

Generation of Rat and Human Induced Pluripotent Stem Cells by Combining Genetic Reprogramming and Chemical Inhibitors

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Conventional mouse and human embryonic stem cells (ESCs) can be typically derived by in vitro culture of blastocysts (Martin, 1981; Thomson et al., 1998), and induced pluripotent stem cells (iPSCs) can be generated by reprogramming somatic cells using defined genetic transduction methods (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). In both cases, different signaling pathways appear to regulate pluripotency with different characteristics in the two species. However, while rat ESC-like cells have been established based on certain traits (Demers et al., 2007; Ruhnke et al., 2003; Schulze et al., 2006; Ueda et al., 2008), to date, these lines fall short of exhibiting true pluripotency (e.g., fail to form teratoma or no/little contribution to chimerism) and thus cannot be considered authentic rat ESCs. Here, we reveal combined genetic reprogramming and chemical conditions that generate and maintain rat iPSCs (riPSCs) that can give rise to teratomas and contribute extensively to chimeric rats. The same strategy is also sufficient to generate atypical human iPSCs (hiPSCs) that exhibit similar colony morphology and self-renewal requirements/signaling responses as those of mESCs.

Pluripotent stem cells have also been derived from the postimplantation egg cylinder stage epiblasts of mouse and rat (Brons et al., 2007; Tesar et al., 2007). These populations have been termed epiblast stem cells (EpiSCs). EpiSCs seem to correspond very closely to conventional hESCs with respect to colony morphology and the culture/signaling requirements that maintain pluripotency but exhibit a range of significant phenotypic and signaling response differences from conventional mESCs. For

example, LIF is insufficient to support either hESCs or EpiSCs, and BMP4, which is also required in conjunction with LIF by mESCs for self-renewal, induces hESC/EpiSCs to differentiate into trophoblasts or primitive endoderm. In addition, bFGF and Activin A are dispensable for mESC self-renewal and induce mESC differentiation, but required by EpiSCs and hESCs. Finally, both rat and mouse EpiSCs show little or no ability to be incorporated into preimplantation embryos and subsequently contribute to chimeras (Brons et al., 2007; Tesar et al., 2007).

Collectively, we hypothesized that, by altering the growth conditions used to select and maintain pluripotent rat cells, lines that exhibit mESC traits and contribute to chimeric animals might be isolated. Given that similar cocktails of transcription factors have been successful in reprogramming somatic murine and human cells to pluripotency, we employed this strategy to generate rat iPSCs. Rat liver progenitor cells (diploid cell line, WB-F344 [Grisham et al., 1993]) were transduced with *Oct4*, *Sox2*, and *Klf4* using retroviral vectors and then split onto MEF feeder cells and cultured in conventional mESC medium containing knockout serum replacement and LIF (see Supplemental Data for detailed Experimental Procedures). Compact, alkaline phosphatase (ALP)-positive colonies that exhibit morphological similarity to mESCs were observed 10 days after transduction with an efficiency of ~0.4% (Figure 1A). When the ESC-like colonies were isolated and subcultured in the same medium, however, they quickly differentiated and lost ESC morphology (Figure 1B), suggesting that conventional mESC medium conditions are not sufficient to

maintain the putative riPSCs in a pluripotent state.

Given the notion that small molecules can inhibit key differentiation-inducing pathways, we and others have previously identified and used small molecules to support mESC self-renewal in a more robust manner (Chen et al., 2006; Schugar et al., 2008; Xu et al., 2008). We used this chemical strategy, combined with the knowledge of signaling differences required for maintenance of self-renewal of mESC versus EpiSC/hESC, to design candidate cocktails to select and maintain pluripotent riPSCs in culture. mESCs require BMP4, in part to inhibit ERK activation, whereas hESCs/EpiSCs exhibit high levels of ERK activation in response to FGF signaling. In addition, GSK3 inhibition by specific small molecules to elevate Wnt signaling seems to contribute favorably to self-renewal of both mESCs and hESCs when combined with appropriate modulation of other pathways (Ludwig et al., 2006; Umehara et al., 2007). Therefore, we supplemented the standard mESC conditions with a combination of a MEK inhibitor (0.5 μ M PD0325901) and a GSK3 β inhibitor (3 μ M CHIR99021) and observed that riPSCs can be maintained short-term in the presence of these chemicals but still exhibit extensive spontaneous differentiation (Figure 1C). Indeed, after serial passages, the growth of putative riPSCs treated with only PD0325901 and CHIR99021 declined, and the culture deteriorated due to the expansion of differentiated cells. Recent studies demonstrated that addition of the FGFR inhibitor PD173074 to the above cocktail is sufficient to maintain mESC pluripotency in the absence of LIF (Ying et al., 2008). However, our MEK and GSK3 β

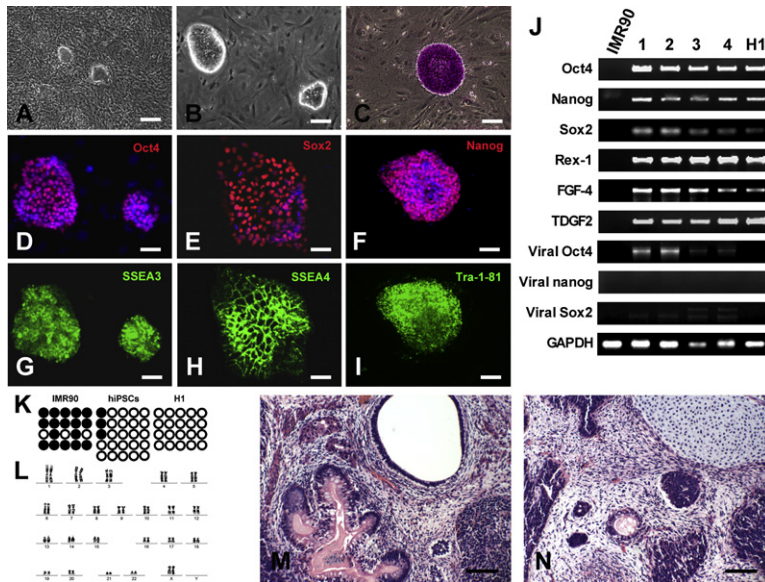


Figure 2. Human iPSCs Are Generated under the Same Conditions that Give Rise to rPSCs IMR90 human fibroblasts were transduced with *Oct4*, *Sox2*, *Nanog*, and *Lin28* by lentiviruses. The hiPSC colonies were observed 3 weeks after transduction (A), picked up at the fourth week after transduction, and were stably maintained under the cocktail of hLIF, 0.5 μ M PD0325901, 0.25 μ M A-83-01, and 3 μ M CHIR99021. Such hiPSCs formed domed colonies similar in appearance to mESCs (B). Under such conditions, the expression of pluripotency markers by hiPSCs was analyzed by ALP staining (C), immunostaining (D–I), and RT-PCR (J). Four clonal hiPSC lines were subjected to RT-PCR analysis, and parental IMR90 cells were used as controls. The methylation status of the *Oct4* promoter in hiPSCs, IMR90 cells, and H1 hESCs was analyzed using bisulfite sequencing (K). Open circles indicate unmethylated, and filled circles indicate methylated CpG dinucleotides. (L) A karyotype analysis of hiPSCs is provided. Hematoxylin and eosin staining of teratoma sections of hiPSCs appears in (M) and (N). Scale bars, 50 μ m.

derivatives. Next, the *in vivo* developmental potential of rPSCs was assessed. After transplantation into Severe Combined Immunodeficient (SCID) mice, rPSCs formed teratomas, which consisted of all three germ layers, including neuroepithelium structures (ectoderm, Figure 1L), ciliated epithelium of airway (endoderm, Figure 1M, arrow), and cartilage structures (mesoderm, Figure 1N). Most remarkably, after injection into Brown-Norway rat (black fur) blastocysts ($n = 18$), three rats were born and all exhibited extensive coat-color chimerism (Figures 1O and 1P). However, no germline transmission has been detected yet.

Taken together, the above results indicate that the addition of the combined inhibitor cocktail to standard mESC growth conditions is sufficient to support and maintain the pluripotency of rPSCs with traits that resemble mESCs and with distinct morphological, signaling, and functional properties from EpiSCs and hESCs. Consistent with this pattern, standard bFGF-containing culture conditions that support conventional hESCs/

EpiSCs and hiPSCs are not sufficient to yield our described rPSCs but do give rise to colonies that resemble EpiSCs (data not shown). To assess whether distinct hiPSCs that share colony morphology and key signaling responses with conventional mESCs could be generated using the rPSC-supporting chemical inhibitor cocktail, we transduced IMR90 human fibroblasts with *Oct4*, *Sox2*, *Nanog*, and *Lin28* in mESC medium containing hLIF. Remarkably, we found that primary hiPSC colonies could be effectively generated with a similar kinetics and efficiency (Figure 2A, 15~20 iPSC colonies from 1×10^5 transduced cells on average) as has been reported using conventional hESC conditions (Yu et al., 2007), and these colonies could be subsequently selected/stabilized and further expanded with the addition of the chemical cocktail of ALK5, GSK3, and MEK inhibitors. The resulting hiPSCs appear to be homogenous and self-renew over long-term periods (>20 passages) in the presence of hLIF and the chemical cocktail of PD0325901, A-83-01, and CHIR99021 (Figure 2B). In contrast to

conventional hESCs and previously isolated hiPSCs, the chemically selected hiPSCs form more compact, ALP-positive domed colonies similar in appearance to mESCs (Figure 2C). In addition, these colonies were clearly resistant to differentiation in response to the MEK and ALK5 inhibitors, whereas the conventional hESC line H1 differentiated rapidly under the same conditions (data not shown). hiPSCs isolated under the rPSC-defined conditions homogeneously express typical pluripotency markers, such as *Oct4*, *Sox2*, *Nanog*, *SSEA3*, *SSEA4*, and *TRA-1-81* (Figures 2D–2I), and RT-PCR analysis of four clonal hiPSC lines confirmed the expression of the endogenous human *Oct4*, *Sox2*, *Nanog*, *Rex-1*, *TDGF2*, and *FGF4* (Figure 2J). By using transgene-specific primers, RT-PCR analysis revealed that the transduced *Oct4*, *Sox2*, and *nanog* genes were largely silenced (Figure 2J). Moreover, these hiPSCs showed similar DNA methylation patterns at the *Oct4* promoter, as observed in H1 human ESCs and distinct from parental IMR90 fibroblasts (Figure 2K). Karyotyping analysis revealed the normal 46, XX chromosomes (Figure 2L). Similar to rPSCs, the inhibitor cocktail was required to maintain the domed colony morphology and long-term *in vitro* self-renewal of the hiPSCs, in that removal of the inhibitor cocktail led to the loss of colony morphology within a single passage. In contrast, removing hLIF from the medium did not show immediate/dramatic effects on the cells. However, hLIF seems to be useful for the long-term culture of hiPSCs; when maintained in medium containing the chemical inhibitors but without hLIF, hiPSC cultures deteriorate gradually and have not been homogeneously maintained beyond ten passages. Nevertheless, the exact signaling mechanism responsible for self-renewal of the chemically selected hiPSCs remains to be determined.

Importantly, immunocytochemical analyses confirmed that hiPSCs generated under rPSC conditions have the capacity to differentiate into endoderm (Albumin, Figure S3A), neuroectoderm (β III-tubulin, Figure S3B), and mesoderm (brachyury, Figure S3C) derivatives *in vitro*. Furthermore, after transplantation into SCID mice, hiPSCs formed teratomas, which consisted of all three germ layers, including neuroepithelium-like structure (ectoderm, Figure 2M), epithelial tube

structure (endoderm, Figure 2M), and cartilage-like structure (mesoderm, Figure 2N). Taken together, the above results suggest that atypical human pluripotent stem cells that exhibit the characteristic morphology and key signaling responses of conventional mESCs can be generated and maintained long-term if the appropriate growth conditions are provided.

Although embryonic stem cells have been derived from mice since 1981 (Martin, 1981), attempts to derive their counterparts from other rodents, such as rat, have not, to date, completely succeeded (Demers et al., 2007; Ruhnke et al., 2003; Schulze et al., 2006; Ueda et al., 2008). By combining genetic reprogramming with the addition of chemical inhibitors, we were able to generate rat and human pluripotent stem cells that exhibit some characteristics of conventional mESCs, such as colony morphology and culture requirements/signaling responses. Under this specific cocktail of small molecules, riPSCs grow stably in culture. In addition, riPSCs are capable of contributing extensively to chimerism in vivo, and thus they demonstrate a degree of pluripotency that had yet to be shown for cultured cells from this species. Rats are better suited for physiological and behavioral studies than mice and have the potential to provide excellent models for multigenic human diseases. However, realizing the experimental potential of this species has been hindered by the unavailability of rat stem cells that are pluripotent in vivo. Our establishment of pluripotent rat cells and the strategy to identify appropriate chemical cocktails that maintain them will pave the way to generate gene-targeted rats for biomedical research. In addition, hiPSCs

grown in these conditions appear to be more robust and exhibit traits that suggest that they occupy a pluripotent state that is similar to that defined by conventional mESCs and distinct from conventional hESCs. These findings also underscore the combined importance of the TGF- β , WNT, and FGF pathways in regulating pluripotency in different species.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, one table, and three figures and can be found with this article online at [http://www.cell.com/cell-stem-cell/supplemental/S1934-5909\(08\)00616-4](http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(08)00616-4).

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