The revolutionary induced pluripotent stem cell (iPSC) technology provides a new means for cell replacement therapies and drug screening. Small molecule compounds have been found extremely useful to improve the generation of iPSCs and understand the reprogramming mechanism. Here we report the identification of a novel chemical, CYT296, which improves OSKM-mediated induction of iPSCs for >10 folds and enables efficient reprogramming with only Oct4 in combination with other small molecules. The derived iPSCs are genuinely pluripotent and support the development of two 'All-iPSC' mice by tetraploid complementation. CYT296 profoundly impacts heterochromatin formation without affecting cell viability. MEFs treated with CYT296 exhibit de-condensed chromatin structure with markedly reduced loci containing heterochromatin protein 1a (HP1α) and H3K9me3, which is very similar to the chromatin configuration in embryonic stem cells (ESCs). Given that an open chromatin structure serves as a hallmark of pluripotency and has to be acquired to fulfill reprogramming, we propose that CYT296 might facilitate this process by disrupting condensed chromatin, thereby creating a more favorable environment for reprogramming. In agreement of this idea, shRNA targeting HP1α also promotes the generation of iPSCs. Thus current findings not only provide a novel chemical for efficient iPSC induction, but also suggest a new approach to regulate somatic cell reprogramming by targeting chromatin de-condensation with small molecules.

**Keywords:** induced pluripotent stem cells, reprogramming, CYT296, small molecule compound, chromatin de-condensation, chromatin remodeling, HP1α

---

**Introduction**

Direct reprogramming of somatic cells via defined factors (Oct4 (O), Sox2 (S), Klf4 (K), c-Myc (M), etc.) not only provides cells useful for regenerative medicine, but also greatly expands our understanding of the molecular events behind cell fate determination (Plath and Lowry, 2011). The resulting induced pluripotent stem cells (iPSCs) are very similar to embryonic stem cells (ESCs) and are able to generate ‘all-iPSC’ mice via tetraploid complementation (Zhao et al., 2009). iPSCs can be easily manipulated genetically and might circumvent immune rejection and ethic issues, and are thereby considered as a prominent source for cell replacement therapy. iPSCs generated from patients can also be differentiated into disease-associated cell types, which can be used to screen for potential drugs.

Reprogramming to pluripotency requires reprogramming factor binding, transcription, and chromatin states changing during the transitions. Despite the extraordinary fidelity of the iPSC technology, the transcription factor-mediated reprogramming typically occurs in <1% of the starting cells at an extended latency (1–2 weeks) (Hochedlinger and Plath, 2009). Although the heterogeneity and differentiation state of the starting cell population (Hochedlinger and Plath, 2009), the expression level and stoichiometry of reprogramming factors (Carey et al., 2011), the open and close chromatin configuration at pluripotency and differentiation genes (Soufi et al., 2012), etc., have been suggested to affect reprogramming efficiency (Plath and Lowry, 2011), we are...
still far away from pinpointing the rate-limiting transition steps or factors in reprogramming. Increasing evidence has suggested that epigenetic priming events early in the process that affects the chromatin accessibility may be critical for the induction of pluripotency.

The genome of eukaryotic cells is organized into euchromatin, which is generally permissive for gene activation, and heterochromatin, which is highly compacted and largely transcriptionally silenced, presumably by limiting the accessibility to transcription factors and DNA interactions (Dillon, 2004). ESC chromatin exists in an unusual configuration with widely dispersed open chromatin, while differentiation leads to extensive reorganization and formation of large compact chromatin domains (Efroni et al., 2008). In ESCs, architectural chromatin proteins such as the heterochromatin component HP1, the linker histone H1, and core histones display hyperdynamic and looser binding to chromatin when compared with differentiated cells (Meshorer and Misteli, 2006). These observations have led to the idea that an open chromatin structure may be required for the maintenance or induction of pluripotency. Indeed, altered expression of chromatin-remodeling proteins in ESCs results in the accumulation of compact chromatin, disruption to self-renewal and reduced ESC differentiation potential (Gaspar-Maia et al., 2011). In the case of factor-mediated reprogramming, changes in histone modifications proceed and are much more widespread than the initial gene expression changes (Koche et al., 2011), indicating genome-wide chromatin remodeling as an immediate early response of reprogramming. Rapid, genome-wide changes in the euchromatic histone modification, H3K4me2, at more than a thousand loci including the promoters and enhancers of large subsets of pluripotency-related genes could be observed, in combination with focused depletion of H3K27me3 specifically at positions where H3K4 methylation is gained (Koche et al., 2011). Apart from histone modification, DNA methylation is also an important factor in restricting reprogramming. Demethylation at the promoters of key pluripotency genes, including Nanog and Oct4, occurs at late stage in reprogramming, indicating that demethylation of these promoters is a rate-limiting step (Mikkelsen et al., 2008). In agreement with the above findings, early screening studies have identified that a panel of epigenetic modulators, including inhibitors of histone deacetylase and DNA methyltransferases, potently facilitate reprogramming (Zhang et al., 2013). Here we report the identification of a novel small molecule compound 90-D3 and its derivative CYT296, which facilitate reprogramming by inducing an open chromatin state in MEFs.

Results

Screening and discovery of a small molecule 90-D3 (compound 1) that promotes the generation of iPSCs from MEFs

We established a 96-well-plate-based chemical screening system for four-factor (4F, OSKM)-induced reprogramming in OG2 MEFs that carry a transgenic Oct4 promoter driven-GFP reporter (Wang et al., 2011) (Supplementary Figure S1A). Successful reprogramming of the MEFs will lead to the generation of GFP⁺ iPS colonies. The HDAC inhibitor VPA was used as a positive control. More than 20000 compounds were screened and a small molecule, designated 90-D3 (compound 1, Supplementary Figure S1B and Table S1), was found to significantly increase the number of GFP⁺ iPS colonies. At Day 15, >40 GFP⁺ colonies could be observed in 90-D3 treated wells (5000 MEFs/well), while the control well had only 1–2 colonies (Supplementary Figure S1C). 90-D3 displayed clear dose response with the best effect observed at 1 μM (Supplementary Figure S1D). 90-D3 seemed to exert its effect at the early stage of reprogramming, since treatment with 90-D3 after Day 6 had no obvious effect on the overall efficiency. In contrast, there was a significant increase in the number of GFP⁺ colonies in the cultures treated with 90-D3 for Day 3–6 (Supplementary Figure S1E). We also found prolonged treatment with 90-D3 did not further increase the efficiency (Supplementary Figure S1F). In fact, prolonged treatment with 90-D3 reduced the number of GFP⁺ colonies, indicating a possible toxic effect of this compound.

Structure modification and discovery of a more effective molecule CYT296

To identify a better small molecule with higher induction efficiency and lower toxicity, structural modification and structure-activity relationship (SAR) studies based on the hit compound 90-D3, which has 4,5-dihydropyrrolicazole-3,3′-thiochroman]-4′-one skeleton, were carried out (Supplementary Table S1). Structural modification at site A, B, C, and D of the spiro skeleton led to a series of racemic analogs of 90-D3 and their efficiency in facilitating iPSC generation is shown in Supplementary Table S1. The o-methoxy derivative 5 and 2, 4-dimethoxy derivative 6 are less effective than 90-D3. Replacement of the methoxyl group of 90-D3 with methyl group, chlorine atom, or bromine atom decreased the iPSC generation efficiency in various degrees (compounds 2–4). We then simplified the 4,5-dihydropyrrolicazole part of 90-D3 to a cyclopropane moiety to give compound 7 (referred to as CYT296), which led to a 4-fold improvement in potency compared with 90-D3. Further structural modifications based on the spiro [cyclopropane-1,3′-thiochroman]-4′-one skeleton were performed. Replacement of the p-methoxyl group with a methyl group at the 4-position (compound 8) significantly decreased the efficacy. And the introduction of a benzoxyl at the 4-position (compound 10) led to a total loss of efficacy. Compound 9, the diastereomeric isomers of 8, did not show any potential for iPSC induction, indicating that the relative stereochemistry is crucial to their efficacy. To investigate the SAR of site B, several analogs were synthesized (compounds 11–16). Replacement of the sulfur in thiochroman-4-one with an oxygen atom (compound 13) retained the iPSC generation efficacy. While the 2,3-dihydro-1H-inden-1-one and the 3,4-dihyronaphthalen-1(2H)-one derivatives (compounds 11 and 12) suffered several-fold decrease or total loss of activity. Oxidation of CYT296 to sulfoxide and sulphone (compounds 14–16) also resulted in total loss of potency. In addition to the above, derivatization of the left-hand aromatic ring was carried out to study the SAR of site A. Introduction of aliphatic substituents or bromine atom to the 8′-position (compounds 20–24) retained the efficacy. And fluorine, methoxy, or trifluoromethyl substitution at 6′-position (compounds 17, 19, and 25) also retained the iPSC
generation efficacy, while the introduction of various substitute groups at 5′- or 7′-position resulted in complete loss of function. Among all the analogs synthesized, compound CYT296 (Figure 1A) turned out to be the best in enhancing 4F-mediated reprogramming with the most effective concentration at 250 nM. At Day 15, >70 GFP+ colonies could be observed in CYT296 treated wells. In contrast, only 2–3 colonies were observed in the control wells (Figure 1B). Like 90-D3, CYT296 was most effective when added at the early stage of reprogramming (Day 3–6). Adding after Day 6 showed no obvious effect (Figure 1C). A more detailed treatment duration study from Day 3 to 6 indicated CYT296 could be significantly effective even if it was only added for 1 day at the early stage (Day 3–4, Figure 1D). Although prolonged CYT296 treatment did not further increase the reprogramming efficiency, it did not significantly affect the growth and maintenance of the iPS colonies, indicating a better safety profile than the original 90-D3 (Figure 1E). CYT296 also facilitated reprogramming mediated by reduced number of factors in combination with chemicals. In 2F (OK), CHIR99021 and RepSox induced reprogramming, CYT296 (125 nM) led to a 2-fold increase in the number of GFP+ colonies at Day 18. In 1F (O), VPA, CHIR99021, parnate, and RepSox mediated reprogramming, CYT296 (125 nM) further enhanced the generation of GFP+ iPS colonies by 6 folds at Day 20 (Figure 1F).

The iPSCs generated with CYT296 are pluripotent

PCR of genomic DNA of 4F-, 2F (Oct4 and Klf4)- and 1F (Oct4)-iPS clones confirmed the integration of the indicated but not the other retroviral factors (Figure 2A). Real-time PCR analysis confirmed the

Figure 1 CYT296 enhances transcription factor-mediated reprogramming of MEFs. (A) Chemical structure of CYT296. (B) Representative images and corresponding statistical analysis show CYT296 promotes somatic cell reprogramming. 5000 4F-infected MEFs were seeded and iPS (GFP+) colonies were counted on Day 15. (C) 4F-infected MEFs were treated with CYT296 (250 nM) for 3 days starting from Day 3, 6, or 9 post-infection and GFP+ colonies were counted on Day 15. (D and E) 4F-infected MEFs were treated with CYT296 (250 nM) for various durations and GFP+ colonies were counted at Day 15. (F) In 2F condition, MEFs infected with Oct4 and Klf4 were treated with 3 μM CHIR99021 and 1 μM RepSox supplemented with or without 125 nM CYT296. In 1F condition, MEFs infected with Oct4 were treated with 3 μM CHIR99021, 1 μM RepSox, 0.5 mM VPA, and 2 μM parnate supplemented with or without 125 nM CYT296. Number of GFP+ colonies was counted on Day 18 for 2F and Day 20 for 1F. Data are mean ± SEM of a representative experiment with triplicates. *P < 0.05, **P < 0.01, ***P < 0.001, vs. control.
reactivation of the endogenous Oct4, Sox2, Nanog, and Rex1 and the silencing of viral genes in these IPS lines (Supplementary Figure S2A). Bisulfite genomic sequencing analyses of the Oct4 and Nanog promoters revealed that both were demethylated in iPSCs, similar to mESCs and in contrast to the parental MEFs (Supplementary Figure S2B). These iPSCs maintain GFP expression and ES like morphology, and express typical pluripotency markers, including alkaline phosphatase, SSEA1, and Nanog (Figure 2B). Subcutaneous injection of the iPSCs into NOD-SCID mice led to teratoma formation in 3–4 weeks, with tissues derived from all three germ layers (Figure 2C). We also examined the ability of CYT296-induced iPSCs to produce adult chimeras. Two iPSC lines (clone 4F-1, 2F-1) were injected into ICR-derived blastocysts, which were transplanted into the uteri of pseudopregnant mice. Adult chimeras were successfully obtained from both lines as determined by the coat color (Figure 2D); 6 out of 10 born mice from clone 4F-1 and 9 out of 15 born mice from clone 2F-1 were chimeras. One of the male chimeras from clone 2F-1 was crossed with female ICR mice, and 20 out of 69 F1 offspring showed dark coat color, confirming the germline transmission of iPSC clone 2F-1 (Figure 2E). This iPSC clone even passed the most stringent tetraploid complementation assay and supported the generation of two ‘all-iPSC’ mice, demonstrating that the iPSCs generated with CYT296 are genuinely pluripotent (Figure 2F).

**CYT296 induces chromatin de-condensation in MEFs**

To investigate the mechanisms underlying the effect of CYT296 on reprogramming, gene expression in MEFs and MEFs treated...
with CYT296 for 72 h were analyzed with microarray. Fifty-one genes were upregulated and 133 were downregulated for >2 folds after CYT296 treatment (Supplementary Table S3). Gene ontology (GO) term enrichment analyses indicated that many biological processes, especially chromatin remodeling-related pathways, were significantly altered upon CYT296 treatment (Supplementary Figure S3A and Table S4). Since electron microscopy (EM) has been widely applied to study the chromatin structure, we checked the CYT296-treated MEFs with EM. EM demonstrated a significant reduction of the electron-dense region, especially under the nuclear envelope (perinuclear heterochromatin), in CYT296-treated MEFs. And this lack of electron-dense region was also observed in mESCs (Figure 3A and B).

DNA from CYT296-treated MEFs was more prone to digest by micrococcal nuclease (MNase), which preferentially digests linker DNA and produces a ladder of bands corresponding to the multiples of the nucleosomal core plus linker DNA (Golob et al., 2008). In DNA isolated from CYT296-treated MEFs, ladder appeared after 3 min treatment with MNase. In contrast, the ladder only started to appear after 5 min treatment with MNase in the control group (Figure 3C). Statistical analysis showed CYT296 treatment significantly facilitated Mnase digestion in short-term treatment (3–7 min). At later time points, there was no obvious difference between the control group and the CYT296 treated ones since the reaction reached plateau (Figure 3D).

We then rechecked genes related to chromatin assembly in the microarray data. We noticed that genes encoding proteins related to the heterochromatin packaging and transcriptional silencing, such as HP1α, HP1γ, dmnt3a, dmnt3b, hdac2, etc., were downregulated by CYT296 treatment (Supplementary Table S5). We performed gene set enrichment analyses (GSEA) on genes that have been reported to play a role in heterochromatin formation (Supplementary Table S5) after CYT296 treatment. Interestingly, the majority of the heterochromatin related genes were inactivated after CYT296 treatment (Supplementary Figure S3B). qPCR analysis also demonstrated that the expression levels of the genes involved in heterochromatin packaging decreased significantly after CYT296 treatment (Supplementary Figure S3C). In contrast, the expression of Chd1, a gene encoding an enzyme that is associated with euchromatin, was not altered (Supplementary Figure S2C).

Lower DNA methylation levels are generally associated with more open chromatin structure and elevated gene expression. Using dot blot analysis, we found CYT296 (250 nM) treatment significantly reduced the global DNA methylation level in MEFs (Figure 3E). Western blot analysis also showed that CYT296 speed up the expression of Nanog during 4f-Mediated reprogramming; Nanog appeared as early as Day 8 (Figure 3F). We also found CYT296 might facilitate the MET by upregulating epithelial markers such as E-cadherin (E-cad) and Epithelial cell adhesion molecule (EpcAM) and downregulating the mesenchymal markers such as fibronectin (fn) and snail. The early reprogramming gene Esrb (Feng et al., 2009) was also upregulated by CYT296. However, another gene involved in early reprogramming, Uft1 (Buganım et al., 2012), was not altered by CYT296 treatment. The methylcytosine dioxygenase Tet1, which is critically involved in somatic cell reprogramming (Gao et al., 2013), was also not affected by CYT296 (Supplementary Figure S3D).

Other small molecule epigenetic modulators such as HDAC inhibitors (HDACi) have been reported to induce chromatin de-condensation (Marchion et al., 2005). We also observed that HDACi VPA reduced nuclei periphery electron-dense regions in MEFs (Supplementary Figure S4A). HDAC inhibitors increase the acetylation level and reduce the positive charge of histones, thereby decreasing the interaction of histones with the negatively charged DNA. Although CYT296 induced a similar level of chromatin de-condensation, it did not affect the acetylation level of histones. Instead, it reduced the protein levels of core histones with a mechanism yet to be identified (Supplementary Figure S4B).

Taken together, these data indicate that CYT296 treatment changes the MEF chromatin toward an ‘open’ state in a global way, which allows easier access of Yamanaka factors and facilitates the reprogramming process.

Blocking heterochromatin assembly in somatic cells facilitates reprogramming

To further confirm the effect of CYT296 on chromatin packaging, we studied two structural components and important markers of heterochromatin, the HP1α and H3K9me3 (Lachner et al., 2003; Maison and Almouzni, 2004). Immunofluorescent staining showed CYT296 treatment significantly reduced the number and total intensity of HP1α+ and H3K9me3+ foci in the nuclei of MEFs. The number and intensity of DAPI+ foci were also significantly reduced, but not as dramatic as those of the HP1α+ and H3K9me3+ foci (Figure 4A–D). Western blot analysis also revealed that during reprogramming, the protein level of HP1α reduced in a time-dependent manner. Significant reduction of HP1α could be observed at/after Day 10 (Figure 4E). In CYT296 treated group, significant reduction of HP1α could be observed at Day 6, and after that, HP1α maintained at a relatively low level (Figure 4E). ESCs expressed lower level of HP1α when compared with MEFs. And CYT296 treatment reduced the protein level of HP1α in MEFs and made it resemble the expression pattern of HP1α in ESCs (Figure 4F).

Knockdown of HP1α has been associated with reduced heterochromatin content (Chan et al., 2011). To confirm our hypothesis that CYT296 might facilitate reprogramming by reducing the heterochromatin content, we tested the effect of HP1α knockdown or overexpression in iPSC generation. Protein levels of HP1α in the knockdown or overexpression experiments were determined by western blot analysis (Supplementary Figure S5). EM observation confirmed the reduction of nuclei periphery electron-dense regions in MEFs after HP1α knockdown, indicating a more open state of the chromatin structure (Figure 5A and B). In contrast, overexpression of HP1α led to a more compact structure of the chromatin (Figure 5E and F). OG2 MEFs were infected with virus encoding 4f and shRNA targeting HP1α, and GFP+ colonies were counted at Day 16. As demonstrated in Figure 5C, knockdown of HP1α significantly enhanced the reprogramming efficiency. In contrast, overexpression of HP1α led to a significantly reduced generation of iPSCs (Figure 5G). Apart from HP1α, a number of genes involved...
in chromatin remodeling have been found to be downregulated by CYT296 (Supplementary Figure S3C). Among them, the *hdac1*, *hdac2*, *suv39h1*, *suv39h2*, *dnmt3*, and *dnmt3b* have been reported to play a role in reprogramming. The functions of others genes (including *HP1α*, *smarca1*, *smarca5*, *cbx2*, and *cbx4*) in reprogramming have yet to be elucidated. To further support our
hypothesis that reducing heterochromatin assembly facilitates reprogramming, we applied shRNA targeting cbx2, a gene involved in heterochromatin formation (Bernstein et al., 2006), in 4F-mediated iPSC generation. As shown in Figure 5D, cbx2 knockdown also significantly improved reprogramming efficiency.

Since CYT296 induced an open chromatin state, the pretreatment of MEFs with CYT296 (before initiating reprogramming by other factors) might also increase reprogramming efficiency. To test this hypothesis, we used the Dox-inducible viral vectors (Sommer et al., 2009) to rule out the possibility that CYT296

---

Figure 4 CYT296 reduces heterochromatin foci formation. (A) Immunofluorescent staining of HP1α before and after CYT296 treatment in MEFs. Scale bar, 50 μm. (B) Statistical analysis of the number and total fluorescence intensity of HP1α+ and DAPI+ foci presented in A. (C) Immunofluorescent staining of H3K9me3 in MEFs treated with CYT296 or vehicle. Scale bar, 50 μm. (D) Statistical analysis of the number and total fluorescence intensity of H3K9me3+ foci presented in C. Data are mean ± SEM; ~40 cells from each group were analyzed. (E) Representative western blot and statistical data of HP1α protein level during 4F-mediated reprogramming with or without CYT296 (250 nM) treatment. Data are mean ± SEM (n = 3). (F) Representative western blot and statistical data of HP1α in mESCs (E14) and MEFs treated with CYT296 (250 nM) for various duration. Data are mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, vs. control.
might affect the viral infection efficiency. MEFs were infected with Dox-inducible viral vectors encoding 4F first and then treated with CYT296 for 2 days. After removal of CYT296, Dox was added to induce reprogramming. The overall iPSC induction efficiency was much less than our routine system, but as demonstrated in Figure 5H, pretreatment of CYT296 before the addition of Dox significantly enhanced the generation of iPSCs. Taken together, these data indicate that chromatin remodeling plays a critical role in somatic cell reprogramming and iPSC generation can be dramatically improved by small molecules blocking heterochromatin assembly.

**Discussion**

Similar to differentiation, somatic cell reprogramming is a change of cell fate governed by global changes of gene expression pattern. Genes in eukaryotic cells tend to stay inactive unless they are specifically turned on by activators in response to environmental or developmental cues. Generally, transcriptional activation begins with the binding of transcription factors to promoter and enhancer regions, which initiates the recruitment of coactivators, chromatin modifying/remodeling factors and facilitates the assembly of the RNA polymerase-II-containing preinitiation complex (PIC) or transcription machinery at the core promoter (Ma, 2011).
The packaging of eukaryotic DNA into nucleosomes affects all aspects of transcription, from transcription factor binding to PIC formation and transcriptional elongation (Li et al., 2007). The binding of transcription factors is modulated by changes in chromatin structure, including ATP-dependent chromatin remodeling and posttranslational modifications of histones, such as methylation, acetylation, phosphorylation, and ubiquitination (Ma, 2011). Chromatin therefore plays a critical role in the establishment of cell-type-specific expression pattern of genes and the maintenance of cellular identity under physiological conditions.

ESC chromatin exists in an unusual configuration with global de-condensation, looser binding of architectural chromatin proteins, and enrichment of active histone modifications, all of which lead to a higher transcriptional activity than differentiated cells (Meshorer and Misteli, 2006; Efroni et al., 2008). Differentiation or development leads to down-regulation of key pluripotency transcription factors with extensive chromatin reorganization and formation of large compact chromatin domains (Efroni et al., 2008). These observations suggested that an open chromatin structure might be required for the maintenance or induction of pluripotency. Indeed, massive nuclear swelling accompanied by global chromatin de-condensation and transcription activation is one of the hallmarks of nuclear reprogramming observed in Xenopus laevis cloning (Gurdon et al., 1979). Nucleolarplasin (a chaperone of histones H2A and H2B) (Tamada et al., 2006) and oocyte-specific linker histone B4 (Jullien et al., 2010) have been reported to facilitate reprogramming by nuclear transfer by inducing chromatin de-condensation. The chromatin remodeling enzyme Chd1 has been shown to be important for the maintenance and factor-induced pluripotency by promoting an open chromatin state (Gaspar-Maia et al., 2011). Chromatin remodelers Brg1 and Baf155 have also been found to increase the efficiency of Oct4 reactivation during induction of pluripotency from MEFs (Hansis et al., 2004; Singhal et al., 2010). Thus, opening the chromatin structure with small molecules seems to offer an alternative approach to facilitate the induction and establishment of pluripotency.

CYT296, modified from the original 90-D3, improves OSKM-mediated generation of iPSCs for >10 folds and enables efficient reprogramming with only Oct4 in combination with other small molecules. With bioinformatics analysis, we noticed that CYT296 treatment influenced many genes involved in chromatin organization. In particular, many genes involved in heterochromatin assembly were downregulated by CYT296 treatment. EM revealed dramatic loss of electron-dense heterochromatin regions in CYT296-treated MEFs, a phenomenon also observed in mESCs. In MEFs, HP1α, an essential component for heterochromatin organization and maintenance (Maison and Almouzni, 2004), was reduced by CYT296 treatment. It has been proposed that the loss of chromosomal proteins is a primary event that is required, but not sufficient for reprogramming and counteracts differentiation-related resistance (Pasque et al., 2011). By knocking down HP1α with shRNA, we were also able to increase the efficiency of OSKM-mediated reprogramming. A recent report also showed evidence that the inhibition of heterochromatin protein 1γ, a protein known to recognize H3K9 methylation, enhances reprogramming (Sridharan et al., 2013). Apart from HP1α, CYT296 also significantly decreased the expression of DNA methyltransferases including DNMT1, DNMT3a and 3b, leading to a reduction of global DNA methylation. This inhibitory effect is beneficial to reprogramming as demonstrated by 5-azacytidine, a well-known DNA methyltransferase inhibitor, which promotes iPSC generation (Huangfu et al., 2008). CYT296 also repressed Uhrf1 (Supplementary Table S5), which is required for DNA methylation maintenance (Zhang et al., 2011).

In summary, CYT296 was identified to improve factor-mediated generation of iPSCs by inducing chromatin de-condensation in somatic cells. We also demonstrated for the first time that knockdown of HP1α and Cbx2, two genes involved in heterochromatin assembly, reduced heterochromatin content and greatly enhanced iPSC generation. Our study not only provides a new chemical to be used in iPSC induction, but also suggested that targeting chromatin de-condensation with small molecules might be a new approach to regulate somatic cell reprogramming. Although the exact target/targets of CYT296 is still not clear, the preliminary structure-activity relationship of CYT296 and its analogs present in this work provides us opportunity to design activity-based probes for further identification of upstream targets involved in chromatin remodeling and pluripotency induction.

Materials and methods

Retroviral-mediated iPSCs generation

Generation of mouse iPSCs using pMXs retroviral vectors containing cDNAs of mouse Oct4, Sox2, Klf4, and c-Myc was described previously (Wang et al., 2011). Briefly, MEFs carrying an Oct4-GFP reporter were isolated from OG2 mice and only early passages of MEFs were used for reprogramming. MEFs were infected with 4f containing viruses at Day 0. Two days after viral transduction, MEFs were reseeded at a density of 5000 cells per well onto 96-well plates pre-seeded with feeders and cultured in mES medium for 3 days followed by the replacement with KSR medium. GFP+ colonies were photographed and counted using Olympus IX71 microscope and Image Pro Plus software after Day 14. GFP+ colonies were also trypsinized and then analyzed using a Guava EasyCyte 8HT flow cytometer. Chemicals were added at the day after reseeding (Day 3) and the treatment lasted for various durations.

Doxycycline (Dox)-inducible reprogramming

Dox-inducible reprogramming was performed as previously described (Sommer et al., 2009) with slight modification. Equal volumes of the pHAGE-STEMCCA virus encoding the four factors and the rTA virus were mixed and used to infect OG2 MEFs by centrifugation. To investigate whether CYT296 pretreatment could improve reprogramming, 2 days after infection, MEFs were reseeded at a density of 5000 cells per well onto 96-well plates pre-seeded with feeders. Twenty-four hours later, MEFs were treated with CYT296 (500 nM) for 2 days. After removal of CYT296, reprogramming was induced by the addition of Dox (1 μg/ml). GFP+ colonies were counted 14 days after the addition of Dox.
**Immunofluorescent and alkaline phosphatase staining**

For AP staining, iPSCs were fixed with 4% paraformaldehyde (PFA) in PBS for 45 sec, rinsed once with PBS and detection was performed using a leukocyte AP kit (Sigma, catalog No 85L3R) according to the manufacturer’s protocol. For immunofluorescent staining, cells were fixed with 4% PFA and incubated with primary antibodies against mSSEA-1 (Santa Cruz, sc-21702), mNanog (Millipore, AB5731), and HP1α (Millipore, MAB3584) followed by the appropriate secondary antibodies conjugated to Alexa Fluor 555 or 556. Nuclei were counterstained with Hoechst 33342. Images were taken with an Olympus IX71 inverted fluorescent microscope or an Olympus FV10i confocal microscope. Statistical analysis of images was performed using the Image Pro Plus software.

**Western blot**

Cells were lysed, sonicated, and boiled at 95°C–100°C for 5 min in sample buffer [50 mM Tris–HCl, 2% w/v SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenyl blue (pH 6.8)]. Cell lysates were separated on SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were first incubated with blocking buffer (TBS with 5% nonfat milk) for 1 h at room temperature and then incubated overnight at 4°C in buffer containing mouse anti-HP1α (1:1000; Millipore MAB3446), rabbit anti-mNanog (1:1000; Millipore AB5731), rabbit anti-GAPDH (1:10000; CST 2118). The membranes were washed thrice and incubated with goat anti-rabbit IgG HRP (1:10000) or goat anti-mouse IgG HRP (1:10000) for 1 h. After washing, immunostaining was visualized using Western Lightning Ultra and ChemiDoc imaging system (Bio-Rad).

**Teratoma formation, chimera generation, and tetraploid complementation**

About 1 x 10⁶ iPSCs were suspended in 200 µl mES medium and injected into the thigh muscle of NOD-SCID mice. Four to six weeks after the injection, teratomas were surgically dissected from the mice. Tissues were fixed in 4% PFA, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

For production of chimeric mice, female ICR mice were superovulated and mated. Embryos at 2-cell stage were flushed from the uteri on Day 1.5 and incubated in EmbryoMax® KSOM medium (Millipore) supplemented with amino acids for 2 days. Ten to fifteen iPSCs were injected into each blastocyst, which was then transferred into the uteri of pseudopregnant ICR mice. Chimeras were identified from the pups based on their coat color. One of the male chimeras was then crossed with wide-type female ICR mice to test germline transmission.

To generation ‘all-iPSC’ mice by tetraploid complementation, two-cell embryos were collected from the superovulated ICR females and electrofused to produce one-cell tetraploid embryos which were then cultured in EmbryoMax® KSOM medium to allow development into blastocysts. Approximately 10–15 iPSCs were injected into the cavity of tetraploid blastocyst. Ten to fifteen injected blastocysts were transferred into the uteri of 2.5-day postcoitum pseudopregnant ICR females. Pregnant recipients with tetraploid embryos were subjected to cesarean section on Day 18.5 of gestation.

**Quantitative RT–PCR**

Total RNA was isolated using Trizol (Gibco) according to the manufacturer’s instructions. About 1.5 µg of RNA was used to synthesize cDNA using random hexamer primer and Moloney Murine Leukemia Virus reverse transcriptase. Quantitative PCR was performed using JumpStartTM Taq ReadyMix™ (Sigma, D7440) with Eva Green (Biotium) and analyzed with a Stratagene Mx 3000P thermal cycler. For semi-quantitative PCR analysis, the cDNA solution was amplified for 35 cycles at an optimal annealing temperature. Primers sequences are supplied in Supplementary Table S2. Gene expression value was normalized to that of β-actin.

**Bisulfite sequencing**

Bisulfite sequencing was used to detect DNA methylation in the promoter regions of Oct4 and Nanog. In brief, genomic DNA was isolated and bisulfite conversion was performed in agarose beads as described (Hajkova et al., 2002). Nested PCR was performed to amplify the promoter regions after bisulfite conversion and the PCR products were cloned into pMD19-T vectors (Takara). Ten randomly selected clones from each sample were sequenced and analyzed.

**Dots blot analysis of total DNA 5-mC**

MEFs were incubated in medium supplemented with 250 nM CYT296 for 24, 48, and 72 h. Genomic DNA was isolated using the DNA Isolation Kit (Promega) according to the manufacturer’s instructions and 200 ng DNA from each sample was dotted onto the nitrocellulose membrane (GE Amersham). Membrane was exposed to UV light for 5 min and then incubated in blocking buffer for 1 h at room temperature. Membrane was incubated overnight with the 5-mC antibody (Active motif 39649) in blocking buffer at 4°C. After through washing, membrane was probed with HRP-conjugated secondary antibody and visualized using Western Lightning Ultra and ChemiDoc imaging system (Bio-Rad).

**Gene expression microarray and GSEA analyses**

Gene expression microarray analyses of control MEF and MEF treated with CYT296 for 72 h were performed using Affymetrix GeneChip Mouse Genome 430 2.0 arrays. Genes displayed 2-fold or greater changes in expression level were selected for GO term enrichment analysis and Gene Set Enrichment Analysis (GSEA). For GO term enrichment analysis, conditional single-sided hyper geometric tests were used to calculate the P-value (http://david.abcc.ncifcrf.gov/). In order to determine whether an a priori defined set of genes show statistically significant concordant difference between CYT296 treated and untreated samples, Java GSEA software was used (available online, http://www.broadinstitute.org/gsea/index.jsp).

**Micrococcal nuclease analysis**

MEF cells were harvested and nuclei were prepared using the SimpleChIP® Enzymatic Chromatin IP Kit according to the
Transmission electron microscopy

Cell samples were fixed in 1% glutaraldehyde for 1 h and then post-fixed in 1% osmium tetroxide for 1 h. Samples were dehydrated in a series of acetone dilutions before being embedded in Epon 812 resin. Ultrathin sections (70 nm) were stained with 4% uranyl acetate solution and lead citrate, and examined using a JEOL 812 resin. Ultrathin sections (post-fixed in 1% osmium tetroxide for 1 h, and then treated with 400 μg/ml proteinase K for 1 h) were then purified by phenol/chloroform extraction and ethanol precipitation. The purified nucleosomal DNA and input DNA (corresponding DNA preparations from nuclei omitting MNase treatment) were analyzed by electrophoresis using 1% agarose gel.

Statistical analysis

All data were expressed as mean ± SEM and analyzed using two-tailed Student’s t-test. P < 0.05 was regarded as statistically significant.

Supplementary material

Supplementary material is available at the Online Journal of Molecular Cell Biology online.

Funding

This work was supported by grants from Chinese Academy of Sciences (XDA01040301), Ministry of Science and Technology of China (2009CB940900, 2011DFB300010, 2011CB965104), and the National Natural Science Foundation of China (91213303, 31371511, 30725049). Enhance reprogramming via chromatin de-condensation | 419

Conflict of interest: none declared.

References


Enhance reprogramming via chromatin de-condensation | 419


