Generation of Zero-footprint iPSCs in Xeno Free or Feeder Free with CytoTune™ -iPS Reprogramming Kit

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ABSTRACT

The generation of induced pluripotent stem cells (iPSC) from fibroblasts or other somatic cells enables the possibility of providing unprecedented access to patient-specific iPSC cells for drug screening, disease modeling, and cell therapy applications. However, a major obstacle to the use of iPSC for therapeutic applications is the potential of genomic modifications caused by insertion of DNA virus and resulting in multiple proviral integrations that pose the danger of insertional mutagenesis. A second are of concern is that reprogramming often requires the use of animal feeder layers to support the generation of iPSCs, which hinders clinical translation due to the presence of animal materials or pathogens. Finally, the current media used for reprogramming contain serum, which is unsuitable for the generation of clinical grade iPSCs. Here we report the generation of zero-footprint iPSCs by RNA Sendai virus that does not integrate in cellular's genome, thus enabling complete footprint free iPSCs. We demonstrate that iPSC generation can be performed in the absence of feeders and in feeder free StemPro® hESC SFM medium. We also show that iPSCs can be generated in completely Xeno-free (XF) conditions. The iPSCs generated in this system are able to proliferate and maintain markers of pluripotency. Further these cells are able to give rise to embryoid bodies (EBs) that can differentiate to all the three lineages - ectoderm, endoderm and mesoderm. Generation of a footprint free iPSCs under Xeno free conditions should facilitate the safe clinical translation of iPSC-based therapies, and the ability to generate iPSCs in the absence of feeders will simplify the workflow and reduce cost significantly during reprogramming.

INTRODUCTION

Takahashi and Yamanaka first demonstrated that induced pluripotent stem cells can be generated from somatic cells by transducing four transcription factors by integrating DNA viruses (3). The major limitation for potential clinical application is the integration of viral transgenes into the host genome that can result in multiple insertions and risk of tumorigenicity (5). Multiple methods have been developed to address these problems, including episomal vectors, mRNAs, or delivery of proteins, to address these problems, however, these methods often result in low efficiency or are difficult to repeat. In addition, due to poor reprogramming efficiency, reprogramming has been performed in the presence of animal feeders to maximize colony formation. Moreover, generation of iPSCs has been performed in the presence of serum and xeno containing products which are not ideal for clinical applications.

To address these issues, we generate iPSCs with a RNA virus based, Sendai virus, and show that it has much higher reprogramming efficiency (0.1-1%) than conventional methods (2). Sendai virus replicates in the cytoplasm of infected cells, and does not go through a DNA phase or integrate into the host genome (1). In addition, Sendai virus can infect a broad host range and is non-pathogenic to humans. We demonstrate that iPSCs generated by Sendai virus are free of genomic integration of exogenous genes by immunocytochemical staining and RT-PCR. They able to proliferate and maintain markers of pluripotency. Further these cells are able to give rise to EBs that can differentiate to all the three lineages - ectoderm, endoderm and mesoderm. Existing reprogramming protocols commonly require animal feeders to support the generation of iPSCs due to the low reprogramming efficiency of conventional methods. Since Sendai virus results in much higher efficiency, we are able to generate iPSCs with Sendai virus in feeder free StemPro® hESC SFM medium. Colonies arise from feeder free can be expanded in StemPro ® hESC SFM on Geltrex™ coated plates and express normal pluripotent markers. In addition, we also demonstrate that the resulting EBs also differentiate to all three lineages. Thus, generation of iPSCs in the absence of feeder simplifies the workflow and cost of reprogramming.

Finally, the major limitation of using serum containing media or xeno products is that it can hinder clinical translation. Here, we demonstrate that iPSCs can be generated with Sendai virus in xeno-free conditions. Colonies arising from xeno-free conditions were expanded and expressed pluripotent markers. In summary, we demonstrate the use of a Sendai virus-based reprogramming method that does not integrate in the host genome, providing zero footprint iPSCs, in either feeder free or xeno-free conditions. Generation of a footprint free iPSCs under these conditions should facilitate the safe clinical translation of iPSC-based therapies.

MATERIALS AND METHODS

Transduction of human dermal fibroblasts

BJ fibroblasts (ATCC) were transduced with four individual Sendai virus expressing Oct4, Sox2, Klf4, and cMyc overnight. Media were replaced the next day with fibroblasts media for 7 days, and cells were transferred to feeder free or Xeno free conditions on the 8th day.

Generation of feeder free iPSCs with Sendai Virus

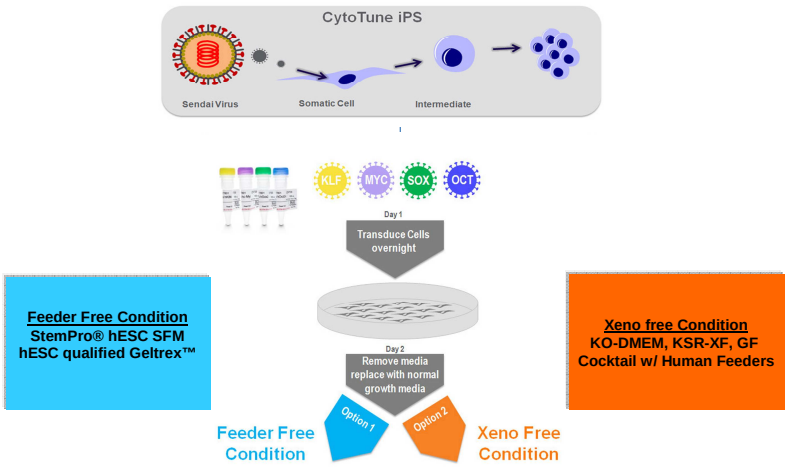
Eight days post transduction, cells were transferred to Geltrex coated plates and incubated with StemPro® hESC SFM medium. Colonies were picked and expanded on feeder free conditions.

Generation of Xeno Free iPSCs with Sendai Virus

Eight days post transduction, cells were transferred to xeno free human fibroblasts and cultured with KO-DMEM with 20%KSR and Xeno-free Growth Factor Cocktail. Colonies were picked and expanded in Xeno-free conditions and human feeders.

Reagents and media are available at www.invitrogen.com

CytoTune™ -iPS Reprogramming Workflow



Colony formation 21-28 days post transduction

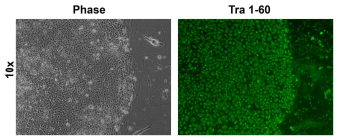


Figure 1. iPSC colony formation in feeder free conditions. BJ fibroblasts were transduced with Sendai virus over night and incubate in fibroblast media for 7 days. 8th post transduction, cells were transferred to StemPro® hESC SFM and Geltrex™. Colonies were stained with Tra1-60 antibody on the 28th day post transduction.

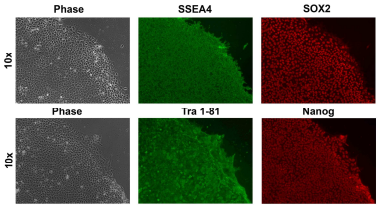


Figure 2. Expression of pluripotent markers of iPSC colonies in feeder free conditions. Colonies were expanded and maintained onto StemPro® hESC SFM and Geltrex™. Colonies were stained with Tra1-81, SSEA4, Sox2, and Nanog antibody.

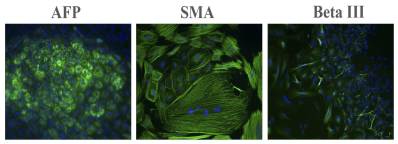


Figure 3. Differentiation potential of iPSC colonies in feeder free conditions. iPSC colonies give rise to EBs and can differentiate into all three lineages, endoderm, ectoderm and mesoderm. 21 days post EB differentiation, cells were stained with AFP, SMA, and Beta III antibody.

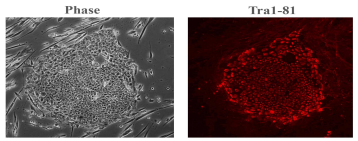


Figure 4. iPSC colony formation in xeno-free condition. BJ fibroblasts were transduced with Sendai virus over night and incubate in fibroblast media for 7 days. 8th post transduction, cells were transferred to KO-DMEM, KSR-XF, Growth factor cocktail with human feeders. Colonies were stained with Tra1-60 antibody on the 28th day post tranduction.

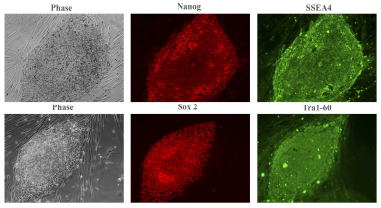


Figure 5. Expression of pluripotent markers of iPSC colonies in xeno free condition. Colonies were expanded and maintained onto KO-DMEM, KSR-XF,Growth factor cocktail with human feeders. Colonies were stained with Tra1-60, SSEA4, Sox2, and Nanog antibody.

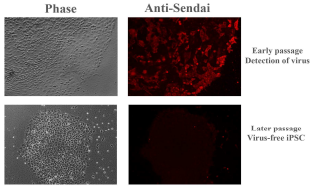


Figure 6. Generation of virus free iPSCs. An iPSC colony was stained with antibody against Sendai virus at early and late passage. At early passage, Sendai virus can be detected. However, at later passage, colony is free of Sendai virus as shown the panel below. RT-PCR were performed to confirm the absence of virus.

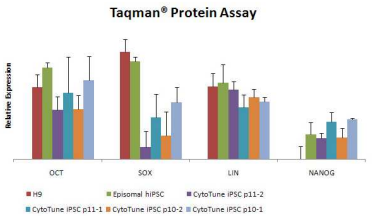


Figure 7. TaqMan® Protein Assays to detect expression of pluripotent markers of iPSC lines generated on feeder free and xeno-free condition. H9, an episomal hiPSC, and CytoTune iPSC lines were subjected to TaqMan® protein assays to detect for Oct3/4, Sox2, Lin28, and Nanog marker. Expression of pluripotent markers varied between lines and at different passages.

CONCLUSIONS

Direct reprogramming of somatic cells by over expression of four transcription factors, Oct3/4, Sox2, Klf4, and cMyc as demonstrated by Yamanaka and colleagues has open the possibility of providing an unlimited source of pluripotent stem cells for biomedical research and regenerative medicine (3). However, currently there are several limitations to iPSC generation, including low efficiency of the process of between 0.01-2% and the limitations of generating iPSCs in conditions amenable to clinical applications(5). Another major concern is that the current reprogramming method involves viral vector-mediated transduction of reprogramming genes which results in random integration of exogenous sequences into the genome.

Here we demonstrate the generation of iPSCs from a RNA Sendai virus that replicates in the cytoplasm and does not integrate into the host genome. In addition, Sendai virus can infect a broad host range and is non-pathogenic to humans. Comparison with other methods, Sendai virus mediates reprogramming with much higher efficiency than conventional methods (2). We demonstrate that iPSCs generated by Sendai virus are free of genomic integration of exogenous genes by ICC staining and RT-PCR. Reprogrammed cells able to proliferate and maintain markers of pluripotency. Further these cells are able to give rise to EBs that can differentiate to all three lineages - ectoderm, endoderm and mesoderm.

Current methods of reprogramming require feeder layers to support and maximize the generation of iPSCs due to low reprogramming efficiency by conventional methods. Here we demonstrate that iPSCs can be generated in feeder free conditions with StemPro® hESC SFM medium on Geltrex™ coated dish. Colonies arising from feeder free can be expanded in StemPro® hESC SFM and expressed normal pluripotent markers. In addition, we also demonstrate that EBs also differentiate to all three lineages. Thus, generation of iPSCs in the absence of feeder will simplify the workflow and cost of reprogramming.

Finally, an additional major obstacle to generate clinical –grade iPSCs is that current reprogramming protocols reported to date involve the use of animal-derived products at several steps, making them unsuitable for clinical applications. Exposure of human cells to animal origin product may increase the non-human pathogen transmission and immune rejection of grafted cells. Here we show that reprogramming of human fibroblasts can be achieved under xeno-free conditions with Sendai virus. We derived iPSCs with xeno-free human fibroblasts and cultured with KO-DMEM with 20%KSR and Xeno-free Growth Factor Cocktail (20ng/ml FGF). Colonies were picked, expanded in xeno-free conditions and expressed normal pluripotent markers. TaqMan™ protein assays can be used to characterize and compare to H9 and other non-integrating iPS lines. Thus, the generation of a footprint free iPSCs under these conditions should facilitate the safe clinical translation of iPSC-based therapies.

Note: all products mentioned are For Research Use Only.

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