

A Murine ESC-like State Facilitates Transgenesis and Homologous Recombination in Human Pluripotent Stem Cells

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SUMMARY

Murine pluripotent stem cells can exist in two functionally distinct states, LIF-dependent embryonic stem cells (ESCs) and bFGF-dependent epiblast stem cells (EpiSCs). However, human pluripotent cells so far seemed to assume only an epiblast-like state. Here we demonstrate that human iPSC reprogramming in the presence of LIF yields human stem cells that display morphological, molecular, and functional properties of murine ESCs. We termed these hLR5 iPSCs because they require the expression of five ectopic reprogramming factors, *Oct4*, *Sox2*, *Klf4*, *cMyc*, and *Nanog*, to maintain this more naive state. The cells are “metastable” and upon ectopic factor withdrawal they revert to standard human iPSCs. Finally, we demonstrate that the hLR5 state facilitates gene targeting, and as such provides a powerful tool for the generation of recombinant human pluripotent stem cell lines.

INTRODUCTION

Embryonic stem cells (ESCs) were first derived in 1981 from the inner cell mass (ICM) of murine preimplantation blastocyst embryos (Evans and Kaufman, 1981; Martin, 1981). ESCs are pluripotent, meaning they are able to expand indefinitely in vitro while retaining the capacity to generate derivatives of all three germ layers both in vitro and in vivo. The discovery of murine ES cells (mESCs) was a major breakthrough in developmental biology, because it enabled the study of mammalian gene function in vivo, via transgenic and knockout technologies. The subsequent derivation of human ES cells (hESCs) raised the expectation that these cells would similarly revolutionize our

insights into human development and disease. Unfortunately, human pluripotent stem cells are remarkably resilient to nonviral genetic manipulation and to date only a handful of human knockin or knockout cell lines exist. As a result, the application of human pluripotent stem cells has been more limited than previously anticipated.

Although both human and murine ESCs are derived from blastocyst-stage embryos, they demonstrate profound differences (Thomson et al., 1998). Murine ESCs grow in three-dimensional, tightly packed colonies with a population doubling time of approximately 16 hr and their maintenance is dependent on LIF and BMP4 growth factor signaling (Smith et al., 1988; Xu et al., 2005; Ying et al., 2003). In contrast, human ESCs form flattened two-dimensional colonies and are maintained in a bFGF and Activin A/TGF- β signaling-dependent manner (Thomson et al., 1998). hESCs proliferate slowly, with a population doubling time averaging 36 hr. Epigenetically, human and murine ESCs display a different X chromosome inactivation pattern and promoter occupancy by pluripotency transcription factors (Boyer et al., 2005; Silva et al., 2008; Tesar et al., 2007). In addition, hESCs are passaged as small clumps of cells, and most hESC lines cannot be passaged as single cells by trypsin digest. The inability of hESC lines to grow from single cells greatly impedes genetic modification of these cells, because the introduction of transgenes is typically followed by clonal selection.

Two reports on the derivation of murine epiblast stem cells (EpiSCs) recently provided a new perspective on the nature of human ESCs (Brons et al., 2007; Tesar et al., 2007). EpiSCs are derived from postimplantation murine epiblast embryos under culture conditions similar to hESC culture conditions. EpiSCs display many of the characteristics of human ESCs including their dependence on bFGF/Activin A signaling, their flattened colony morphology, their slower proliferation rate compared to murine ESCs, their X-inactivation status, and their requirement to be passaged as small clumps of cells (Brons et al., 2007; Tesar et al., 2007).

The culture dynamics and the specific characteristics of murine ESCs and EpiSCs appear to be largely determined by the growth factor conditions under which these cell types are derived and maintained. Indeed, recent work from our group demonstrates that culture growth factor conditions play a critical role in defining the pluripotent stem cell state (Chou et al., 2008). Intriguingly, although pluripotent stem cells can be stably derived and propagated from multiple species in an epiblast-like state, including the rat and “nonpermissive” mouse strains, the LIF-dependent pluripotent state appears to be unstable in these species (Buehr et al., 2008; Hanna et al., 2009; Li et al., 2009; Liao et al., 2009). However, the LIF-dependent pluripotent state can be stabilized through the constitutive ectopic expression of one or more of the reprogramming factors (*Oct4*, *Sox2*, *Klf4*, *cMyc*), which induce the generation of induced pluripotent stem cells (iPSCs) from somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). In the nonpermissive NOD mouse strain, for example, the constitutive ectopic expression of either *Klf4* or *cMyc* is sufficient to allow the derivation of ES-like cells from blastocyst embryos (Hanna et al., 2009). Small molecule inhibitors of glycogen synthase kinase 3 beta (GSK3 β) and the mitogen-activated protein kinase (MAPK) signaling pathway can replace some of the reprogramming factors during iPSC generation (Li and Ding, 2009). These inhibitors can similarly stabilize the LIF-dependent mES-like pluripotent stem cell state from both the nonpermissive NOD mouse strain and the rat (Buehr et al., 2008; Hanna et al., 2009; Li et al., 2009; Liao et al., 2009). Thus, it appears that the LIF-dependent pluripotent state is metastable in these species, meaning that it is dependent on either the constitutive expression of ectopic reprogramming factors or the continued inhibition of GSK3 β and/or the MAPK signaling pathways.

Although distinct pluripotent stem cell states are known to exist in mouse and rat, they have thus far not been described for human stem cells. A recent report demonstrates that stable human iPSCs (hiPSCs) can be derived in the presence of LIF and inhibitors of GSK3 β and the TGF- β and MEK/ERK signaling pathways (Li et al., 2009). However, these cells appear to be molecularly identical to conventional hiPSCs. We used hiPSC derivation as a tool to investigate the influence of growth factor signaling on the human stem cell pluripotent state. Here we demonstrate the derivation of human cell lines that display many characteristics of murine ESCs including a dome-shaped colony morphology, the ability to be propagated by trypsin digest and to clonally grow from single cells, and the activation of LIF downstream signaling pathways. We demonstrate that in this state, the human cells are more amenable to the introduction of transgenes and allow homologous recombination-mediated gene targeting. The LIF state is metastable, as indicated by the fact that it depends on the constitutive expression of ectopic reprogramming factors. Yet a combination of growth factors and inhibition of MEK-kinase signaling allows the conversion of the human LIF-iPSCs to a stable, pluripotent human iPSC state.

Our findings support the idea that, analogous to mouse strains and the rat, human iPSCs adopt murine ESC properties, when the cells are derived in the presence of LIF and ectopic reprogramming factors. Importantly, this novel state facilitates homologous recombination-mediated gene targeting in human stem cells. As such, the intermediate iPSC state

described here can be a useful tool in research and future cell therapies.

RESULTS

Derivation of Metastable Human iPSC Lines with Murine ESC Characteristics

We used the recently reported induced pluripotent stem cell (iPSC) strategy to explore the possibility of deriving human iPSCs in the presence of LIF. The five reprogramming factors *OCT4*, *SOX2*, *NANOG*, *c-MYC*, and *KLF4* were expressed in human fibroblast via a recently reported doxycycline-inducible lentiviral system (Figure 1A; Maherali et al., 2008). Fibroblasts were reprogrammed either directly from the primary fibroblasts or from so-called secondary fibroblasts, derived from differentiated “primary” hiPSCs (Maherali et al., 2008). With either approach, reprogramming of human fibroblasts in the presence of LIF resulted in the formation of two types of colonies: transient, irregularly shaped colonies that deteriorated a few days after their first appearance (Figure S1A available online) and smaller, tightly packed colonies (Figure S1B). We picked individual colonies of the latter for further clonal analysis. These clones displayed the hallmark, tightly packed, bright, dome-shaped morphology of mESCs (Figure 1B), contrasting the flattened two-dimensional colony morphology of hESCs (Figure 1B). We designated these cells human LR5-iPSCs (hLR5) (human LIF + the constitutive expression of 5 reprogramming factors).

Next we investigated the cell surface marker profile of the hLR5 cells. Murine and human pluripotent stem cells express a mutually exclusive complement of cell surface markers. SSEA1 is expressed on undifferentiated murine pluripotent stem cells whereas human pluripotent stem cells express the SSEA3, SSEA4, TRA-1-81, and TRA-1-60 cell surface markers. Flow cytometry analysis of the hLR5 cells revealed a marker profile that resembles the surface marker profile of mESCs (Figure 1C). hLR5 cells do not express the TRA-1-81 cell surface marker (Figure 1C) but demonstrated high expression of the SSEA1 cell surface marker, which is typically expressed on murine pluripotent stem cells (Figure 1C). A fourth clone, which was derived through direct reprogramming of human fibroblasts from a different genetic background, also lacked TRA-1-81 expression but expressed low SSEA1 levels, suggesting that the level of SSEA1 expression is heterogeneous between hLR5 clones of different genetic backgrounds. In addition to the general lack of TRA-1-81 expression, hLR5 cells also do not express SSEA3, SSEA4, and TRA-1-60, as tested by flow cytometry and immunohistochemistry (not shown). Unlike hESCs, the hLR5 cells can be propagated by trypsin digest. This result suggested that similar to mESCs, hLR5 cells are tolerant to passaging as single cells. Indeed, upon single cell sorting of hLR5 cells into 96-well plates, hLR5 clones re-emerged in approximately 22% of the wells ($n = 10$), similar to the efficiency of single-cell sorted mESCs (~30%), whereas upon single-cell sorting of the trypsin-adapted HUES3 hESC line (Cowan et al., 2004), no colonies re-emerged ($n = 10$). In addition, the hLR5 cells displayed a much higher proliferation rate than human ESCs or iPSCs, with a cell doubling time of approximately 22 hr (Figure 1D). The hLR5 proliferation rate is close to the mESC or miPSC proliferation rate (doubling time ~16 hr) and

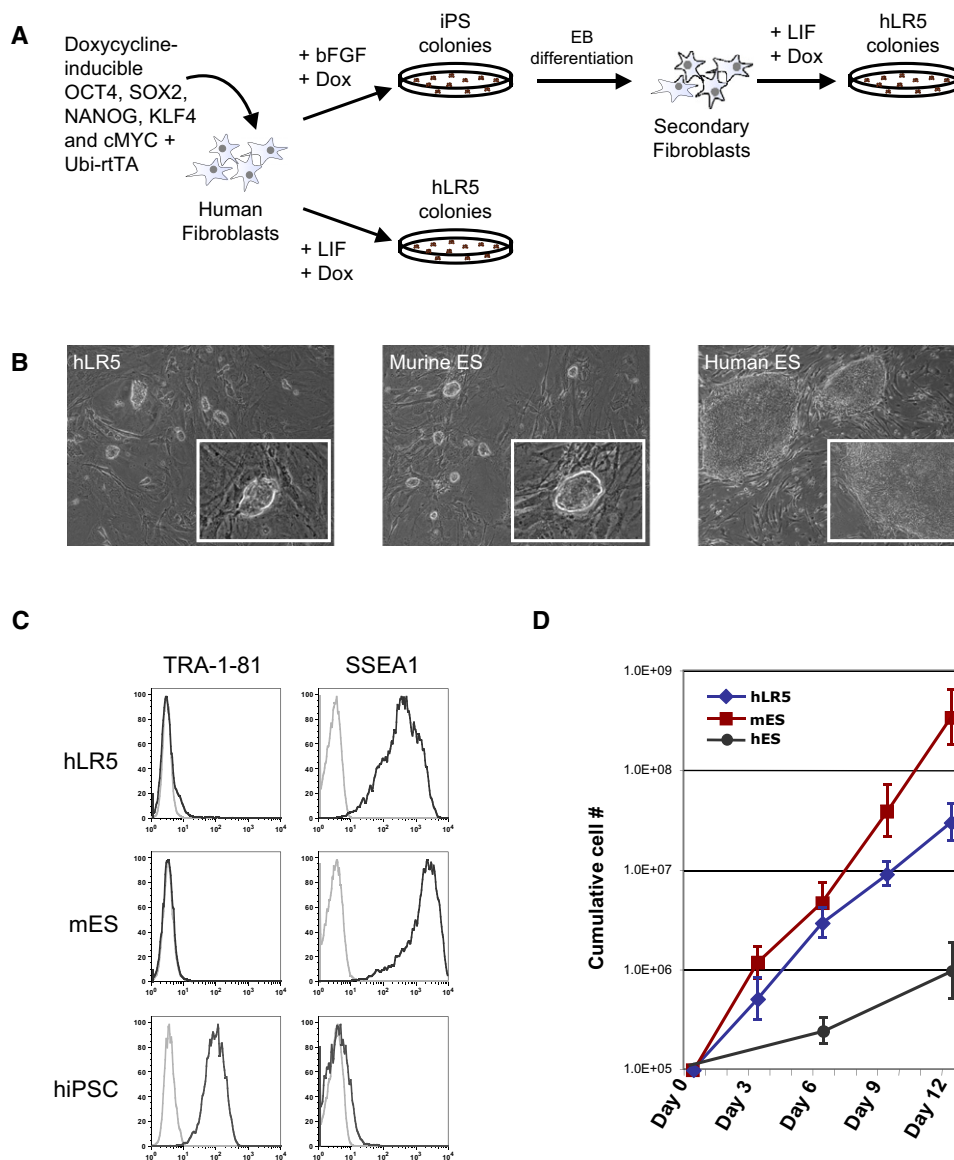


Figure 1. A Metastable Human iPSC State with Murine ESC Properties

(A) Schematic representation of the used strategy. Doxycycline-inducible lentiviral vectors were added either as individual vectors (Maherali et al., 2008) or the polycistronic human STEMCCA virus (Sommer et al., 2009) and inducible NANOG as indicated in the text.

(B) Colony morphology of hLR5 cells (left), murine ESCs (middle), and human ESCs. See also Figure S1.

(C) FACS analysis of cell surface marker expression on hLR5 cells, murine ESCs, and human iPSCs. Black lines, cell surface marker with the indicated primary antibody; grey line, no primary antibody control.

(D) Growth curve of hLR5 cells, mESCs, and hESCs over a period of 12 days. Cumulative cell number is plotted against days (n = 3, SD).

much higher than the proliferation rate of hESCs or hiPSCs (doubling time ~36 hr).

Activation of the JAK-STAT Pathway and Downstream Target Genes in hLR5 Cells

The growth factor environment is known to be an important determinant of the stem cell pluripotent state (Brons et al., 2007; Chou et al., 2008; Tesar et al., 2007). In mESCs, LIF activates the JAK/STAT3 and the RAS/MEK/MAPK signaling pathways, which have opposing roles in mESC maintenance and

differentiation. Activation of the JAK-Stat3 signaling pathway has been shown to be important for long-term self-renewal of mESCs, whereas the RAS/MAPK pathway drives mESC differentiation. Indeed, pharmacological inhibitors of the RAS/MEK/MAPK pathway have been shown to enhance mESC self-renewal and in combination with inhibitors of GSK3β allow growth factor-independent maintenance of pluripotent stem cells (Ying et al., 2008). hLR5 cells display many characteristics of murine ESCs, so we investigated the effect of LIF and its signaling pathways on these cells.

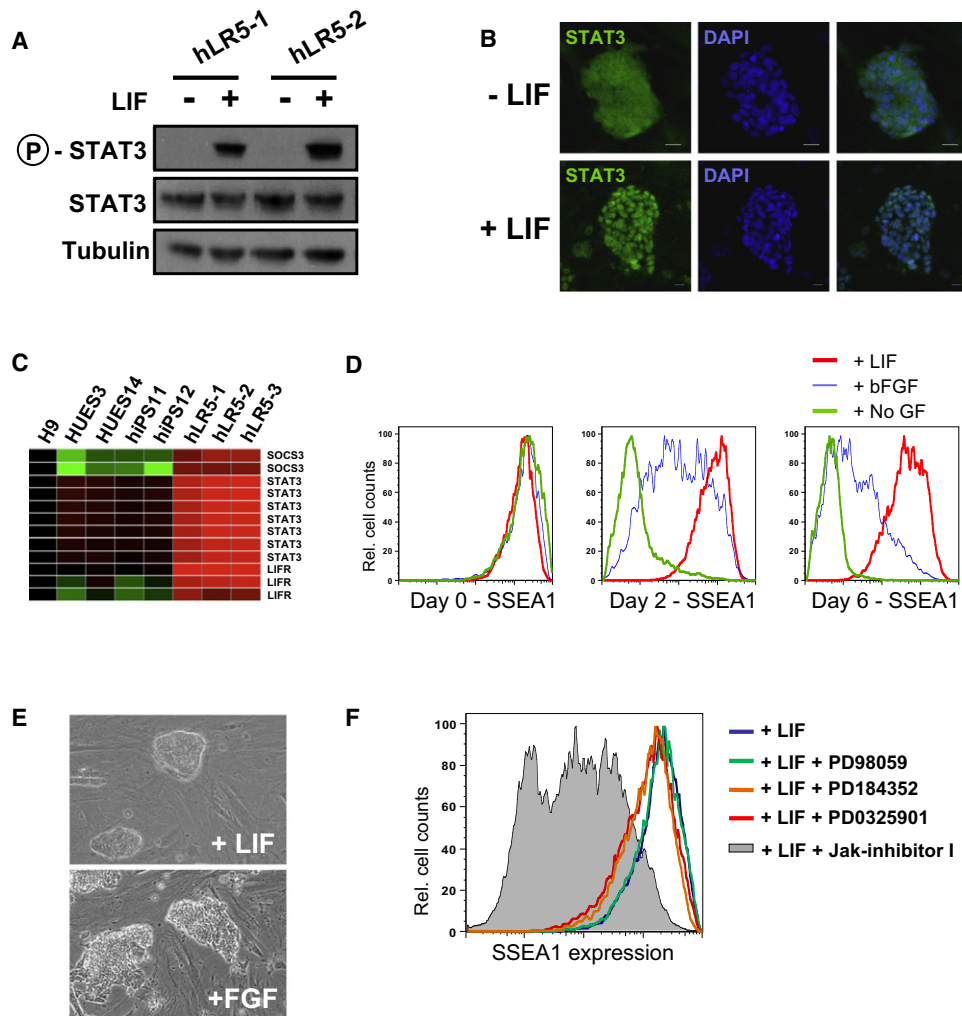


Figure 2. LIF Responsiveness of hLR5 Cells

(A) Western blot analysis of STAT3 phosphorylation in two independent hLR5 clones, with or without LIF stimulation as indicated. (B) Immunostaining of STAT3 subcellular localization in hLR5 cells before (top) or after (bottom) LIF stimulation. Note the translocation of STAT3 from the cytoplasm (top) to the nucleus (bottom). Cell nuclei were visualized with DAPI. (C) Gene expression analysis of downstream target genes of the JAK/STAT signaling pathway in three hLR5 clones as well as human ESC lines and conventional human iPSC lines. (D) Flow cytometry analysis of SSEA1 surface marker on hLR5 cells upon LIF removal. Red lines, LIF control; blue line, LIF substitution with bFGF; green lines, no added growth factor. (E) Colony morphology of the hLR5 cells before (top) and after LIF substitution with bFGF. (F) Flow cytometry analysis of SSEA1 cell surface marker expression on hLR5 cells in the presence of small molecule inhibitors. hLR5 cells were maintained in hLR5 media for 1 week in the presence of inhibitors. Grey shaded area, Jak inhibitor; colored lines, MEK inhibitors (PD98059, PD184352, and PD0325901, as indicated); blue line, no inhibitor control.

As shown in Figure 2A, STAT3 phosphorylation is robustly stimulated in hLR5 cells in a LIF-dependent manner. Upon LIF activation of the JAK-STAT signaling cascade, STAT3 translocates from the cytosol to the nucleus and directly activates downstream target genes. Immunofluorescence staining of STAT3 in hLR5 cells revealed nuclear translocation in response to LIF stimulation (Figure 2B). This translocation results in activation of STAT3 downstream target genes, including STAT3 itself as well as SOCS3 and the LIF receptor, indicating that this pathway is functionally active in response to LIF stimulation of the hLR5 cells (Figure 2C).

Upon removal or substitution of LIF from the hLR5 culture media, SSEA1 expression waned (Figure 2D). In addition, we noticed a change in colony morphology (Figure 2E). Next we used specific inhibitors of JAK/STAT signaling or the MAPK/MEK signaling pathway to examine the roles of these pathways in hLR5 cells. SSEA1 cell surface marker expression was used as a readout. As shown in Figure 2F, inhibition of the JAK/STAT3 pathway resulted in a marked decrease of SSEA1 on hLR5 cells (Jak-inhibitor I [0.6 μM], gray shaded area), whereas specific inhibition of the MEK/ERK1/ERK2 pathway did not affect cell surface marker expression (PD98059 [50 μM], PD184352

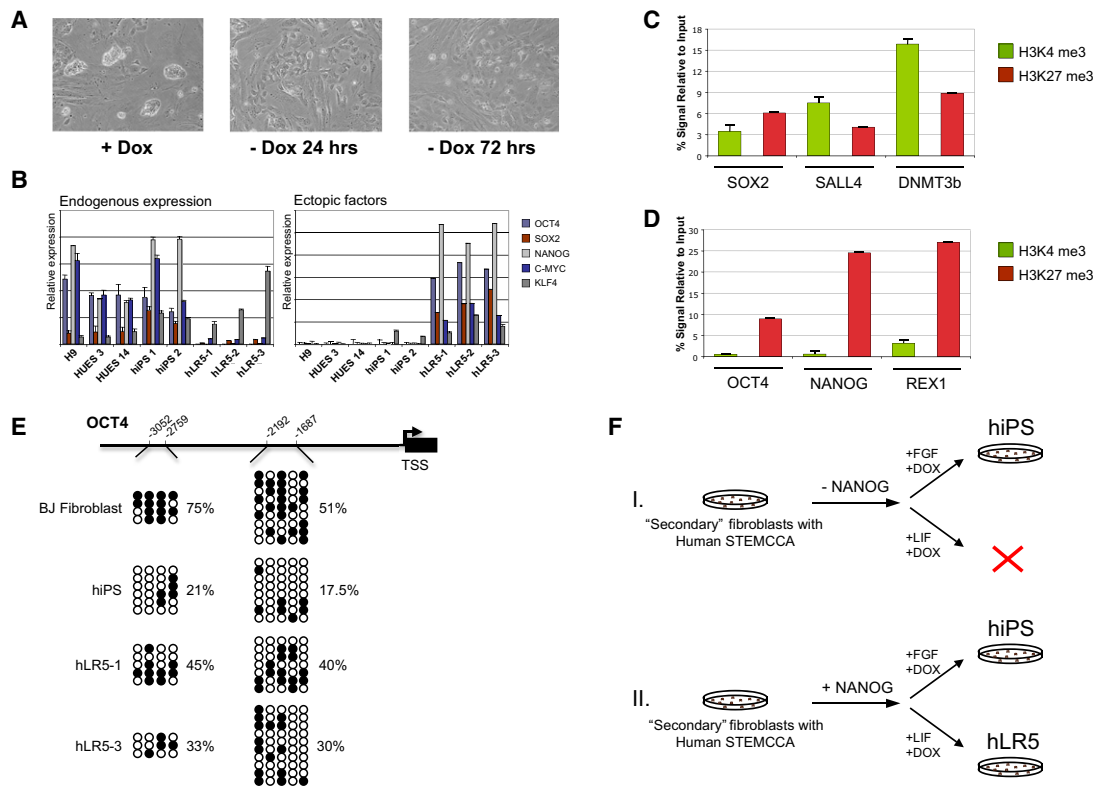


Figure 3. The hLR5 State Depends on Ectopic Pluripotency Factors but Is Poised for Reactivation of Endogenous Pluripotency Genes

(A) Ectopic factor dependence of hLR5 cells. Upon doxycycline withdrawal, hLR5 colony morphology is lost and cells adopt a fibroblast-like appearance. Days of differentiation are indicated.

(B) Quantitative RT-PCR analysis of the expression of reprogramming factors used for the derivation and maintenance of hLR5 cells. Left panel: expression of endogenous genes. Right panel: expression of the doxycycline-inducible ectopic reprogramming factors. Human ESC strains (H9, HUES3, HUES14) and human iPSC strains (hiPS1, hiPS2) were used as controls. Color coding of the genes is indicated (n = 3, SD).

(C) ChIP-qPCR analysis of the presence of histone 3 lysine 4 (H3K4, green bars) marks and histone 3 lysine 27 (H3K27, red bars) marks at the promoter regions of the pluripotency genes SOX2, DNMT3b, and SALL4 as indicated in hLR5 cells (n = 3, SD). See Table S1 for primers.

(D) ChIP-qPCR analysis of the presence of histone 3 lysine 4 (H3K4, green bars) marks and histone 3 lysine 27 (H3K27, red bars) marks at the promoter regions of the pluripotency genes OCT4, NANOG, and REX1 as indicated in hLR5 cells (n = 3, SD).

(E) DNA methylation analysis of two CpG islands in the OCT4 promoter as indicated in the schematic of the OCT4 promoter region. Open circles indicate unmethylated and filled circles indicate methylated CpG dinucleotides. Shown are representative sequenced clones from BJ fibroblasts, human iPSCs, and two independent clonal hLR5 cell lines. The percentage of CpG methylation at each CpG island in the respective cell lines is indicated. TSS, transcription start site.

(F) Schematic representation of the generation of hLR5 cells in the absence (top, I) or presence (bottom, II) of NANOG. Although in the absence of NANOG expression traditional hiPSCs can be derived, no hLR5-like colonies form. Addition of ectopic NANOG results in the formation of hLR5 colonies. See also Figure S2.

[0.8 μ M], and PD0325901 [1 μ M], colored lines). Together these results indicate that LIF stimulation of hLR5 cells results in activation of the JAK-STAT3 signaling cascade and upregulation of downstream target genes whereas LIF withdrawal results in changes in hLR5 colony phenotype. However, LIF withdrawal does not result in hLR5 differentiation, perhaps because of the persistent doxycycline-induced ectopic expression of reprogramming factors.

The hLR5 State Requires Continued Ectopic Expression of Five Reprogramming Factors

Next we examined whether hLR5 cells could be stably propagated in the absence of ectopic reprogramming factors. As shown in Figure 3A, doxycycline withdrawal resulted in the rapid loss of hLR5 colony morphology, with all cells adopting a fibroblast-like appearance within 3 days. A similar dependence was

found for rat iPSCs (Liao et al., 2009) and for iPSCs from the nonpermissive NOD mouse strain (Hanna et al., 2009). The need for ectopic reprogramming factors suggested that in hLR5 cells, the endogenous pluripotency genes have not yet been fully activated. Q-PCR analysis of the expression of endogenous and ectopic pluripotency factors revealed that hLR5 cells fail to reactivate endogenous OCT4 and NANOG expression, while the expression of endogenous SOX2 and c-MYC are low (Figure 3B). Endogenous KLF4 was expressed at similar levels in hLR5 cells and human ESCs or iPSCs.

We then analyzed the presence of activating and silencing histone marks at the promoter regions of critical regulators of pluripotency. By using chromatin immunoprecipitation (ChIP) and Q-PCR, we tested the presence of two histone marks: histone 3 lysine 4-trimethylation (H3K4me3), a histone mark that activates transcription (Pray-Grant et al., 2005; Santos-Rosa

et al., 2003; Sims et al., 2005; Wysocka et al., 2005), and histone 3-lysine 27-trimethylation (H3K27me3), which promotes stable transcriptional repression (Francis et al., 2004; Ringrose et al., 2004). Unexpectedly, ChIP-qPCR analysis revealed the presence of both marks at the promoter regions of *SOX2*, *DNMT3b*, and *SALL4* (Figure 3C). The H3K4 and H3K27 methylation marks are simultaneously present in so-called bivalent domains, which in ESCs are often found at promoters of important transcriptional regulators of development (Bernstein et al., 2006). Bivalent domains result in transcriptional repression, but lead to a “poised” state that allows rapid activation or permanent repression by removing either one of the opposing histone marks and is consistent with the low-level endogenous expression of these genes in hLR5 cells (Figure 3B).

ChIP-qPCR analysis of the H3K4me3 and H3K27me3 marks at the *OCT4*, *NANOG*, and *REX1* promoters revealed the presence of the repressive H3K27me3 mark in the hLR5 cells, corroborating the absence of endogenous expression of these genes (Figure 3D). In somatic cells, *OCT4* and *NANOG* are silenced through additional epigenetic mechanisms including DNA methylation, which is thought to be a permanent transcriptional barrier. Indeed, DNA methylation has been shown to be a limiting step during iPSC reprogramming, and inhibition of DNA methylation can complete the reprogramming of partially reprogrammed iPSCs (Mikkelsen et al., 2008). Bisulfite sequencing showed that, unexpectedly, the *OCT4* promoter regions are hypomethylated in the hLR5 cells as compared to the parental BJ fibroblasts (Figure 3E).

Together, these results suggest that hLR5 cells exist in a poised state of near pluripotency, in which some pluripotency genes, including *SOX2*, *DNMT3b*, and *SALL4*, are in a bivalent histone methylation state, whereas others, such as *OCT4*, *NANOG*, and *REX1*, still carry the transcriptionally repressive H3K27 methylation mark, but already display hypomethylation at the *OCT4* promoter region.

Surprisingly, in addition to the four common reprogramming factors, ectopic expression of *NANOG* is also required for the maintenance of the hLR5 state. By using the “secondary fibroblasts,” we analyzed hLR5 derivation in the presence or absence of ectopic *NANOG* expression (Figure 3F). iPSC reprogramming was induced with doxycycline via either conventional hESC medium (with bFGF) or in hLR5 conditions (LIF). Whereas in the presence of bFGF hiPSC colonies formed with or without ectopic *NANOG* (Figures 3F, part I), under hLR5 culture conditions colony formation is dependent on ectopic *NANOG* expression (Figure 3F, part II), demonstrating that *NANOG* is required for the de novo derivation of hLR5 cells. In addition, we explored the effect of ectopic *NANOG* expression when reactivating reprogramming factors in existing hiPSCs in hLR5 medium. By using hiPSCs derived with the STEMCCA lentivirus (Sommer et al., 2009), we induced ectopic factor expression in the presence of human LIF with or without ectopic *NANOG* expression (Figure S2A). In the presence of *NANOG* expression, colonies appeared after 2–3 passages that showed the typical hLR5 morphology (Figure S2B, left), whereas without ectopic *NANOG* expression, hiPSC colony morphology rapidly deteriorated in hLR5 conditions (Figure S2B, right). However, hLR5 cell cultures directly derived from hiPSCs remained heterogeneous, indicating that direct conversion of hiPSCs into hLR5 cells was

incomplete and may require prolonged passaging and/or selection. Indeed, in similar manner, the conversion of murine EpiSCs into mES-like cells requires prolonged culture and passaging in combination with selection for mES-like cells (Bao et al., 2009).

Conversion of hLR5 Cells to a Stable Pluripotent State

Previous reports have demonstrated that, similar to our hLR5 cells, rat iPSCs and miPSCs from the NOD background are unstable. However, NOD-derived iPSCs can be converted to a stable, epiblast-like pluripotent state by simultaneously removing the ectopic reprogramming factors and altering the culture growth factor conditions (Hanna et al., 2009). We examined whether changes in the growth factor environment could similarly induce the conversion of hLR5 cells into a stable pluripotent state.

Figure 4A outlines the procedure of converting hLR5 cells into stable iPSCs. Clonal hLR5 cell lines were plated at a density of 5000 cells per cm² in media containing human LIF and doxycycline. The next day, doxycycline was withdrawn from the hLR5 cultures and cells were further maintained in the presence of bFGF alone (Figure 4A). Withdrawal of ectopic reprogramming factors resulted in the rapid differentiation of most of the hLR5 colonies (Figure 3A). However, after 7–10 days, stable colonies emerged that required mechanical passaging and displayed a typical hiPSC-like colony morphology (Figure 4B). We termed these cells hLR5-derived human iPSCs (LD-hiPSCs). The conversion frequency of hLR5 cells into LD-hiPSCs was approximately 0.01%, similar to the conversion of murine metastable iPSCs into stable EpiSC-like iPSCs (Hanna et al., 2009). Pharmacological inhibitors of GSK3 β and/or MAPK signaling were shown to stabilize the LIF-dependent pluripotent state in iPS and ICM-derived stem cell lines from NOD mice and rat. We analyzed whether LIF and/or small molecule inhibitors could positively influence the conversion of hLR5 cells (Li and Ding, 2009). Addition of LIF or the MEK inhibitor PD98059 (50 μ M) alone resulted in a slight increase in hLR5 conversion rate (Figure S3), but in combination resulted in a near 8-fold increase in conversion frequency compared to FGF alone. Emerging converted colonies displayed the typical hiPSC morphology and were subsequently maintained with bFGF alone, indicating that the resulting iPSCs are not LIF dependent. Characterization of three independent LD-hiPSC lines revealed a normal (2n = 46, XY) karyotype of these cells (Figure 4C). ChIP analysis of the H3K4 and H3K27 histone methylation marks at the promoter regions of key pluripotency mediators suggested reactivation of pluripotency genes in LD-hiPSCs (Figure S4). Indeed, Q-PCR analysis of pluripotency regulators demonstrated reactivation of endogenous *OCT4*, *SOX2*, *NANOG*, *KLF4*, and *cMYC* in LD-hiPSCs and the absence of ectopic factors (Figure 4D). The expression and nuclear localization of *OCT4*, *SOX2*, and *NANOG* was further confirmed via immunofluorescence staining of LD-hiPSCs (Figure 4E). LD-hiPSCs displayed a cell surface marker profile characteristic of human pluripotent stem cells, with expression of TRA-1-60, TRA-1-81, and SSEA4 cell surface markers (Figure 4F), whereas the SSEA1 cell surface marker was absent (not shown). In addition, E-Cadherin, a cell-cell interaction protein that is important for pluripotent stem cell maintenance and differentiation, is induced upon conversion of hLR5 cells into LD-hiPSCs (Figure S5). Hierarchical cluster analysis

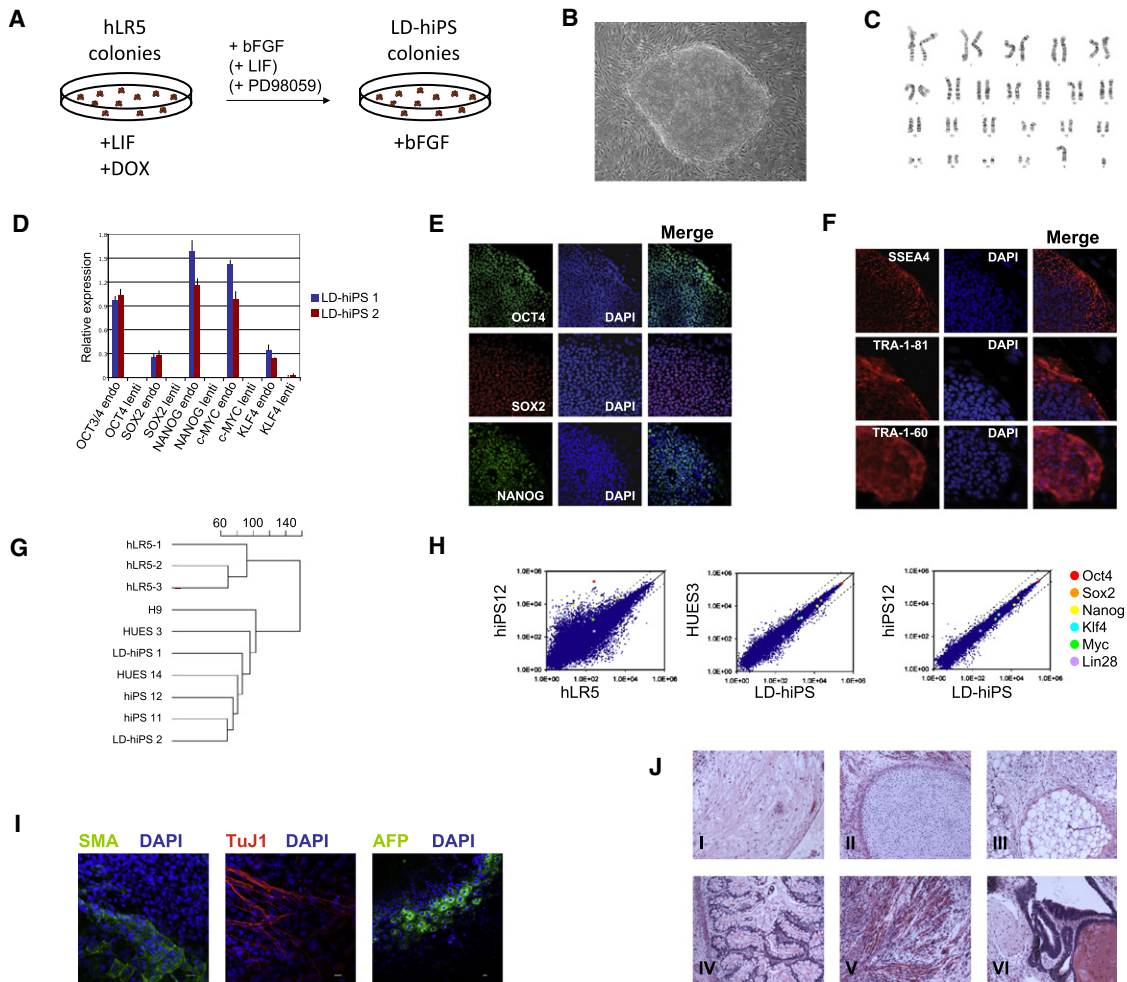


Figure 4. Conversion of hLR5 Cells to a Stable Pluripotent State

(A) Schematic representation of the conversion of hLR5 cells. See also Figure S3.
 (B) Representative image of the LD-hiPS colony morphology.
 (C) LD-hiPSCs have a normal 46 XY karyotype.
 (D) Quantitative RT-PCR analysis of the expression of endogenous pluripotency factors and the silencing of the doxycycline-inducible ectopic reprogramming factors in two independent LD-hiPSC clones (n = 3, SD). See also Figures S4 and S5.
 (E) Immunofluorescence analysis of OCT4 (top), SOX2 (middle), and NANOG (bottom) protein expression and nuclear localization in LD-hiPSCs. DAPI was used to visualize the cell nuclei.
 (F) Immunofluorescence staining of characteristic cell surface markers of human pluripotent stem cells: SSEA4 (top), TRA-1-81 (middle), and TRA-1-60 (bottom). DAPI was used to visualize the cell nuclei.
 (G) Unbiased cluster analysis of global gene expression profiles of three independent hLR5 clones, two independent LD-hiPSC clones, three human ESC lines, and two human iPSC lines.
 (H) Scatter plots of microarray data on the global gene expression patterns of hLR5 cells, human iPSCs of the same genetic background (hiPS12 [Maherali et al., 2008]), human ESCs (HUES3), and LD-hiPSCs as indicated. The position of individual pluripotency genes listed in the legend is indicated with colored circles.
 (I) Immunostaining of differentiated LD-hiPSC lines with markers for mesoderm (SMA, left), ectoderm (Tuj1, middle), and endoderm (AFP, right) as indicated. DAPI was used to visualize cell nuclei. See also Movie S1.
 (J) H&E staining of teratomas generated from clonal LD-hiPSCs. Derivatives of all three germ layers are observed: I, ganglion; II, cartilage; III, adipose tissue; IV, gut; V, muscle; and VI, respiratory epithelium and squamous epithelium.

of the global gene expression profiles of hLR5 cells, LD-hiPSCs, human ESCs, and human iPSCs revealed that LD-hiPSCs are highly similar to human ESCs and iPSCs, whereas hLR5 cells form a separate cluster of unrelated cells (Figure 4G). Scatter plots of the microarray expression analysis of hLR5 cells and LD-hiPSCs further highlight the differences between hLR5 cells and the genetically identical hiPS#12 cell line. In contrast, there

is high similarity between LD-hiPSC and the genetically identical hiPS#12 cell line, but also between LD-hiPSC and the genetically unrelated HUES3 human ESC line (Figure 4H; Maherali et al., 2008). Finally, we examined the ability of LD-hiPSCs to generate derivatives of the three embryonic germ layers. Embryoid bodies (EBs) were generated from LD-hiPSCs and plated onto gelatin-coated coverslips. Immunofluorescence staining revealed the

Table 1. Introduction of Transgenes into Human ESCs and hLR5 Cells by Electroporation

Experiment	Cell Type	# Cells per Electroporation	# Electroporations	Size of the Construct	Drug Selection	Average # Colonies per 10×10^6 Cells
#1	human ES	1×10^7	10	20 kbase	hygromycin	6.8
#2	human ES	1×10^7	10	20 kbase	hygromycin	10.6
#3	human ES	1×10^7	6	20 kbase	hygromycin	12
#4	human ES	5×10^6	3	14 kbase	puromycin	5.7
#5	hLR5	1×10^7	2	20 kbase	hygromycin	> > 1500
#6	hLR5	1×10^7	2	20 kbase	hygromycin	> > 1500
#7	hLR5	1×10^7	1	14 kbase	puromycin	1300
#8	hLR5	1×10^7	1	14 kbase	puromycin	1100

presence of cells expressing TuJ1, a neural marker, Smooth Muscle Actin, a mesoderm lineage marker, and alpha-fetoprotein, a marker of endoderm differentiation (Figure 4I). In addition, we observed that some of the EBs started beating, indicating the development of cardiac tissue with pacemaker function (Movie S1). Finally, subcutaneous injection of LD-hiPSCs into immunocompromised mice resulted in the formation of teratomas containing differentiated derivatives of the three embryonic germ layers (Figure 4J), demonstrating that the LD-hiPSCs are indeed pluripotent.

hLR5 Cells Facilitate Transgenesis and Gene Targeting in Human Stem Cells

A major obstacle for the application of human pluripotent stem cells in modeling human development and disease is the difficulty these cells have displayed in allowing the introduction of foreign genetic elements (Ptaszek and Cowan, 2007). Although such basic molecular manipulations are mainstay in mESCs, generation of transgenic human stem cells is very inefficient and labor intensive.

Because the human hLR5 cells display many characteristics of murine ESCs, we examined whether these cells are more amenable for transgene insertion by using standard electroporation procedures. We tested the transfection efficiency of hLR5 cells with either a 10 kb vector constitutively expressing a red fluorescent protein (tdTomato) and a puromycin selection cassette or a 20 kb vector expressing tdTomato driven by the *ISL1* promoter (Bu et al., 2009) and a hygromycin selection cassette. hLR5 cells or control human ESCs were electroporated with linearized constructs, and after antibiotic selection, the number of colonies was counted. Table 1 summarizes the result of 6 independent electroporations in 2 independent clonal hLR5 lines (hLR5-1 and hLR5-3) and 29 independent electroporations of hESC lines (H9 and HUES3). Electroporation and selection of the same number of hLR5 cells with the same amount of vector yields more than 200-fold more colonies that had incorporated the transgene compared to human ESC electroporation. The high efficiency in which hLR5 cells incorporate transgenes is particularly important when large constructs such as BAC clones are used. Indeed, the same electroporation protocol allowed the introduction of a 250 kb BAC clone with a puromycin selection cassette, albeit at a slightly lower efficiency, approximately 1 colony per 10^6 electroporated cells.

Finally, we tested the possibility of targeting specific loci in the hLR5 cell genome. We argued that because the hLR5 cells

display many characteristics of mESCs, gene targeting may be facilitated in these cells. We chose to target the hypoxanthine phosphoribosyltransferase (*HPRT*) locus, because it offers the benefit of both positive and negative drug selection. Cells lacking *HPRT* are resistant to the drug 6-thioguanine (6-TG) but sensitive to HAT selection, whereas wild-type cells and cells with random integrations are HAT resistant and 6-TG sensitive. Figure 5A shows a schematic representation of the human *HPRT* locus and the targeting construct, which disrupts the second exon by inserting a puromycin selection cassette. A combination of three primers (indicated, Figure 5A) can be used to distinguish between homologous recombination events and random integration of the targeting construct. Because the *HPRT* gene is located on the X chromosome and therefore has only one copy in male hLR5 cells, a single targeting event can generate a knockout cell line. Upon electroporation of hLR5 cells with the *HPRT* targeting construct, cells were treated with puromycin to select for positive clones and subsequently treated with 6-TG to select for homologous recombination events. With this strategy, we determined the targeting efficiency to be approximately 1% ($n = 3$). PCR analysis confirmed the disruption of the wild-type gene (Figure 5B, top). In addition, we tested the absence of functional *HPRT* expression in our targeted clones by culturing the cells in the presence of HAT supplement. As shown in Figure 5C, the *HPRT* knockout cells did not form colonies in the presence of HAT supplement while they were resistant to the positive-selection drug 6-TG.

DISCUSSION

Stable and Metastable Pluripotent States

Recent reports have demonstrated that stem cells can exist in several distinct pluripotent states, which are defined, in part, by the culture growth factor conditions (Brons et al., 2007; Chou et al., 2008; Tesar et al., 2007). bFGF and Activin A allow the derivation of EpiSCs from murine postimplantation embryos (Brons et al., 2007; Tesar et al., 2007). EpiSCs display many characteristics of human ESCs and iPSCs, which are derived and maintained under similar growth factor conditions. In contrast, LIF or a combination of GSK3 β and MEK inhibitors (2i inhibitors) allows for the derivation of murine ESCs from preimplantation blastocysts, which display a characteristic, dome-shaped colony morphology and differ from EpiSCs in their culture dynamics, molecular and epigenetic characteristics, and

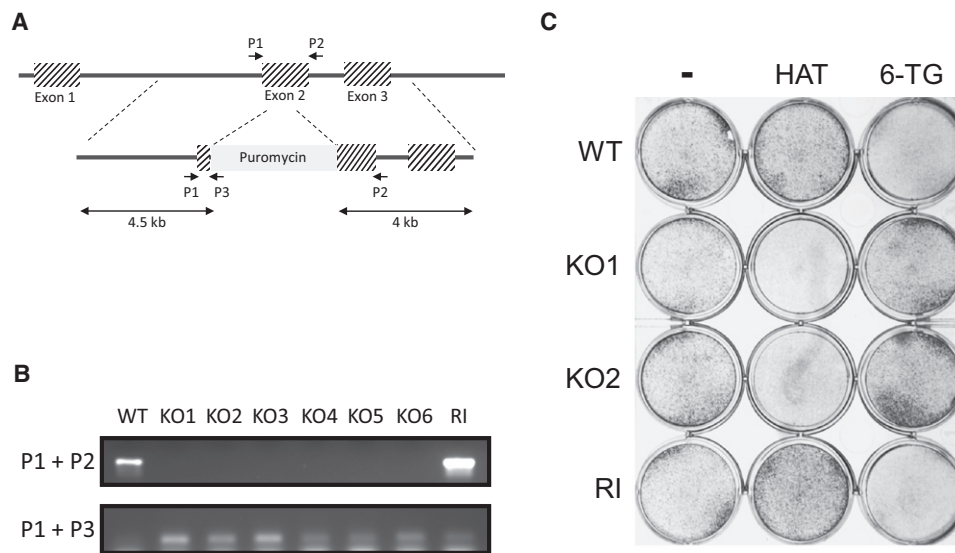


Figure 5. Homologous Recombination-Mediated Gene Targeting in hLR5 Cells

(A) Schematic representation of the human *HPRT* locus and the targeting construct. The PCR primers used to detect the wild-type locus and the targeting construct (P1, P2, P3) are indicated.

(B) PCR detection of six independent clones in which the *HPRT* locus was successfully targeted (KO1-6) and one clone with random integration of the targeting construct (RI). Wild-type cells (WT) were used as control. Top, presence of the wild-type allele. Bottom, detection of the targeting construct.

(C) Confirmation of functional knockout of the *HPRT* gene in targeted hLR5 cells. WT, wild-type; KO1 and KO2, knockout clones; RI, clone with random integration; 6-TG, 6-thioguanine; HAT, HAT supplement.

notably, the ability to generate chimeric mice (Evans and Kaufman, 1981; Martin, 1981; Ying et al., 2008).

Yet, only a few inbred mouse strains can spontaneously give rise to stable ESC lines, whereas other mouse strains and other species such as rat and primates give rise only to pluripotent stem cells with epiblast-like properties (Brons et al., 2007; Thomson et al., 1998; Thomson and Marshall, 1998). However, the combined use of LIF and 2i inhibitors recently allowed researchers to derive true LIF-dependent ESCs from rat blastocysts and rat iPSCs (Buehr et al., 2008). Remarkably, in the absence of 2i inhibitors, these rat ESCs convert to an EpiSC-like pluripotent state (Buehr et al., 2008). In the mouse, EpiSCs can be converted into ESCs through the overexpression of *Klf4* in the presence of 2i inhibitors (Guo et al., 2009). The derivation of ESC-like iPSCs from the nonpermissive NOD mouse strain is similarly dependent on either the constitutive expression of *Klf4* or *c-Myc* or the addition of 2i inhibitors to the culture media (Hanna et al., 2009). It appears therefore that the EpiSC pluripotent state is the common stable pluripotent state for most strains of mice as well as other species, whereas the unique murine ESC-like pluripotent state is “metastable” in these genetic backgrounds.

We found that upon iPSC reprogramming of human fibroblasts in the presence of human LIF, colonies appear that display hallmark characteristics of mESCs, including the dome-shaped tightly packed mESC morphology, the high proliferation rate, the activation of downstream targets of STAT signaling, and the tolerance of trypsin passaging and single cell cloning. These hLR5 cells could be derived either through direct reprogramming of primary human fibroblasts or by using a more efficient “secondary” fibroblast system (schematic Figure 1A). hLR5 cells are metastable, because they depend on the constitutive expression of ectopic reprogramming factors. Upon removal of these

factors, hLR5 cells convert to a stable pluripotent state that is indistinguishable from previously described hiPSC lines. The conversion frequency of hLR5 cells into LD-hiPSCs was similar to the frequencies of the conversion of murine metastable iPSCs of the NOD strain into stable EpiSC-like iPSCs (Hanna et al., 2009), about 0.01%, and is improved to almost 0.1% when LIF and the MEK inhibitor PD98059 are added during the conversion process. Several arguments support the notion that the emerging LD-hiPSCs are the result of conversion of hLR5 cells into a stable hiPSC state rather than selection of pre-existing hiPSCs in the hLR5 population. First, the hLR5 cells were clonally derived and maintained for more than 30 passages before conversion. Second, the hLR5 cells were continually passaged by trypsinization; and third, LD-hiPSCs can be derived from hLR5 cells generated directly from primary fibroblasts, which have never before existed in a hiPSC state. In hLR5 cells, the JAK/STAT3 signaling pathway is activated in a LIF-dependent manner, resulting in the expression of STAT3 downstream target genes. In addition, hLR5 cells respond to LIF withdrawal with changes in cell morphology and surface marker expression. Because hLR5 cells in themselves do not form differentiated derivatives upon LIF withdrawal, probably because of the forced ectopic expression of the five reprogramming factors, the cells are not LIF dependent to the same degree as mESCs are. However, continued maintenance of hLR5 in the presence of LIF is critical for the efficient conversion of hLR5 cells into pluripotent LD-hiPSCs, in particular in combination with the MEK inhibitor PD98059.

The conversion of hLR5 cells into hiPSCs is accompanied by epigenetic changes at the promoter regions of critical pluripotency regulators. Unexpectedly, these pluripotency factors, although transcriptionally silent, appear to be in a “poised” state in hLR5 cells, from which they can be rapidly activated. SOX2,

DNMT3b, and *SALL4* display the bivalent H3K4me3 and H3K27me3 histone methylation marks, which allow rapid conversion to the transcriptionally active H3K4me3 methylation state. *OCT4*, *NANOG*, and *REX1* are silenced by H3K27me3, yet the *OCT4* promoter region is hypomethylated in the hLR5 cells, which greatly facilitates *OCT4* reactivation. Similarly, the metastable iPSCs derived from the NOD mouse strain display hypomethylation at the *Oct4* promoter (Hanna et al., 2009), suggesting that demethylation of promoter regions of critical pluripotency regulators is an essential property of the metastable state that allows gene reactivation and stable conversion to the epiblast-like pluripotent state.

Interestingly, the number of ectopic factors that is required to stabilize the mESC-like state differs between the murine NOD strain, rat, and human. Although murine metastable NOD-iPSCs can be maintained with the constitutive expression of a single factor (either *cMyc* or *Klf4*) (Hanna et al., 2009), rat metastable iPSC lines require the full complement of reprogramming factors (Liao et al., 2009), and in the case of the hLR5 cells, this repertoire is expanded with the addition of *NANOG*. Genetic background has been shown to be a critical determinant in defining murine (meta)stable pluripotent states (Hanna et al., 2009), and it is possible that genetic background affects the reactivation of pluripotency genes in human hLR5 cells in a similar manner.

Gene Targeting in Human Pluripotent Cells

Murine ESCs have been instrumental in the discovery of gene function in the context of mammalian development and disease. The standard techniques that readily allow the introduction of transgene and reporter gene constructs in mESCs work poorly in human pluripotent stem cells. As a result, our ability to introduce foreign genetic elements into human cells is largely limited to the use of virus or site-specific zinc-finger nucleases, which are expensive and can have off-target effects. Recent studies in metastable pluripotent stem cells in the NOD mouse strain and the rat demonstrate that the LIF-dependent, mES-like pluripotent state allows for the genetic manipulation of these cells via standard electroporation-based techniques. We demonstrate that in a similar fashion, large reporter constructs and even BAC clones can be introduced into hLR5 cells. hLR5 cells even allow homologous recombination-based gene targeting. Until now, the (targeted) introduction of genetic elements into human pluripotent stem cells was highly inefficient and largely impractical. The intolerance of hESCs to grow from single cells resulted in very low yields upon antibiotic selection and the low proliferation rate made the process time consuming and labor intensive. Recently, Song et al. reported a recombinant BAC-based strategy for gene targeting in hESCs (Song et al., 2010). Although a BAC-based system has the advantage of high homologous recombination efficiency, the system still suffers from the same practical difficulties associated with introducing a BAC clone into human pluripotent stem cells, the low numbers of clones, and the added technical difficulties in identifying homologous recombination events. In contrast, hLR5 cells are tolerant to clonal selection and they have a high proliferation rate, which further facilitates clonal outgrowth and selection. Finally, hLR5 cells allow researchers to perform gene targeting with small (4 kb) homologous arms via standard electroporation procedures that have been well established for the targeting of

mESCs. Combined with the ability of hLR5 cells to convert into a stable iPSC state, we demonstrate here that the hLR5 intermediate provides an efficient platform for targeted gene modification and/or correction in human pluripotent stem cells (Figure 6). As such, it may find use in the generation of recombinant human cell lines for biomedical research, drug development, and perhaps in future cell or gene correction therapies.

EXPERIMENTAL PROCEDURES

Culture of Human ESCs and iPSCs

Human ESCs or iPSCs were maintained on γ -irradiated MEFs in hESC medium (DMEM/F12 containing 20% knockout serum replacement [KOSR], 2 mM L-glutamine, 1% NEAA, 100 U of penicillin, 100 μ g of streptomycin [all from Invitrogen], 0.1 mM β -mercaptoethanol [Sigma], and 5 ng/ml bFGF [R&D Systems]). Cells were routinely passaged every 5–7 days. For EB derivation, colonies were picked and collected in EB medium (IMDM containing 15% FBS, 2 mM L-glutamine, 1% NEAA, 1 mM sodium pyruvate, 100 U of penicillin, 100 μ g of streptomycin [all from Invitrogen], 200 μ g/mL iron-saturated transferrin [Sigma], 4.5 mM monothioglycerol [Sigma], and 50 μ g/mL ascorbic acid [Sigma]). Colonies were cultured with gentle agitation. After 7–9 days, colonies were transferred to gelatin-coated chamber slides, allowed to adhere, and incubated for 3–5 more days. For teratoma formation, LD-hiPSCs were injected subcutaneously into NOG mice (Jackson Laboratories, Bar Harbor, ME) in accordance with institutional guidelines and approval. After ~10–12 weeks, teratomas were dissected, washed, and fixed. Paraffin sections were stained with H&E.

Derivation and Maintenance of hLR5 Cells

Clone#12 hiPSC colonies were differentiated as described (Maherai et al., 2008). The fibroblasts were induced to generate hLR5 cells by passaging the cells onto MEF feeders in hLR5 media (DMEM/F12 containing 20% KOSR, 100 U of penicillin, 100 μ g of streptomycin, 2 mM L-glutamine, 1% NEAA [all from Invitrogen], 0.1 mM β -mercaptoethanol [Sigma], 10 ng/ml human LIF [Sigma], and 2 ng/ml doxycycline [Sigma]). Emerging colonies were individually picked and subcultured by trypsin digest in hLR5 media on MEF feeders. For the direct reprogramming of human fibroblasts (HS27, ATCC) into hLR5 cells, 10^5 cells per 1 cm² were transduced with the STEMCCA lentivirus (containing doxycycline-inducible human *OCT4*, *SOX2*, *KLF4*, and *cMYC*) and rTA with or without a doxycycline-inducible lentivirus for *NANOG* (Maherai et al., 2008; Sommer et al., 2009; Stadtfeld et al., 2008). After approximately 30 days, emerging colonies were individually picked and expanded further by trypsin digest in hLR5 media with MEF feeders. hLR5 cells are passaged every 2–3 days, depending on culture density.

Microarray and Q-PCR Analysis

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. cDNA synthesis (Superscript III First-Strand synthesis system, Invitrogen) was performed with random primers. qRT-PCRs were carried out with Brilliant II SYBR Green mix (Stratagene) and a Stratagene MXPro4000 real-time thermocycler. Primer sequences for the analysis of endogenous and ectopic pluripotency gene expression were reported previously (Maherai et al., 2008).

For genome-wide expression analysis, total RNA was labeled and hybridized to Agilent Whole Human Genome Oligo 4X44K Microarrays (one-color platform) according to the manufacturer's protocols. The gene expression results were analyzed with GeneSifter microarray analysis software.

FACS Analysis

Cells were incubated with the antibodies against the indicated surface antigens for 30 min at 4°C in RPMI + 0.5% FBS. The following antibodies were used for cell surface marker profiling: SSEA1 (BD Biosciences), TRA-1-81, TRA-1-60, SSEA3, and SSEA4 (Millipore). Cells were washed twice and incubated with the relevant fluorophore-conjugated secondary antibody (BD Biosciences) for 30 min at 4°C. Cells were washed, resuspended in RPMI/0.5% FBS, and analyzed on a Becton Dickinson FACSCalibur cell analyzer.

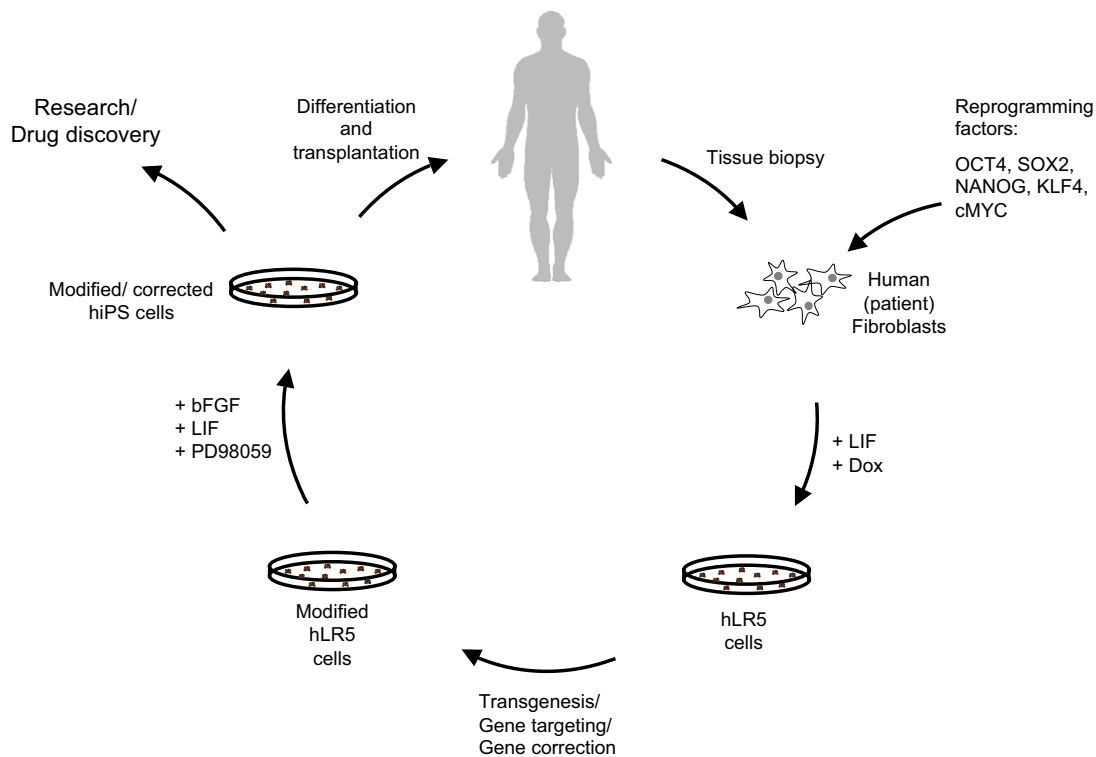


Figure 6. Application of the Intermediate hLR5 State to Generate Recombinant Human Pluripotent Stem Cells for Research, Drug Development, and Potential Gene Correction Therapy

Schematic model summarizing the procedure for gene targeting in human (patient) cells via the metastable hLR5 intermediate state. Human primary fibroblasts are collected and transduced with the five reprogramming factors *OCT4*, *SOX2*, *NANOG*, *KLF4*, and *cMYC*. Upon induction of reprogramming in the presence of human LIF, hLR5 colonies emerge. hLR5 cells are genetically modified via standard. The modified hLR5 cells are subsequently converted into pluripotent LD-hiPSCs, which in turn can be used in research or differentiated for use in future cell/gene correction therapies.

Electroporation and Gene Targeting

Cells were trypsinized, strained, resuspended in 700 μ l PBS containing 15–30 μ g linearized DNA, and transferred to a 0.4 cm gap electroporation cuvette (Biorad). The electroporation was carried out with a single 320V, 200 μ F pulse. Upon electroporation, cells were replated onto gelatinized dishes with DR4 MEFs. Antibiotic selection was started 48–72 hr later with either 25 μ g/ml hygromycin (Invitrogen) or 0.25 μ g/ml puromycin (Invivogen) as indicated. For *HPRT* KO, cells were selected with puromycin followed by treatment with 6-thioguanine (6-TG, Sigma) to select for homologous recombinants. HAT selection was carried out by adding 1 \times HAT supplement (Invitrogen) directly to the hLR5 medium.

ChIP

Cells were fixed in 1% formaldehyde for 10 min, quenched with glycine, and washed three times with PBS. Cells were then resuspended in lysis buffer and sonicated 10 \times 30 s in a Bioruptor (Diagenode, Philadelphia, PA) to shear the chromatin to an average length of 600 bp.

Supernatants were precleared with protein-A agarose beads (Roche, Mannheim, Germany) and 10% input was collected. Immunoprecipitations were performed with polyclonal antibodies to H3K4trimethylated, H3K27trimethylated, and normal rabbit serum (Upstate, Temucula, CA). DNA-protein complexes were pulled down with Protein-A agarose beads and washed. DNA was recovered by overnight incubation at 65 $^{\circ}$ C to reverse cross-links and purified with QIAquick PCR purification columns (QIAGEN, Maryland). Enrichment of the modified histones in different genes was detected by quantitative real-time PCR with the primers in the [Supplemental Experimental Procedures \(Table S1\)](#).

Immunostaining

Primary antibodies used were: α -TRA-1-60, α -SSEA-3, α -SSEA-4, α -TRA-1-81, α -Sox2 (all from Millipore), α -SSEA1, α -Stat3 (both from Cell Signaling),

α -Oct4, α -Nanog (both from Abcam), α -TuJ1 (Covance), α -SMA (Sigma), and α -AFP (Santa Cruz). All secondary antibodies were from Invitrogen. The nuclei were visualized with DAPI.

Western Blotting

Cells were lysed with RIPA buffer containing proteinase inhibitors. The protein concentration was estimated with Bradford reagents and equal amounts of protein were run on 4%–12% Bis-Tris Gels (Invitrogen) and transferred to PVDF membranes (Millipore). Primary antibodies used were: Phospho-Stat3, Stat3, E-Cadherin (all Cell Signaling), and Tubulin (Sigma). HRP-coupled secondary antibody was from Cell Signaling.

ACCESSION NUMBERS

Microarray data have been deposited in the GEO database with the accession number GSE21792.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and one movie and can be found with this article online at [doi:10.1016/j.stem.2010.05.003](https://doi.org/10.1016/j.stem.2010.05.003).

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