 in the presence of only OCT4 and LIN-28 (Yu et al., 2007). This may indicate that Klf4 is required for Nanog to function optimally in reprogramming and suggests that either they or the genes they modulate interact during the reprogramming process.

It is well known that ~90% of genes with promoters bound by OCT4 and SOX2 in human ESCs are also bound by NANOG (Boyer et al., 2005). Our result suggests that either Nanog or Sox2 may be sufficient for collaborating with Oct4 to modulate these genes and drive reprogramming. Although Nanog is not required for pluripotency, it safeguards ESCs against neuroectodermal and, to a more limited extent, mesodermal differentiation (Chambers et al., 2007; Vallier et al., 2009). Therefore, it is possible that Nanog functions in reprogramming by repressing differentiation signals, thereby assisting in the transition to an undifferentiated state.

Interestingly, we found that RepSox is also able to functionally replace cMyc in reprogramming. Together, these observations highlight the fact that small molecules may functionally replace reprogramming transcription factors at either early or late stages of the process and that they can act by different mechanisms—by inducing the expression of the gene itself, a closely related family member, or an unrelated gene that can functionally rescue the omission of the reprogramming transcription factor.

Our observation that a 1 day treatment with RepSox can relieve the requirement for transgenic Sox2 indicates that unlike reprogramming with transgenic Oct4, Klf4, and Sox2, in which each transgene must be expressed for several days (Sridharan et al., 2009; Stadtfeld et al., 2008), small molecules can act as switches to induce stable gene expression changes that promote the completion of reprogramming. This could be an important concept for achieving purely chemical reprogramming given that our data show that chemicals such as RepSox can affect cellular processes differently depending on the timing of administration.

As we have shown here, there need not always be a discrete, one-to-one mapping between the functions of the reprogramming factors and their chemical replacements. Thus, it may be that reiterative screening in the presence of Sox2 replacement molecules will be required for identifying compounds that can act in concert to replace Oct4 and Klf4. However, it will be of significant interest to determine whether the novel reprogramming compounds we have identified can collaborate with those previously described (Marson et al., 2008; Shi et al., 2008a; Silva et al., 2008) to replace the remaining reprogramming genes, opening a route to purely chemical reprogramming.

**EXPERIMENTAL PROCEDURES**

**Retroviral Infection**

Retroviral infections were performed as previously described with the pMXs vector (Takahashi et al., 2007a). MEFs were infected with two to three pools of viral supernatant during a 72 hr period. The first day that viral supernatant was added was termed “day 1 post-infection.” For quantification, Oct4::GFP+ colonies were counted at day 30 postinfection unless otherwise stated. All animal research was performed under the oversight of the Office of Animal Resources at Harvard University.

**Small-Molecule Screens**

On day 4 postinfection, infected MEFs were trypsinized and reseeded on irradiated feeders in 96-well plates at 2000 cells/well and cultured in mouse ESC media (Knockout DMEM, 15% FBS, L-glutamine, penicillin/streptomycin, nonessential amino acids, 50 mM 2-mercaptoethanol, and 1000 U/ml LIF). The next day, compound stock solutions diluted in DMSO and VPA (Sigma) were added at a final concentration of 1 µM and 2 mM, respectively. VPA was removed after 1 week, and the compound was reapplied every other day with each media change. Plates were scored for GFP+ colonies after 11 days of compound treatment.

**Quantification of Oct4::GFP+ iPSCs Generated with Small-Molecule Hit Compounds, SB431542, and Tgf-β Antibodies**

Retroviral infection and compound or antibody treatment was performed as in the original chemical screen. For quantification of the numbers of GFP+ colonies produced in different conditions, the number of colonies in each well was counted and at least two different wells were counted and averaged. Concentrations of compounds and antibodies were as follows: VPA (Sigma), 2 mM; RepSox (Calbiochem), 25 µM or 1 µM as noted; E-616451 (Calbiochem), 3 µM; E1-275 (Biomol), 3 µM; SB431542 (Sigma), 25 µM or 2 mM as noted; TgfβIII-specific antibody (R&D Systems, AB-12-NA), 10 µg/ml; and pan-Tgfβ antibody (R&D Systems, AB-100-NA), 10 µg/ml. Unless otherwise noted, all chemical treatments were continuous from initial administration at day 4–5 postinfection until GFP+ colonies were scored at day 30 posttransduction. Fresh chemical was added at each media change.

**Chemical Reprogramming of Stable Intermediate Cell Lines**

Oct4::GFP-negative colonies in Oct4, Klf4, and cMyc or Oct4+, Klf4+, cMyc-, and Sox2+-infected MEF cultures were picked and plated on irradiated feeders, and single colonies were picked after 1 week. The resulting cell lines were...
passaged with trypsin and grown in mESC media on feeders until passage 4, at which time they were treated with RepSox (25 μM), AZA (500 μM), or both for 48 hr. For 2i treatment, CHIR99021 (Stemgent) was used at 3 μM and PD0325901 (Stemgent) was used at 1 μM. Oct4::GFP+ colonies were scored 12 days after the beginning of chemical treatment. Treatments were performed in mESC media containing FBS unless otherwise noted.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 13 figures, and 2 tables and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00508-6.

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