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Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4

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Contributed by Peter G. Schultz, April 9, 2009 (sent for review February 27, 2009)

Ectopic expression of defined transcription factors can reprogram somatic cells to induced pluripotent stem (iPS) cells, but the utility of iPS cells is hampered by the use of viral delivery systems. Small molecules offer an alternative to replace virally transduced transcription factors with chemical signaling cues responsible for reprogramming. In this report we describe a small-molecule screening platform applied to identify compounds that functionally replace the reprogramming factor Klf4. A series of small-molecule scaffolds were identified that activate Nanog expression in mouse fibroblasts transduced with a subset of reprogramming factors lacking Klf4. Application of one such molecule, kenpaullone, in lieu of Klf4 gave rise to iPS cells that are indistinguishable from murine embryonic stem cells. This experimental platform can be used to screen large chemical libraries in search of novel compounds to replace the reprogramming factors that induce pluripotency. Ultimately, such compounds may provide mechanistic insight into the reprogramming process.

chemical biology | high-throughput screening | Nanog

Recently it has been demonstrated that forced expression of combinations of 4 transcription factors—Oct4, Klf4, Sox2, and c-Myc or Oct4, Sox2, Nanog, and Lin28—can reprogram somatic cells into induced pluripotent stem (iPS) cells that closely resemble embryonic stem cells (ES cells) (1–4). The induction of pluripotency in somatic cells by ectopic expression of these 4 transcription factors has created new opportunities in the stem cell field. For example, patient-derived pluripotent cells ultimately may provide a personalized source of tissue to replace cells lost to degenerative and age-associated diseases (5). However, the clinical use of iPS cells is hindered by the use of virally transduced transcription factors and proto-oncogenes.

Optimization of the original reprogramming method has achieved reprogramming of both mouse and human somatic cells with the 3-factor combination of Oct4, Sox2, and Klf4 in the absence of c-Myc (6–8). Other methods to reduce the number of reprogramming factors have taken advantage of endogenously expressed reprogramming factors, thereby precluding the need for ectopic expression of these factors, such as Sox2 (9–11). More recently, iPS cells have been generated using excisable vectors (12–14), nonintegrating vectors (15, 16), and transient transfection approaches (17). Although these methods have solved some of the limitations posed by the presence of multiple proviruses in the genome of iPS cells, they either remain inefficient or fail to further our understanding of the mechanistic details of epigenomic reprogramming.

Small molecules that induce epigenetic changes also have been used to increase the efficiency of reprogramming and/or replace reprogramming factors (18–22). Recently, the histone deacetylase inhibitor valproic acid was found to allow for 2-factor reprogramming (Oct4 and Sox2) of human fibroblasts in the absence of Klf4 and c-Myc (18, 19). This method relies on the use of known molecules that target known mechanisms. The identification of novel small molecules that induce pluripotency could provide useful chemical tools to unravel and study the molecular mechanisms governing this process. Furthermore, application of such molecules ultimately may lead to useful new therapeutics for treating disease. Toward this end, we have developed a high-throughput screening strategy to identify small molecules that can functionally replace reprogramming transcription factors. In this paper we describe the identification of a compound class that can substitute for the reprogramming factor Klf4 in the formation of iPS cells. Our results provide proof of principle that high-throughput screening is a robust vehicle for identifying small molecules that can replace the transcription factors used to reprogram somatic cells.

Results

Nanog-Luciferase Reporter Mouse Strain. The induction of pluripotency in fibroblasts by the ectopic expression of Oct4, Sox2, Klf4, and c-Myc requires a minimum of 8–12 days and occurs at low frequency, with <0.1% of the somatic cells giving rise to iPS cells (4, 23). The slow kinetics of reprogramming impedes the development of robust assays to screen large chemical libraries with a reliable readout that can be captured on a well-to-well basis in a miniaturized format. To overcome these limitations, we relied on the high sensitivity and quantitative readout capabilities of luciferase to develop a screening platform. To establish a sensitive readout for iPS cell formation, the firefly luciferase gene was inserted into the Nanog locus by homologous recombination. We chose Nanog because it plays important roles in maintaining the undifferentiated state (24, 25), is completely inactivated in somatic lineages, and iPS cells selected for Nanog reactivation demonstrate complete developmental potential (1, 2, 4).

A targeting vector was constructed using 1.2 kb of the Nanog promoter as a 5′ arm and 1.4 kb of Nanog intron 2 as a 3′ arm. The firefly luciferase gene from pGL3-basic and a floxed


Conflict of interest: R.J. is an advisor to Stemgent and Fate.

1C.A.L., R.K.F., and J.S. contributed equally to this work.

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pgk-neo cassette were inserted between the targeting arms (Fig. L4). Nanog-luciferase (NL) ES cell clones that carried the targeting construct in the Nanog locus, as confirmed by Southern blot analysis (Fig. 1B), gave rise to a signal 10–30 times greater than that of wild-type V6.5 ES cells (Fig. 1C). The NL ES cells were introduced into blastocysts to generate chimeric mice, which were bred to generate both transgenic mice and mouse embryonic fibroblasts (MEFs). NL-MEFs produced negligible amounts of luciferase (Fig. 1C) and were used for subsequent experiments.

**High-Throughput Chemical Screening Strategy: Klf4 Replacement.** In this work, we customized our screening strategy to identify compounds that could replace Klf4 from those that simply activate Nanog expression, colony formation was used as a secondary assay. Representative compounds from each lead structural class were added to OSM-transduced MEFs and analyzed for the presence of colonies 10 days later. Members of each structural class induced the conversion of somatic cells to iPS cells, it does not prove the existence of a chemical replacement for Klf4. To distinguish compounds that replace Klf4 from those that simply activate Nanog expression, colony formation was used as a secondary assay. Representative compounds from each lead structural class were added to OSM-transduced MEFs and analyzed for the presence of colonies 10 days later. Members of each structural class induced the conversion of somatic cells to iPS cells, it does not prove the existence of a chemical replacement for Klf4.

To identify a chemical replacement for Klf4, NL-MEFs were transduced with a subset of reprogramming factors comprising Oct4, Sox2, and c-Myc (OSM). To obtain the ~10^6 cells needed to screen large chemical libraries required expansion of OSM-transduced NL-MEFs for 3 passages before screening. At passage 4, OSM-transduced NL-MEFs were seeded into 1,536-well plates at 500 cells/well in mouse ES cell growth media supplemented with leukemia inhibitory factor (LIF; 20 ng/mL) and screened against a large (500,000 compounds), chemically diverse small-molecule library (30). Compounds were added at a final concentration of 2.2 μM (20 nL of 1 mM stock solutions in 9 μL of media) immediately after plating, and Nanog-driven luciferase expression was assayed 10 days later (Fig. 1D). Analysis of a focused library in pilot experiments demonstrated a high degree of homogeneity of luciferase response from well to well—10^6 molecules in triplicate [supporting information (SI) Fig. S1]. Furthermore, normalization of these data using the “jackknife” z-score method—which accounts for plate-to-plate variation and does not rely on positive control readings—and subsequent analysis using the 1-sample Kolmogorov-Smirnov test (P = .54) indicated little difference between the empirical and theoretical inverse gamma distributions. These statistical analyses confirmed that the screening platform was robust and that the output data were highly reliable (31).

**Activity of Lead Compounds.** To distinguish compounds that replace Klf4 from those that simply activate Nanog expression, colony formation was used as a secondary assay. Representative compounds from each lead structural class were added to OSM-transduced MEFs and analyzed for the presence of colonies 10 days later. Members of each structural class induced the conversion of somatic cells to iPS cells, it does not prove the existence of a chemical replacement for Klf4. To distinguish compounds that replace Klf4 from those that simply activate Nanog expression, colony formation was used as a secondary assay. Representative compounds from each lead structural class were added to OSM-transduced MEFs and analyzed for the presence of colonies 10 days later. Members of each structural class induced the conversion of somatic cells to iPS cells, it does not prove the existence of a chemical replacement for Klf4.
Consistent with observations using NL-MEFs, Nanog expression was activated in kenpaullone-treated OSM-transduced MEFs in a time-dependent manner (Fig. 2C and Fig. S2).

After 20 days of concurrent kenpaullone treatment, colonies expressing Oct4 from their endogenous locus were selected upon supplementation of the culture media with neomycin (Fig. 2C). Kenpaullone was able to replace Klf4, as demonstrated by the appearance of Oct4-neo colonies, although the reprogramming was less efficient than after transduction with a Klf4 vector. Likewise, OSM transduction and kenpaullone treatment of MEFs carrying a GFP reporter in the Oct4 locus, O4G-MEF (33), gave rise to GFP-positive colonies (Fig. 3A) with similar efficiency and kinetics as observed in the O4N-MEFs (Fig. 3B).

The DMSO-treated OSM-transduced O4G-MEFs did not display GFP-positive cells or colony formation even after 40 days in culture (data not shown).

**Characterization of iPS Cells.** iPS cells generated by OSM and kenpaullone treatment were indistinguishable from ES cells by morphological criteria and expressed the pluripotency-associated markers Oct4 and Nanog from the endogenous loci (Fig. 3A). However, the reprogramming efficiency of kenpaullone-treated OSM-transduced O4G-MEFs was 10-fold lower than that of Klf4-infected controls (Fig. 3B). To confirm the activity of kenpaullone in an independent culture system, we used “secondary” MEFs that carry doxycycline-inducible proviruses encoding Oct4, Sox2, and c-Myc (34). The results, shown in Fig. 3C, indicate that kenpaullone induced reprogramming of secondary OSM-MEFs with a similar efficiency as primary infected OSM-MEFs. The reprogramming kinetics of kenpaullone-treated iPS cell formation was delayed, however; doxycycline-independent iPS cells appeared only after 25–30 days, compared with the 15 days observed for the 4-factor control iPS cells. This slight kinetic delay in reprogramming suggests that kenpaullone is less efficient in epigenetic reprogramming than Klf4.

Once established, Klf4-free iPS cells grew in the absence of doxycycline and kenpaullone while maintaining ES cell morphology and Nanog and Oct4 expression. To test for pluripotency, the cells were injected into blastocysts and were found to generate germline-competent chimeras (Fig. 3A and data not shown).

It has been shown that mouse neural progenitor cells (NPCs) that express endogenous Sox2 can be reprogrammed to a pluripotent state with the addition of Oct4 and Klf4 +/- c-Myc (9, 11) or simply Oct4 (10), albeit with delayed kinetics and low efficiency. To determine the functionality of kenpaullone in the reprogramming of neural cells, NPCs were transduced with all combinations of Oct4, Klf4, and c-Myc and treated with 5 μM kenpaullone or vehicle control. Eight days later, discernible...
AP-positive colonies had formed in Oct4/c-Myc/kenpaullone-treated wells that, when passaged, appeared morphologically indistinguishable from ES cells and stained positive for the pluripotency-associated markers Nanog, Sox2, and AP (Fig. 3D). Furthermore, these cells no longer expressed GFP from the virally delivered c-Myc vector (pCMV-Myc-IRES-GFP; Fig. S3), consistent with retroviral silencing observed in reprogrammed cells (4). The efficiency of iPSC cell formation from NPCs was lower with kenpaullone than with Klf4 gene transduction, although not as low as in MEFs (Fig. 3B and E). It is also noteworthy that neither MEFs nor NPCs reprogrammed in the absence of c-Myc when kenpaullone was substituted for Klf4. Collectively, these results suggest that kenpaullone requires transduction of c-Myc for reprogramming and emphasizes that kenpaullone may not completely recapitulate the role of Klf4.

**Biological Activity of Kenpaullone.** Previous work with kenpaullone has demonstrated a wide range of biological utility, extending from maintenance of pancreatic β cell survival and proliferation (35) to the induction of apoptosis in cancer cells (36). Kenpaullone’s diverse range of activity is a direct result of its kinase inhibition promiscuity (SI Text, Tables S1 and S2). It is a potent inhibitor of GSK-3β (23 nM), CDK1/cyclin B (400 nM), CDK2/cyclin A (680 nM), and CDK5/p35 (850 nM) and exhibits inhibitory activity toward various other kinases at higher concentrations (36, 37).

To determine whether kenpaullone’s reprogramming activity results from its well-established role as a GSK-3β or CDK inhibitor, a highly selective small-molecule inhibitor of GSK-3β (CHIR99021), a promiscuous inhibitor of CDKs (purvalanol A), or both were applied to OSM-transduced O4N-MEFs and compared against kenpaullone. As shown in Fig. 3F, known inhibitors of these kinases were unable to replace Klf4 in the reprogramming of murine fibroblasts and had negligible activity in the NL reporter MEF line (Fig. S4). It is interesting that kenpaullone, like CHIR99021, increased the efficiency of 4-factor reprogramming (Fig. 3F). This observation is consistent with reports indicating that GSK-3β inhibitors can help facilitate the self-renewal of mouse ES cells (38) and the reprogramming of mouse NPCs back to the pluripotent state (11). But because other, more specific GSK-3β inhibitors were not able to activate Nanog expression (Fig. S4 and Table S3) or to replace Klf4 (Fig. 3F), this activity likely is independent of the reprogramming activity of kenpaullone.

In addition to small-molecule–mediated inhibition, short hairpin RNA (shRNA)-targeted ablation of each of the canonical kenpaullone kinase targets individually, combinatorially or in a pooled format did not result in colony formation or an increase in Nanog-driven luciferase expression in OSM-transduced MEFs (Fig. S5). Collectively, these data strongly suggest that kenpaullone’s activity does not result from its well-documented role as a GSK-3β or cell cycle inhibitor. Finally, kenpaullone does not directly activate Klf4 expression at the mRNA or protein level (Fig. S6), suggesting that kenpaullone functions through an entirely novel mechanism.

**Discussion**

In this paper we have described the development and application of an unbiased, high-throughput (>500,000 compounds) cell-based screening technology that can be applied to identify small molecules to replace virally transduced reprogramming transcription factors. In a proof of concept application, we have
identified 3 classes of molecules that activate Nanog expression in murine fibroblasts during direct reprogramming in the absence of Klf4, and have characterized one such molecule, kenpaullone, in detail. We have shown that kenpaullone is able to replace Klf4 in the reprogramming of primary and secondary fibroblasts and NPCs. OSM iPS cells derived in the presence of kenpaullone display the characteristics of pluripotent ES cells, including the ability to contribute to chimeric mice.

Previous studies with small molecules that induce epigenetic changes have suggested that chromatin remodeling is a rate-limiting step in the conversion from a somatic to a pluripotent epigenetic state (18–22). For example, treatment of MEFs with a small-molecule inhibitor of DNA methyltransferase (5-azacytidine) facilitates reprogramming during a brief temporal window by removing demethylation marks, thereby lowering the kinetic barrier to the transition to pluripotency (20, 22). Histone deacetylase inhibitors and histone methyl transferase inhibitors also have been shown to increase the efficiency of reprogramming (18, 19, 21). In particular, the histone deacetylase inhibitor valproic acid was shown to dramatically increase 3-factor reprogramming efficiency in the absence of c-Myc in both mouse and human cells and to allow 2-factor reprogramming (Oct4 and Sox2) of human fibroblasts in the absence of Klf4 and c-Myc (18, 19).

Although modulation of known epigenetic control elements has provided significant progress toward reprogramming that does not require genetic manipulations, such techniques rely on specific compounds to target known mechanisms. With the study of induced pluripotency still in its infancy, the mechanisms governing reprogramming remain largely unknown. It is likely that the activation of different alternative pathways by small molecules will enhance the efficiency of vector-free reprogramming. Furthermore, identifying chemicals with novel mechanisms of action to modulate this process is of great interest. This work demonstrates that unbiased, cell-based screens can identify small molecules from large chemical libraries to replace transcription factors for reprogramming. Moreover, the identification of these and other molecules provides novel tools to study the molecular mechanisms at play during epigenome overhauls and ultimately may help bring iPS cell technology one step closer to clinical application.

Materials and Methods

Chemicals. Kenpaullone, purvalanol A, and 7-hydroxyflavone were purchased from Sigma, and CHIR99021 was purchased from Axon MedChem.