Pluripotency

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A Novel Small Molecule Facilitates the Reprogramming of Human Somatic Cells into a Pluripotent State and Supports the Maintenance of an Undifferentiated State of Human Pluripotent Stem Cells**

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Human somatic cells can be reprogrammed to a pluripotent state by forced expression of a combination of four transcription factors: *Oct-4* and *Sox-2* with either *Klf-4* and *c-Myc* (OSKM); or *Nanog* and *LIN28* (OSNL).[1] Because of the groundbreaking cellular reprogramming discovery that pluripotent cells have the ability to differentiate into all cell types found in adults,[1–2] human induced pluripotent stem cells (hiPSCs) generated from a patient's own cells have been considered a highly promising cellular source for basic research, drug development, and toxicity testing, as well as regenerative medicine.[3] However, current reprogramming methods have some known limitations including a low efficiency and slow kinetics of the reprogramming process, and a safety concern needs to be addressed before they can be widely applied in different settings including clinical uses.

Currently, small molecules that modulate specific signaling pathways and epigenetic modifications have been a major focus to control stem cell fate and function, especially for the maintenance and/or reacquisition of pluripotency and differentiation.[4] The various small-molecule inhibitors targeting either DNA methyltransferase (AZA),[4d,5] G9a histone methyltransferase (BIX-01294),[5] histone deacetylase (VPA, TSA, and SAHA),[4d,6] TGF-β (A-83-01, SB431542, and E-616452),[7] MEK (PD0325901),[8] GSK-3 (CHIR99021),[8] mTOR (rapamycin),[4f] or Pan-Src family kinase[9] have been reported to enhance iPSC generation; some of these inhibitors have even been shown to compensate for reprogramming factors. However, only a limited number of small molecules are shown to be effective in the human cell system. Herein, we report a novel synthetic chemical, RSC133, which was found to effectively enhance reprogramming of human somatic cells and maintenance of human stem cell pluripotency.

To identify small molecules that can promote the reprogramming process, our in-house chemical library of heterocyclic compounds (MW < 500) was initially screened using mouse embryonic fibroblasts (MEFs) that are hemizygous for the *Oct4-GFP* transgene (OG-MEFs).[10] The MEFs were reprogrammed by transducing retroviral vectors that encoded the standard four reprogramming factors (OSKM) and then either not treated or treated with chemical compounds (Figure S1 in the Supporting Information). Based on primary screening results, we further synthesized new series of indoleacrylic acid and indolepropionic acid derivatives (Scheme S1 in the Supporting Information) and evaluated their structure–activity relationship (SAR) for reprogramming efficiency (Table S1 in the Supporting Information). Among the candidate compounds examined, indoleacrylic acid analogues ID133 and ID558, which contain an indole ring with a free NH group and a benzoic acid analogue connected through a double bond, were the most potent in enhancing reprogramming efficiency; the enhanced efficiency was confirmed by a substantial increase in the number of GFP-positive colonies compared to the untreated control (Figure S1 and Table S1 in the Supporting Information). Of note, compound ID133 features a simple benzamide in the benzoic acid part, while ID558 contains 4-hydroxy-*N*,*N*-dimethylbenzamide. Because of its ability to promote iPSC generation in both mouse and human (see below), we renamed the novel small molecule ID133 as the reprogramming stimulating compound (RSC) 133 (Figure 1a). The full characterization of the identified RSC133 is shown in Figures S2–S5 in the Supporting Information.

We selected 35 candidates including indole analogues and further explored whether those chemicals could enhance the reprogramming process of human foreskin fibroblasts (hFFs, ATCC# CRL-2097) into hiPSCs. The overall reprogramming procedure we used in this study is a slightly modified version of the published protocols.[1a,6] The OSKM-transduced hFFs not treated or treated with chemical compounds were

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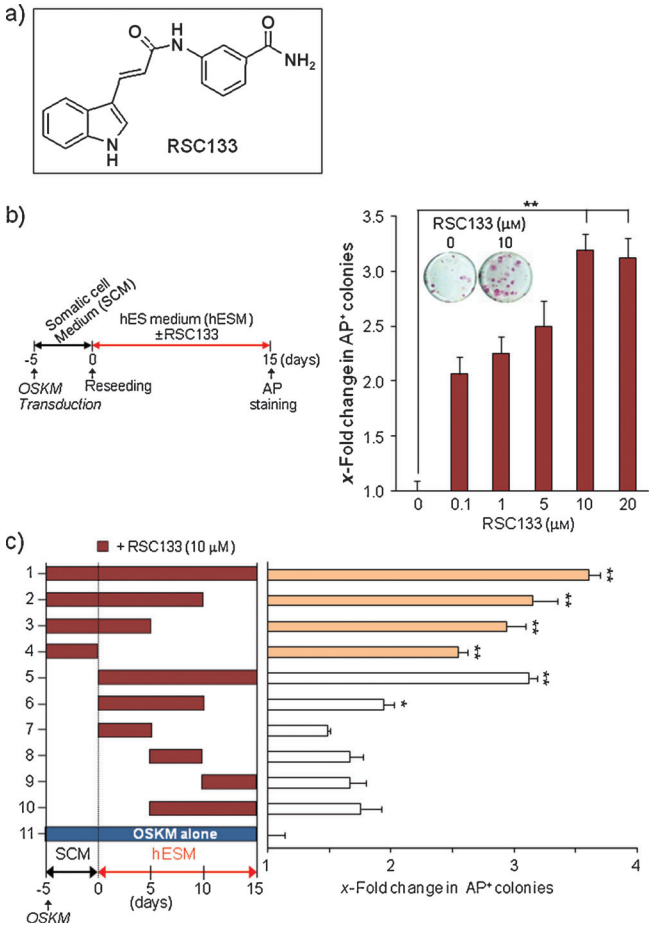

Figure 1. A new indole compound, RSC133, promotes the reprogramming of human somatic cells to pluripotent state. a) Chemical structure of RSC133. b) Dose-dependent enhancement of reprogramming efficiency by RSC133. The OSKM-transduced hFFs were incubated with or without RSC133 at the indicated concentrations. c) Time course study of RSC133 treatment on reprogramming. The OSKM-transduced hFFs were incubated with or without RSC133 (10 μM) at the indicated time periods. The hESC-like colonies were stained and quantified for AP to determine reprogramming efficiency. The relative fold changes in AP^+ colonies compared to the untreated control (designated as 1) were shown (orange bars indicate initial treatment with RSC133 on days −5 to 0, and white bars indicate the treatment started on day 0, 5, or 10 as shown in the scheme on the left). Results (b and c) are shown as the mean ± standard deviation (SD; $n=3$). The statistical significance was determined using Student's t-test. $^*P<0.05$, $^{**}P<0.005$.

monitored daily for morphologic changes culminating in human embryonic stem cell (hESC)-like colonies, and the resulting hiPSCs were verified by alkaline phosphatase (AP) staining on day 15 after treatment. Under our conditions, only compound RSC133 significantly increased the reprogramming efficiency of hFFs in a dose-dependent manner up to 10 μM compared to the untreated control (Figure 1b,c and Table S1 in the Supporting Information). An increase of RSC133 concentration to 20 μM, with no changes in cell viability, did not further enhance reprogramming efficiency (Figure 1b and Figure S6a in the Supporting Information).

To optimize the sequence and duration of RSC133 treatment, we tested 11 different reprogramming culture conditions (Figure 1c) and found that the addition of RSC133 (10 μM) to the culture soon after OSKM transduction (Figure 1c, condition 1–4) was more effective than other conditions, where RSC133 was added after reseeding (Figure 1c, condition 5–10). Out of all the various conditions, the continuous treatment with RSC133 throughout reprogramming was the most effective way to increase reprogramming efficiency (approximately 3- to 4-fold; Figure 1c, condition 1; days −5 to 15). In combination with hypoxic conditions (5% O_2), RSC133 synergistically increased the reprogramming efficiency by approximately 5- to 6-fold compared to the untreated control (data not shown). Under our conditions, the stimulatory effect of RSC133 on reprogramming was 1.5- to 3.3-fold higher than that of well-known chemicals (AZA, VPA, TSA, and SB431542) that enhance iPSC generation (Figure S7 in the Supporting Information). The hFFs reprogrammed with RSC133 displayed a rapid activation of pluripotency-associated genes *Nanog, Oct4,* and *Rex1* up to 2- to 2.5-fold (Figure 2c) and highly proliferative (bromodeoxyuridine (BrdU)-positive) hESC-like colonies that showed positive immunostaining for pluripotency markers Nanog or Tra-1-81 (Figure 2d) on day 10 after treatment compared to the untreated control. These results suggest that RSC133 significantly enhanced both the efficiency and kinetics of the reprogramming process.

Reports have shown that the ectopic expression of four reprogramming factors OSKM inhibited cell proliferation and caused cell-cycle arrest, senescence, and apoptosis during reprogramming and that is significantly related with the up-regulation of *p53*, *p21*, and *p16/INK4A*, which are negative regulators of the reprogramming process.[4c,11] RSC133 constituted a favorable condition for human somatic cell reprogramming through affecting multiple cellular responses. Firstly, RSC133 exerted positive effects on cell proliferation. RSC133 significantly increased the total number of cells, counted by trypan blue exclusion, by approximately 1.7-fold (Figure 2a) and the percentage of proliferating, BrdU-positive cells by approximately 2.5-fold during reprogramming on day 10 compared to the untreated control (Figure 2b). Positive effects of RSC133 on cell proliferation were also observed in normal culture condition of hFFs (Figure S6b,c in the Supporting Information). Cell proliferation is tightly associated with supporting self-renewal and pluripotent properties in ESCs,[12] and the induction of cell proliferation notably contributes to efficient somatic cell reprogramming.[4f,12a]

Secondly, RSC133 ablated pro-senescence phenotypes through down-regulating critical inducers of cellular senescence, such as p53, p21, and p16/INK4A. The up-regulation of *p53*, *p21*, and *p16/INK4A* genes, mediated by OSKM induction, was markedly inhibited by RSC133 up to 2.13-, 3.09- and 2.79-fold, respectively during reprogramming on day 10 compared to the untreated control (Figure 2c). In parallel, the proportion of senescence-associated β-galactosidase (SA-β-gal)-positive cells was decreased by approximately 37% during reprogramming on day 10 compared to

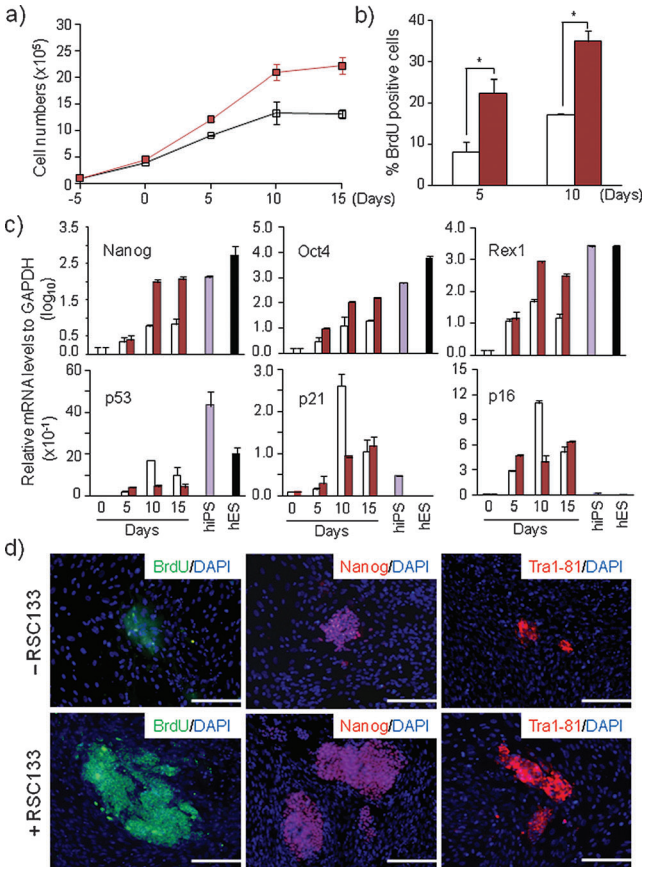

Figure 2. RSC133 ablated reprogramming barriers and resulted in accelerated kinetics of reprogramming. The OSKM-transduced hFFs were incubated with 10 μM RSC133 (brown in (a–c)) or without RSC133 (white in (a–c)) for 10 days unless otherwise indicated. a) RSC133 promoted the cell growth rate during reprogramming. The growth curves were plotted using total cell counts per well. The viable cells were counted by using a trypan blue exclusion assay. b) RSC133 increased the cell proliferation rate during reprogramming. The proliferation rate of the reprogramming cells, determined by the number of $BrdU^+$ cells, was shown as the mean ± SD of three independent experiments. The percentage of $BrdU^+$ cells in each condition (treated with RSC133) was compared with the untreated control. The statistical significance was determined using Student's t-test. $*P<0.05$. c) Quantitative real-time RT-PCR analysis for pluripotency (*Nanog, Oct4*, and *Rex1*)- and cell cycle arrest (*p53, p21, and p16*)-associated genes. All values were calculated with respect to the hFFs control, which was set to 1. hiPS (gray bars) and hES (black bars) indicate the positive control. d) Immunocytochemical analysis of BrdU and pluripotency-associated markers (Nanog and Tra-1-81). Scale bars represent 200 μm. GAPDH = glyceraldehyde-3-phosphate dehydrogenase, DAPI = 4′,6-diamidino-2-phenylindole.

the untreated control (Figure S8 in the Supporting Information).

Thirdly, RSC133 could also regulate the epigenetic modulators, such as DNA methyltransferase (Dnmt) and histone deacetylase (HDAC). To identify specific molecular targets of RSC133, we performed in silico reverse docking calculations. In reverse docking, in contrast to the conventional virtual screening in which one searches chemical space for chemicals that can specifically bind to a specific protein target, one tries to find the protein targets that can bind to a particular ligand.[13] Using AutoDock Vina,[14] one of the most widely used molecular docking programs, we calculated the protein–ligand docking scores between RSC133 and nearly all human protein structures currently available in the Protein Data Bank (total 10886 protein structures), and found that RSC133 most strongly binds to Dnmt1 with the docking score of −9.7. Considering that the second best docking score is −8.8 and the docking score of Dnmt1 (= −9.7) is well separated from the background score distribution (Figure 3a), we concluded that Dnmt1 is indeed a specific binding target of RSC133. Visual inspection revealed that RSC133 is docked into the site corresponding to cofactor *S*-adenosyl methionine binding pocket and stabilized mainly through multiple hydrogen bonds (Figure 3b). In correlation, RSC133 significantly reduced Dnmt1 enzymatic activity by approximately 38% during reprogramming on day 10 compared to the untreated control (Figure 3c).

Epigenetic chromatin remodeling modulated by DNA methylation and/or histone modification plays a critical role in the global transcriptional regulation during the reprogramming process.[4d,6] The inhibition of DNA methylation by treatment with inhibitor AZA or knockdown of Dnmt1 expression induces rapid conversion from a partially to a fully reprogrammed state.[4d] An increased level of H3K9 histone acetylation (ace) is specifically correlated with restoring pluripotency and reprogramming capacity.[15] A report also demonstrated that DNA methylation, mediated by Dnmt1, is closely associated with altered chromatin state through HDAC activity.[16] Interestingly, the total levels of H3K9ace were significantly up-regulated in RSC133-reprogrammed cells compared to the untreated control (Figure 3d). Furthermore, RSC133 reduced HDAC enzyme activity by approximately 13.7% during reprogramming on day 10 compared to the untreated control (Figure 3e). The expression level of HDAC1 proteins but not of HDAC2 was down-regulated by RSC133 on day 10 after treatment, compared to the untreated control, although they share an almost identical catalytic core domain and an extensively similar amino acid sequence (Figure S9 in the Supporting Information). Reports have demonstrated that HDAC1 occupied the gene loci for the four factors: *Oct4, Sox2, Klf4,* and *c-Myc* in ESCs, and the pluripotency-associated genes were up-regulated in the HDAC1 null ES cells.[17] These results collectively indicate that RSC133-mediated inhibition of Dnmt1 and subsequent decrease of HDAC1 activity enhances the reprogramming process by indirectly affecting gene transcriptional modulation through the changed DNA methylation and chromatin states, and increased accessibility of the gene loci for the four defined reprogramming factors.

The RSC133-generated hiPSC lines (1 and 2, chosen randomly) picked up after three weeks post-infection showed silencing of exogenous retroviral four factors (OSKM), expression of the pluripotency markers, normal karyotype, and pluripotency both in vitro and in vivo (Figure S10 in the Supporting Information).

We also confirmed that RSC133 supported the maintenance of human pluripotent stem cells (hESCs and hiPSCs) in an undifferentiated state. H9 hESCs cultured in uncondi-

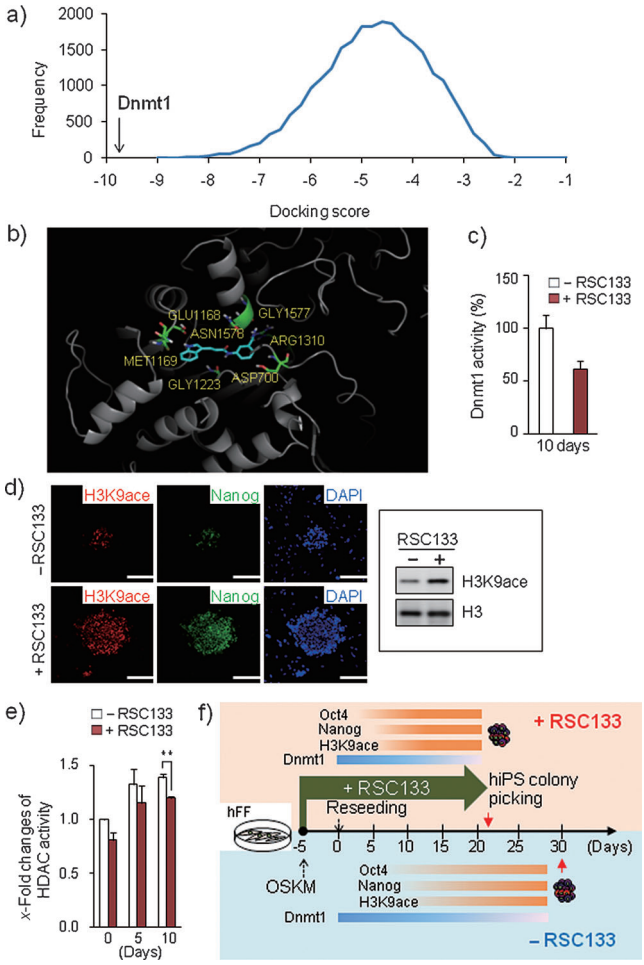

Figure 3. The beneficial effect of RSC133 on reprogramming was closely related to its inhibitory effects on Dnmt1 and HDAC1 enzyme activity. The OSKM-transduced hFFs were incubated with or without RSC133 treatment for 10 days unless otherwise indicated. a) Distribution of docking scores between RSC133 and all human protein structures calculated by AutoDock Vina. Docking score of Dnmt1 (−9.7, arrow) is clearly separated from background score distribution (blue curve), thus indicating that Dnmt1 is a specific molecular target of RSC133. b) RSC133–Dnmt1 docking model. RSC133 is shown in light blue. c) Repression of Dnmt1 enzyme activity by RSC133. The relative activity of Dnmt1 was shown with the value obtained without RSC133 for 10 days taken as 100%. d) Immunocytochemical (left panel) and Western blot (right panel) analysis of H3K9ace and Nanog proteins. The cells were stained with antibodies against H3K9ace (red) and Nanog (green). The DNA was stained with DAPI (blue). H3 was used as an internal control for western blot analysis. e) Repression of HDAC enzyme activity by RSC133. The HDAC activity was determined by using a colorimetric assay. All of the values were calculated with respect to the value for the hFFs (control), which was set to 1. f) Overview of the RSC133-mediated reprogramming process. The RSC133 treatment during the reprogramming process improved the reprogramming efficiency and kinetics (green arrow). The timing of gene activation of the pluripotency markers (Oct4 and Nanog) and of H3K9ace is indicated by orange bars. The timing of gene repression of Dnmt1 is indicated by vanishing blue bars. Results in (c) and (e) are shown as the mean ± SD ($n=3$). The statistical significance was determined using Student's t-test. $*P<0.01$, $**P<0.005$.

tioned medium (UM) failed to maintain their undifferentiated status and underwent differentiation. Addition of RSC133 to UM significantly protected hESCs from the loss of self-renewing undifferentiated states, which was confirmed by hESC-typical morphology and pluripotency marker expressions (Figure S11a,b in the Supporting Information). The undifferentiated hESCs maintained in the UM plus RSC133 displayed significant amounts of H3K9ace on par with the levels in hESCs cultured with the conditioned medium (CM; Figure S11c in the Supporting Information), thus indicating that RSC133 can restore pluripotency of inferior ESCs by increasing the level of H3K9ace.

In conclusion, we present a novel and effective small molecule, RSC133, which facilitates the maintenance of pluripotent hESCs and hiPSCs as well as functions as a potent chemical booster for human somatic cell reprogramming by improving reprogramming efficiency and kinetics (Figure 3 f). The reprogramming of human somatic cells is a comparatively slow and gradual process, and the fundamental molecular mechanisms regulating the reprogramming process are still largely unknown. The identification of small molecules that are effective for somatic cell reprogramming and/or supporting hESC/hiPSC pluripotency would provide a better understanding of pluripotency and improve reprogramming technology that, if coupled with scalable, defined, and safe culture conditions, would result in a higher quality of hiPSCs.

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