Stem Cell Reports
Article

ISSCR

OPEN ACCESS

Simultaneous Reprogramming and Gene Correction of Patient Fibroblasts

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http://dx.doi.org/10.1016/j.stemcr.2015.10.009

SUMMARY

The derivation of genetically modified induced pluripotent stem (iPS) cells typically involves multiple steps, requiring lengthy cell culture periods, drug selection, and several clonal events. We report the generation of gene-targeted iPS cell lines following a single electroporation of patient-specific fibroblasts using episomal-based reprogramming vectors and the Cas9/CRISPR system. Simultaneous reprogramming and gene targeting was tested and achieved in two independent fibroblast lines with targeting efficiencies of up to 8% of the total iPS cell population. We have successfully targeted the *DNMT3B* and *OCT4* genes with a fluorescent reporter and corrected the disease-causing mutation in both patient fibroblast lines: one derived from an adult with retinitis pigmentosa, the other from an infant with severe combined immunodeficiency. This procedure allows the generation of gene-targeted iPS cell lines with only a single clonal event in as little as 2 weeks and without the need for drug selection, thereby facilitating "seamless" single base-pair changes.

INTRODUCTION

Induced pluripotent stem (iPS) cells, generated by introducing defined factors to reprogram terminally differentiated somatic cells, offer enormous potential for the development of autologous or customized cellular therapies to treat or correct many inherited and acquired diseases (Takahashi et al., 2007; Yu et al., 2007). Complications associated with immunorejection can be avoided through the generation and subsequent disease correction of patient-specific iPS cells, which can be differentiated into relevant cell types for the repopulation and regeneration of a defective tissue or organ. Gene targeting by homologous recombination is the ideal approach for the correction of genetic defects as it enables replacement of the defective allele with a normal functional one without disturbing the remaining genome. The generation of a genetically modified iPS cell line typically involved multiple procedures that required the cells to be in culture for an extensive period, drug selection, and several clonal events (Hockemeyer et al., 2009; Howden et al., 2011; Liu et al., 2011; Zou et al., 2011). In the first step, somatic cells are reprogrammed, and several clones are expanded and characterized. Gene targeting constructs are then introduced, and cells are usually subjected to drug selection to isolate and identify correctly modified iPS cell colonies. Once successfully targeted clones are identified, it is preferable to excise the drug selectable marker, commonly flanked by loxP or FRT sites. Taken together, the multiple steps required for the generation of genetically modified iPS cell lines typically require cells to be in culture for several months, which is not compatible for patients for whom urgent medical intervention is imperative. Furthermore, there is evidence to suggest that increased culture times are associated with undesirable changes in genomic integrity, such as duplications of oncogenic genes (Laurent et al., 2011) and other karyotypic abnormalities (Chen et al., 2008). Here we report that reprogramming and gene targeting can be performed together in a one-step procedure that requires only a single electroporation. Multiple gene-targeted iPS cell clones can be generated from patient cells in as little as 2 weeks, requiring only a single clonal event. The procedure also does not require the use of drug selection and permits the generation of clones that contain "seamless" single base-pair changes, without leaving residual loxP or FRT sites in the host genome.

RESULTS

We used an enhanced episomal-based reprogramming system to generate iPS cell lines that would eventually be free of vector sequences. In addition to the seven factors (*OCT4*, *SOX2*, *NANOG*, *c-MYC*, *KLF4*, *LIN28*, and the SV40 Large T-Antigen) encoded by the three oriP-based vectors previously reported to induce pluripotency (Yu et al., 2009), we also forced expression of the micro RNA (miR) 302/367 cluster, which is known to facilitate

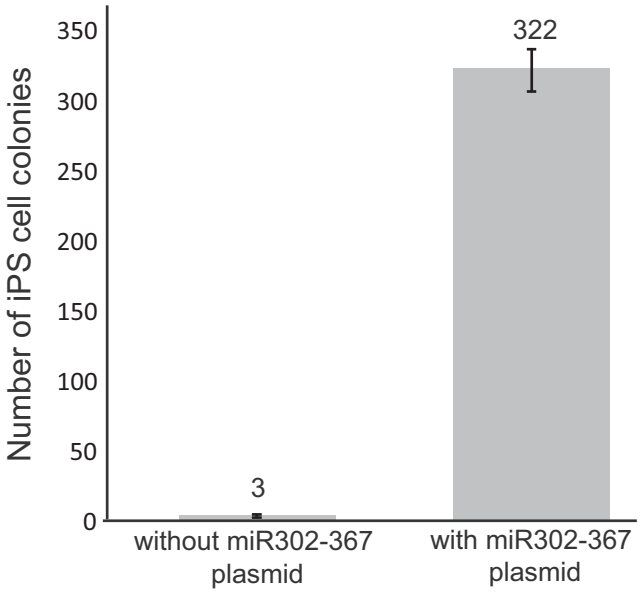

Figure 1. Episomal Reprogramming System Is Enhanced with Inclusion of Plasmid Encoding the miR302/367 Cluster

Reprogramming experiments were performed with and without inclusion of the miR302/367 expression plasmid using a normal male fibroblast line. Data represent an average of three independent experiments ± SD.

reprogramming and maintenance of pluripotency (Lin et al., 2008; Miyoshi et al., 2011). The inclusion of an additional episomal vector encoding miR 302/367 resulted in a substantial increase (more than 100-fold) in the total number of iPS cell colonies in human fibroblasts (Figure 1). This plasmid was included in all subsequent reprogramming experiments and was necessary to obtain sufficient iPS cell colony numbers when combining gene targeting and reprogramming in a single step.

In our initial attempts at simultaneous reprogramming and gene targeting of somatic cells, we chose to target the *DNMT3B* gene with an EGFP reporter, since *DNMT3B* is highly expressed in pluripotent cells and quickly downregulated following differentiation, allowing targeted iPS cell colonies to be easily identified by fluorescent microscopy. To facilitate homologous recombination at the *DNMT3B* locus, we used in vitro transcribed mRNA encoding the Cas9 protein derived from *N. meningitides* (Hou et al., 2013), a plasmid encoding a *DNMT3B*-specific short-guide (sg)RNA and a donor template encoding an EGFP reporter and puromycin resistance gene flanked by 1-kb homology arms specific to sequences upstream and downstream of the *DNMT3B* start codon (Figure 2A). We first evaluated targeting of *DNMT3B* using this system in the embryonic stem cell line H9 and routinely obtained a gene-targeting efficiency of 0.5%–0.9% (Figure 2B). We next co-transfected the reprogramming plasmids along with the *DNMT3B*-specific gene-targeting factors into a fibroblast line derived from a patient with autosomal dominant retinitis pigmentosa. Although iPS cell colonies first emerged as early as 10 days following transfection, we observed the vast majority emerge between 2 and 3 weeks postelectroporation, at which point the culture was routinely analyzed by fluorescent microscopy. Of three independent experiments we identified 8, 13, and 44 iPS cell colonies that stably expressed the EGFP reporter, indicative of a successful gene-targeting event at the *DNMT3B* locus (Figure 2C). We obtained a large number of iPS cell colonies (>1,000) from each of these experiments, making it difficult to accurately assess gene-targeting efficiency. Thus, to estimate targeting efficiency in the pool of iPS cells, we passaged cells from a single representative experiment approximately 3 weeks post-transfection using EDTA to selectively remove iPS cells from the residual fibroblasts before re-plating. As measured by the number of EGFP-expressing cells, targeting efficiency was approximately 3% and 5% following flow cytometric analysis of the total cell population after three and five passages, respectively (Figure 2D). An increase in the number of EGFP-expressing cells is most likely due to a further loss of the residual parental fibroblast population, and we did not observe any further increase in the number of EGFP-expressing cells after five passages. Using the reprogramming experiments that were not passaged, we randomly selected and expanded six EGFP-expressing and six EGFP-non-expressing colonies for further analysis. Gene targeting of the *DNMT3B* locus was confirmed in all six EGFP-expressing clones by PCR using primers that flank the recombination junction site, but not in any of the EGFP-non-expressing clones (Figure 2E). Flow cytometry analysis also revealed a uniform level of EGFP expression in > 95% of the cell population with similar fluorescence intensities observed in all six clones (Figure 2F). Although targeting of *DNMT3B* in H9 cells with the same donor template routinely yielded numerous puromycin-resistant colonies, confirming functionality of the phosphoglycerate kinase (PGK) promoter in pluripotent stem cells, EGFP-expressing iPS cell lines generated by simultaneous reprogramming and targeting of *DNMT3B* exhibited puromycin sensitivity. This suggests the PGK promoter was transcriptionally silenced during the reprogramming process, making drug selection of simultaneously reprogrammed and gene-targeted clones infeasible. Conversely, when we used a one-step procedure to generate iPS cell lines with an EGFP reporter fused to the *OCT4* coding region using the Cas9/CRISPR system described previously (Hou et al., 2013) (Figure S1), these clones did exhibit resistance to puromycin in the culture media. In this case the puromycin resistance gene is fused to the EGFP reporter via a 2A sequence and is thereby