Stem Cell Research (2012) 9, 185–191

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SHORT REPORT

A fluorescent screening platform for the rapid evaluation of chemicals in cellular reprogramming

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Received 9 December 2011; received in revised form 4 June 2012; accepted 20 June 2012
Available online 29 June 2012

Abstract Current strategies to monitor reprogramming into induced pluripotent stem cells (iPSCs) are limited in that they rely on the recognition of advanced stage biomarkers or they involve the transduction of genetically-modified cells. These limitations are particularly problematic in high-throughput screenings where cell availability, low cost and a rapid experimental protocol are critical issues. Herein we report the application of a pluripotent stem cell fluorescent probe (i.e. **CDy1**) as a reporter for the rapid screening of chemicals in reprogramming iPSCs. **CDy1** stains early-stage iPSCs at 7 dpi as well as matured iPSCs; hence it can partially overcome the slow kinetics of the reprogramming process. As a proof of concept, we employed a **CDy1**-based screening in 384 well-plates to examine the effect of newly synthesized hydroxamic acid derivatives in reprogramming mouse fibroblasts transduced with Oct4, Sox2 and Klf-4 without c-Myc. One compound (**1**–**26**) was identified as a reprogramming enhancer by 2.5-fold and we confirmed that **1**–**26** behaves as a histone deacetylase (HDAC) inhibitor. The successful identification of novel small molecules enhancing the generation of iPSCs by means of a rapid and simple protocol demonstrates the suitability of this **CDy1**-based screening platform for the large scale and high-throughput evaluation of iPSC modulators.

Abbreviations: CDy1, compound of designation yellow 1; HDAC, histone deacetylase; MEF, mouse embryonic fibroblast; LIF, leukemia inhibitory factor; 5′-azaC, 5′-azacytidine; TSA, trichostatin A.

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doi:10.1016/j.scr.2012.06.006

Introduction

Induced pluripotent stem cells (iPSCs) hold an enormous potential for biomedical research (e.g. regenerative medicine, drug discovery) (Rubin, 2008; Xu et al., 2008; Deng, 2010) and current stem cell research pursues the generation of iPSCs in an efficient and safe manner. Since the original report of Takahashi and Yamanaka (2006), different strategies with the aim at replacing the viral transduction of exogenous transcription factors by small molecules, proteins, mRNA or microRNA have been described (Feng et al., 2009; Kim et al., 2009; Judson et al., 2009; Warren et al., 2010). The evaluation of small molecules has been probably the most studied approach, partially due to the availability of large chemical collections and the standardized procedures (e.g., high-throughput screens) developed in some areas of medicinal chemistry (Desbordes et al., 2008; Gonzalez et al., 2011; Lyssiotis et al., 2011). Alkaline-phosphatase and immunocytochemistry-based assays are commonly used methods which rely on the recognition of iPSC markers at an advanced stage of the reprogramming process (typically 12–14 days in mouse and longer in human) (Zhu et al., 2010; Lin et al., 2009; Bone et al., 2009). An alternative is the transduction or transfection of genetically-modified cells that express reporters by the promoters of iPSC-specific genes (e.g., Oct4, Nanog) and enable tracking the reprogramming. *Nanog-GFP* (Green Fluorescent Protein) and *Oct4-GFP* have been used to monitor iPSC reprogramming (Mikkelsen et al., 2008; Huangfu et al., 2008) and Lyssiotis et al. (2009) developed a *Nanog-luciferase* reporter that was successfully applied to high-throughput chemical screening. The main drawback of genetically-modified cells is their limited availability, which may be critical for broad usage. Besides, these genetic reporters usually 'turn on' at relatively later stages of reprogramming. Our group recently developed a fluorescent probe (**CDy1**) that selectively stains pluripotent stem cells (Im et al., 2010; Kang et al., 2011). **CDy1** is a fluorescent small molecule (absorption maximum: 535 nm; emission maximum: 570 nm) discovered from a high-throughput screening of 280 rosamine dyes in embryonic stem cells (ESC) and mouse embryonic fibroblasts (MEFs). **CDy1** showed 12.2-fold higher fluorescence intensity in mESC when compared to MEF. Further studies proved that **CDy1** could also stain mouse iPSCs (miPSCs) at early stages, when the signal of an *Oct4-GFP* reporter was not detectable. Time-course studies indicated that the early fluorescent signal of **CDy1** overlapped well with the signal of *Oct4-GFP* at advanced stages of reprogramming (Im et al., 2010). We envisioned that **CDy1** could be used as a fluorescent reporter to develop a high-throughput screening platform for the rapid evaluation of chemicals in mouse iPSCs (miPSCs). In addition to overcoming the slow kinetics of reprogramming, this platform would comprise other advantages, such as the compatibility with both genetically and non-genetically modified transduced cells, the possibility to monitor the reprogramming process at different time points and a low cost that may facilitate its application to large collections of chemicals.

Materials and methods

Cell culture and iPSC generation

MEFs for feeder were cultured in 10-cm dishes coated with 0.1% gelatin using high-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM non-essential amino acids and 0.1% β-mercaptoethanol. For feeder cell preparation, MEFs were treated with mitomycin C (10 μg/mL) for 2.25 h and washed with PBS (×1). For iPSC generation, MEFs from E13.5 B6;CBATg(Pou5f1-EGFP)2Mnn/J mouse (Jackson Laboratory) embryo at passage 3 were infected with pMX-Oct4, Sox2, Klf4 retrovirus with or without cMyc with 10 μg/mL polybrene (Sigma) in 10-cm dishes. After 24 h the media were changed to high-glucose DMEM with 20% knock-out serum replacement (KOSR, Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids and 100 U/mL leukemia inhibitory factor (LIF, Chemicon). Transduced MEFs were seeded as 10,000–30,000 cells/well on feeder cells (30,000 cells/well) in 12 well-plates for *Oct4-GFP*-based experiments or as 4000 cells/well on feeder cells (1000 cells/well) in 384 well-plates for **CDy1**-based screens and incubated in a humidified atmosphere at 37 °C with 5% CO_2.

Chemical treatment and CDy1-based screening

MEFs transduced with Oct4, Sox2 and Klf4 were seeded on 384 well-plates and treated at 2 dpi with chemicals to a final concentration of 5 μM for hydroxamic acids (containing 0.5% DMSO), 600 nM for 5′-azaC, 20 nM for TSA and 2 μM for SB431542 in mESC media containing LIF (100 U/mL). Four replicates were run for every treatment and cells were incubated for 5 days in a humidified atmosphere at 37 °C with 5% CO_2. At 7 dpi, cells were treated with **CDy1** (final concentration: 100 nM) and incubated for 1 h at 37 °C. Cells were washed with mESC media and incubated at 37 °C for 2.5 h, then incubated with mESC media containing Hoechst (1 μg/mL) for nuclear counterstaining (15 min, 37 °C). Fluorescent images of **CDy1** and Hoechst-stained cells were acquired in an ImageXpress^{MICRO} system (TRITC filter for **CDy1**, DAPI filter for Hoechst) at 10× magnification and analyzed using MetaXpress 2.0 software (Molecular Devices, Inc. USA, www.moleculardevices.com).

Chemical and biological characterization of 1–26

The purity and identity of **1**–**26** were confirmed by HPLC–MS and NMR (^{1}H and ^{13}C) (Figure S4). The biological activity of **1**–**26** was evaluated using a HDAC inhibitor drug screening kit (BioVision, catalog #K340-100) according to the manufacturer's instructions and the data analysis was performed using GraphPad Prism 5.0. Cell viability assays were performed using a CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay kit (Promega).

Immunocytochemistry, RT-PCR and in vitro differentiation assays

For immunocytochemistry assays, iPSCs (expanded at least for six passages without chemical treatment) were fixed with 4% paraformaldehyde for 10 min, washed with PBS and blocked with 1% BSA for 30 min. Cells were then incubated with a mouse monoclonal antibody against SSEA-1 (1:100, Millipore) and a rabbit monoclonal antibody against Oct4 (1:100, Abcam) overnight at 4 °C, washed with PBS (×3) and incubated for 3 h with Alexa594-conjugated goat anti-mouse antibody (1:500, Invitrogen) and Cy5-conjugated goat anti-rabbit antibody respectively. Fluorescence microscope images were taken in a Nikon A1R confocal microscope using FITC (*Oct4-GFP*), Texas Red (SSEA-1) and Cy5 (Oct-4) filters and processed using the software NIS-Elements 3.10.

Total RNAs from chemical-treated iPSCs, MEFs and mESCs were extracted with miRNeasy Mini Kit (QIAGEN). The sequences of the primers for RT-PCR are included in the Supporting Information. One-step quantitative RT-PCR was performed on a StepOne™ Real-Time PCR System using a Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, USA, www.appliedbiosystems.com).

Differentiated cells from iPSC lines were obtained upon 10–12 days differentiation of embryoid bodies formed for 3 days in mESC growing media without LIF. Cells were fixed as above mentioned, incubated with mouse monoclonal antibodies against α-SMA (1:200, Abcam) or Nestin (1:200, Millipore) and a goat polyclonal antibody against SOX-17 (1:200, R&D Systems) overnight at 4 °C, washed with PBS (×3) and then incubated for 3 h, respectively, with Alexa 594-conjugated goat anti-mouse antibody (1:200, Invitrogen) and Alexa 594-conjugated donkey anti-goat antibody (1:200, Invitrogen) and Hoechst (0.1%, v/v) for nuclear counterstaining. Fluorescent microscope images were taken in a Nikon Ti epifluorescence microscope using the Texas Red filter (α-SMA, Nestin, Sox17) and the DAPI filter for Hoechst staining. Images were processed with the NIS-Elements 3.10 software.

In vivo teratoma assays

Cells suspended in PBS were injected subcutaneously into the lower flanks of 8 weeks old SCID mice (3×10^6 cells per site). The tumors grown under the skin were excised 6–8 weeks after the injection and fixed in 4% paraformaldehyde for paraffin embedding, sectioning and hematoxylin and eosin staining. All animal experiment procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Results and discussion

In order to evaluate the staining properties of **CDy1** in a format compatible with high-throughput screening, we transduced MEFs with different combinations of Oct4, Sox2, Klf-4 and c-Myc and seeded them in 96 or 384-well plates using a 4:1

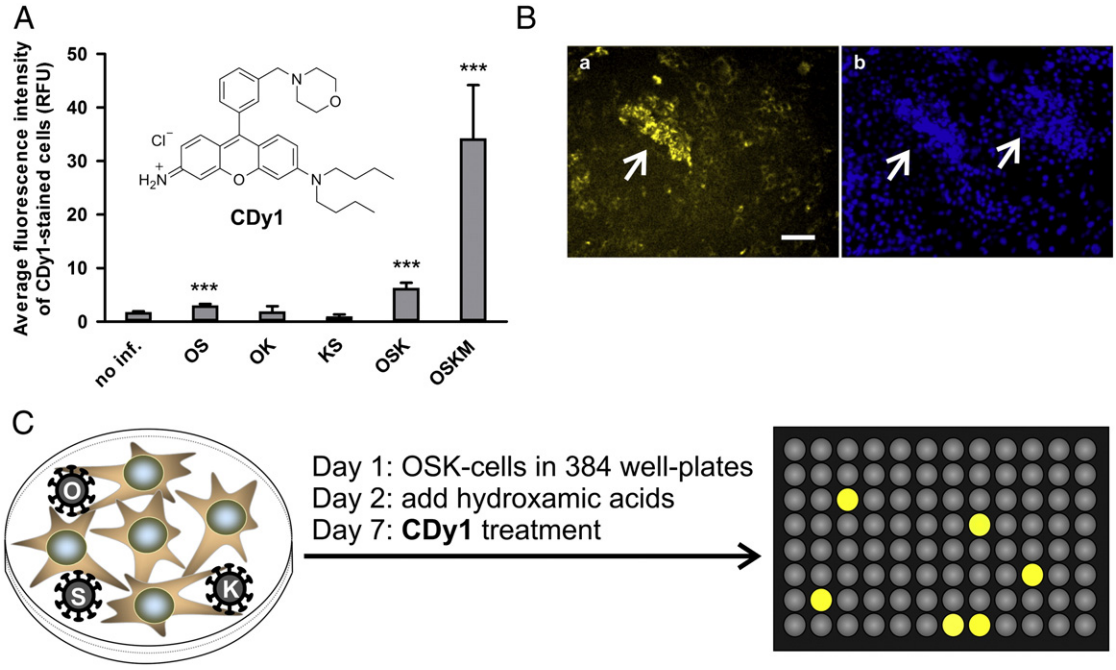

Figure 1 A fluorescence-based high-throughput screening platform. A) **CDy1**-stained early miPSCs (7 dpi) from different reprogramming factors. Values as means ± SD ($n=4$). Student's t-test for unpaired infected cells showed significant differences (*** $p<0.001$) compared to non-infected cells. B) Fluorescent microscope images of **CDy1**-stained OSK-transduced cells at 7 dpi in 384 well-plates (scale bar: 50 μm). Hoechst was used for nuclear counterstaining. White arrows indicate **CDy1**-stained and Hoechst-stained colony-like structures. C) Work flow: OSK-transduced MEFs were seeded in 384 well-plates and incubated with hydroxamic acids. At 7 dpi, cells were treated with **CDy1**, washed and analyzed by high-throughput fluorescence cell imaging.

(transduced:feeder) cell ratio. After 7 days post infection (dpi), we incubated the cells with **CDy1** and observed them with an ImageXpress^{MICRO} system for high-throughput fluorescence cell-imaging data acquisition after washing (Fig. 1A). As shown in Fig. 1A, the average fluorescence intensity in cells infected with Oct4, Sox2, Klf-4 and c-Myc (OSKM) was significantly higher than in cells infected with different combinations of Oct4, Sox2 and Klf-4 (OSK, OS, OK, and KS) and non-infected cells. OSKM-transduced cells may render a significant population of non-iPSC colonies (Nakagawa et al., 2008) so we analyzed the fluorescence cell images and observed that **CDy1** stained cells resembling early iPSC colonies

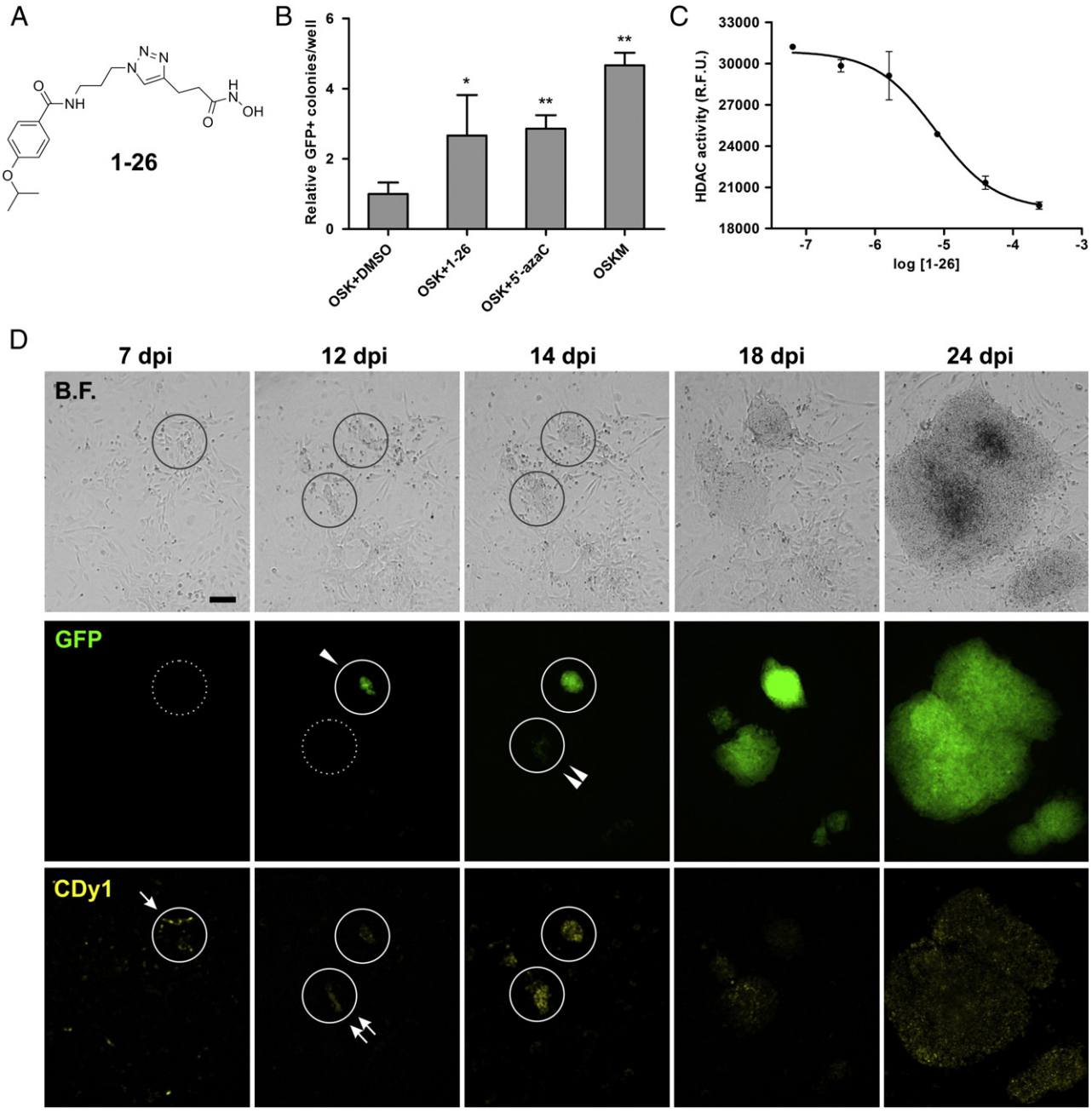

Figure 2 Time-course experiments and evaluation of **1**–**26** in reprogramming mouse fibroblasts. A) Chemical structure of **1**–**26**. B) Reprogramming efficiencies (as relative number of *GFP*$^{+}$ colonies/well at 28 dpi) in *Oct4-GFP* OSK(M)-transduced cells after treatment with **1**–**26** (1 μM) or 5′-azaC (600 nM). Values as means ± SEM (n = 3). Student's t-test for unpaired chemically-treated samples showed significant differences (* $p<0.1$, ** $p<0.01$) compared to untreated samples. C) The activity of HDAC under increasing concentrations of **1**–**26** was determined with a fluorescence-based HDAC drug screening kit. Values as means ± SEM (n = 3). D) Time-course fluorescence microscope images of **1**–**26** treated *Oct4-GFP* iPSCs: *upper*) bright field, *center*) *Oct4-GFP* signal, *lower*) **CDy1** signal (scale bar: 100 μm). Arrows point at **CDy1**-stained cells whose *Oct4-GFP* signal is not detectable at the same time point (dashed circles) but appears at a later stage (arrow heads).

but not all clumps of cells or colony-like structures (Fig. 1B). These results confirmed that **CDy1** could be efficiently used as a fluorescent reporter to discriminate early stages of reprogramming in a high-throughput screening format.

The evaluation of small molecules that can substitute exogenous transcription factors in iPSC generation is focused on the replacement of c-Myc, as its oncogenic role hampers the potential therapeutic application of iPSCs (Huangfu et al., 2008; Shi et al., 2008a, 2008b). Since **CDy1** proved to discriminate well the reprogramming states of OSK and OSKM-infected cells at 7 dpi (Fig. 1A), we designed a **CDy1**-based screening to identify small molecules that could replace c-Myc in reprogramming (Okita et al., 2007) (Fig. 1C). OSK-transduced cells were seeded at 4000 cells/well into 384-well plates with mitomycin-treated MEF as feeder cells (1000 cells/well) and incubated in mouse ESC (mESC) media containing leukemia inhibitory factor (LIF, 100 U/mL) in the presence of a collection of newly synthesized hydroxamic acids at a final concentration of 5 μM (0.5% DMSO). After 5 days of incubation with the hydroxamic acids (i.e. 7 dpi), cells were treated with **CDy1**, washed with mESC media and analyzed by high-throughput fluorescence cell-imaging. OSK-transduced cells treated with 0.5% DMSO and OSKM-transduced cells were included as controls for non- and full-replacement of c-Myc respectively. Upon evaluation of 240 hydroxamic acids, we identified 5 compounds inducing higher fluorescence intensities in OSK-infected cells than DMSO and with comparable responses to various reported chemicals enhancing reprogramming (e.g. epigenetic modulators: 5′-azacytidine (5′-azaC) and trichostatin A (TSA) as well as TGFβ inhibitors: SB431542) (Figure S1 in Supporting Information (SI)). Secondary screenings showed that **1**–**26** yielded the largest population of **CDy1**-stained cells in a reproducible manner and suggested that it could partially replace c-Myc to a similar extent than reported epigenetic modulators, such as 5′-azaC (Huangfu et al., 2008) (Figure S2 in SI).

After identifying **1**–**26** from the **CDy1**-based primary screening, we aimed to confirm the effect of **1**–**26** (Fig. 2A) in the reprogramming efficacy using an alternative reported method. The *Oct4-GFP* transgene has been extensively used to monitor the generation of miPSCs, so we examined OSK-transduced cells harboring an *Oct4-GFP* reporter upon continuous incubation with **1**–**26**. The treatment of *Oct4-GFP* OSK-transduced cells with **1**–**26** or 5′-azaC enhanced the formation of *GFP*$^+$ colonies at an earlier time point than DMSO-treated cells with an overall 2.5-fold increase in the reprogramming efficiency (Fig. 2B). Furthermore, we investigated the biological activity of **1**–**26** that may be responsible for this effect. Some hydroxamic acids (e.g. TSA, suberoylanilide hydroxamic acid) have been reported as HDAC inhibitors (Finnin et al., 1999; Biel et al., 2005), which can improve the generation of iPSC reprogramming by inducing chromatin modifications (Huangfu et al., 2008). **1**–**26** behaved as a HDAC inhibitor at micromolar concentrations (Fig. 2C), which correlated well with its reprogramming effect in OSK-transduced cells.

In addition, we performed time-course experiments to compare the fluorescent signal of **CDy1** and the conventional *Oct4-GFP* reporter on transduced cells treated with **1**–**26** and harboring an *Oct4-GFP* reporter. We treated the cells with **CDy1** at 7 dpi, and monitored both fluorescent signals (i.e. *Oct4-GFP* and **CDy1**) at different time points (i.e. 7, 12, 14, 18 and 24 dpi) (Fig. 2D). Cells undergoing reprogramming at early stages were stained by **CDy1** when the signal of *Oct4-GFP* was not distinguishable, and consistently overlapped with the fluorescence of *Oct4-GFP* at later stages. These experiments confirmed that **CDy1** is an earlier reporter of cells undergoing reprogramming than the *Oct4-GFP* transgene (Im et al., 2010), and that the chemical treatment with **1**–**26** did not affect the properties of **CDy1**. With these results we corroborated that **1**–**26** increases the generation of miPSCs by inhibiting HDACs, and proved the reliability of our **CDy1**-based primary screening to identify small molecules that accelerate the reprogramming process.

To analyze whether the chemical treatment may affect the pluripotency of miPSCs, we established miPSC lines from **1**–**26**-treated OSK-infected cells harboring an *Oct4-GFP* reporter without further chemical addition. As shown in Fig. 3, the immunochemical analysis of **1**–**26**-treated miPSCs showed that *GFP*$^+$ colonies did also express specific mouse embryonic stem cell markers, such as SSEA-1 and Oct4 (Fig. 3A). qRT-PCR analysis of two different miPSC lines indicated that the endogenous levels of Nanog, Oct4, Sox2, Klf4 and c-Myc in **1**–**26**-treated miPSCs were higher than in MEFs and similar to those expressed in mESC (Fig. 3B).

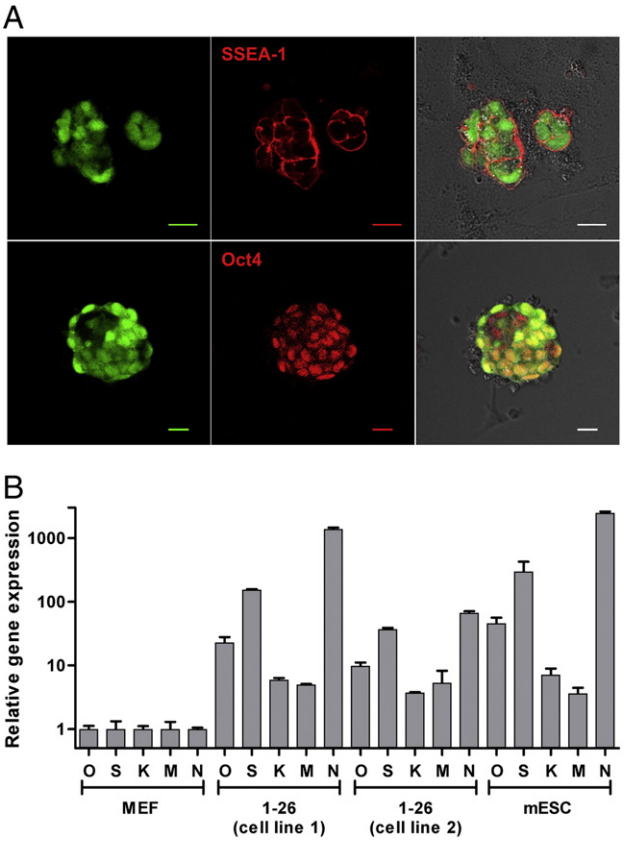

Figure 3 Characterization of iPSCs. A) Immunocytochemistry of SSEA-1 and Oct4 in *Oct4-GFP*$^+$ colonies: *left*) *Oct4-GFP* signal, *center*) SSEA-1 or Oct4 immunostainings, *right*) merged fluorescent and bright field images (scale bar: 20 μm). B) RT-PCR analysis of MEFs, two different **1**–**26**-treated iPSC lines and mESCs. Values as means ± SEM (n = 2) of two independent experiments.

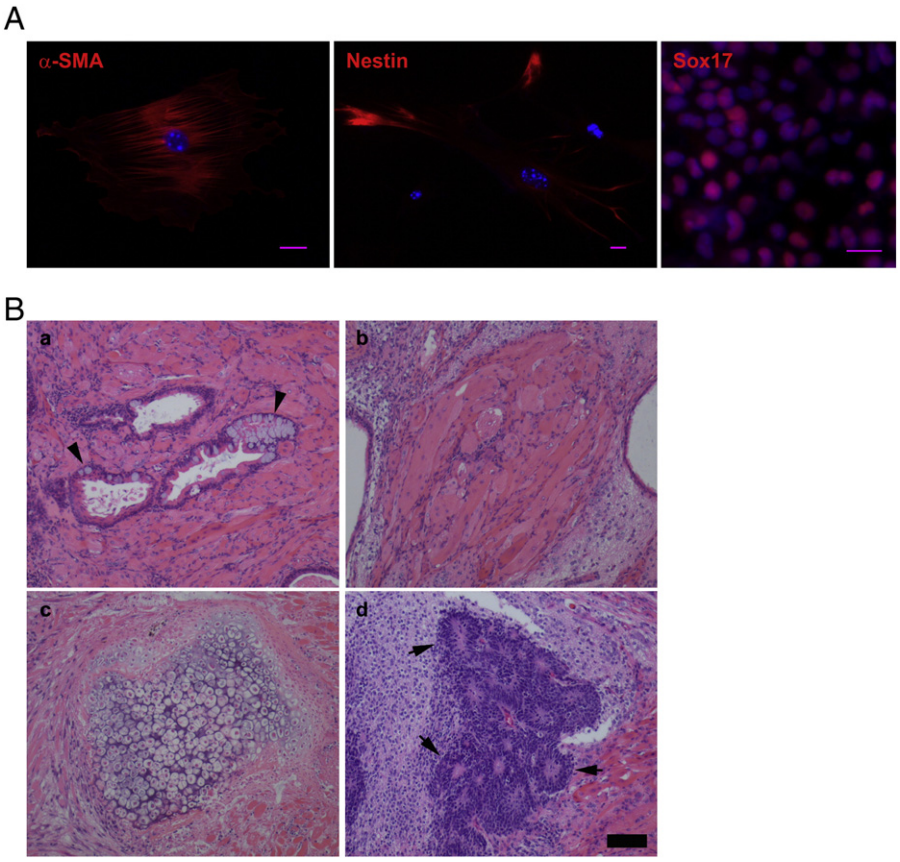

Figure 4 Differentiation of iPSCs. A) Immunocytochemistry of in vitro differentiated cells with anti-α-SMA (mesoderm), anti-Nestin (ectoderm) and anti-SOX17 (endoderm) (scale bar: 20 μm). Hoechst was used for nuclear counterstaining. B) In vivo teratoma assay. Staining with hematoxylin and eosin of three germ layers (endoderm, mesoderm, and ectoderm) differentiated from **1**–**26**-treated iPSCs: a) goblet cells of the endodermal germ layer originated intestine epithelia (arrow heads), b and c) mesodermal smooth muscle cells (b) and cartilages (c), d) neuronal rosettes of ectodermal origin (arrows) (scale bar: 100 μm).

Furthermore, these cells were induced to form embryoid bodies and further differentiated under culturing conditions without LIF into a mixed population of cells including the three embryonic germ layers (e.g. mesoderm, ectoderm and endoderm) as confirmed by immunochemical analysis (e.g. α-smooth muscle actin (α-SMA), Nestin and Sox 17 respectively) (Fig. 4A). Lastly, we demonstrated the capability of **1**–**26**-treated miPSCs to differentiate into all three germ layers by means of teratoma assays (Fig. 4B), which illustrates the pluripotent nature of the generated lines.

Conclusions

We developed a high-throughput screening platform to evaluate small molecules enhancing the generation of miPSCs using the fluorescent signal of **CDy1** as an earlier reporter (i.e. 7 dpi) than previously described methods. In a proof of concept application, we examined a collection of synthesized hydroxamic acids in OSK-transduced cells and identified **1**–**26** as a small molecule capable to induce a large population of **CDy1**-stained cells. Further experiments indicated that **1**–**26** behaved as a HDAC inhibitor and confirmed a 2.5-fold increase in the reprogramming efficiency of *Oct4-GFP* OSK-transduced cells. The versatility, speed and simplicity of this **CDy1**-based high-throughput screening make it a suitable platform for the rapid evaluation of iPSC reprogramming modulators.

Acknowledgments

The authors gratefully acknowledge the financial support from the intramural funding and grant 10/1/21/19/656 from A*STAR (Agency for Science, Technology and Research, Singapore) Biomedical Research Council and Singapore Polytechnic and Totalisator Board (11-27801-36-M113).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2012.06.006.

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