Application Note

Single transfection of a synthetic polycistronic self-replicative RNA yields high numbers of transgene-free human iPSCs

Introduction

A ready source of induced pluripotent stem cells (iPSCs) is critical to the effective study of differentiation pathways and the investigation of the therapeutic potential of iPS cells. Since the discovery that human iPSCs could be generated by inducing expression of the four reprogramming factors (OCT-4, SOX-2, KLF-4 and c-MYC)[1], many different reprogramming technologies have emerged to generate iPSCs, each possessing their own advantages and disadvantages[2]. First-generation technologies, based on retroviral and lentiviral systems, allowed for highly efficient reprogramming events but lacked the necessary control over host genome integrations. Cre-excisable lentiviral systems offered a solution to genome integration but required lengthy subcloning procedures and screening to ensure excision of the reprogramming factors. Second-generation technologies used non-integrating episomal DNA plasmids, which were transgene-free but lacked the high reprogramming efficiencies of earlier retroviral and lentiviral techniques. Third-generation technologies used negative sense, non-integrating RNA viruses, termed Sendai Viruses (SeV), which originated from highly transmissible respiratory tract infections in mice, hamsters, guinea pigs, rats, and pigs. These RNA viruses produced integration-free iPSCs, produced high reprogramming efficiencies and were easy to use, but residual Sendai virus was difficult to clear from cells, resulting in the requirement for multiple rounds of clonal expansion and analysis.

The Simplicon™ RNA Reprogramming Technology is a next generation reprogramming system that uses a single synthetic, polycistronic self-replicating RNA strand engineered to mimic cellular RNA to generate human iPS cells[3,4]. The single RNA strand contains the four or five reprogramming factors, OCT-4, KLF-4, SOX-2, GLIS1 or c-MYC, and enables extremely efficient reprogramming using a single transfection step without any viral intermediates or host genome integration. Once iPSCs are generated, the RNA can easily be selectively eliminated by removing the interferon-alpha/beta inhibitor, B18R, from the cell culture medium.

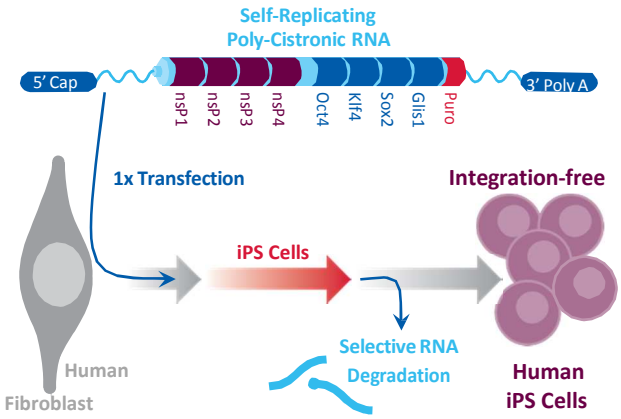

Figure 1.

Human iPS cells can be generated with a single transfection of polycistronic RNA. Once created, the reprogramming RNA can be selectively degraded by removing B18R from the culture media, creating transgene-free, replicon-free iPSCs.

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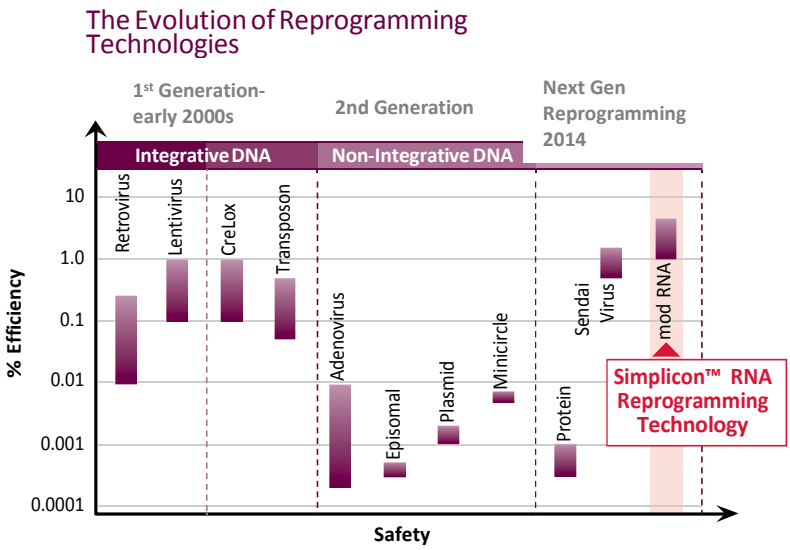

Figure 2.

The evolution of reprogramming technologies has culminated in the development of synthetic RNA-mediated reprogramming (extreme right), representing the safest and most efficient method for iPS cell generation: Simplicon™ RNA Reprogramming Technology combines the efficiency of retroviral and lentiviral reprogramming technologies with the safety of non-viral based reprogramming methods.

Materials and methods

Human Reprogramming Timeline

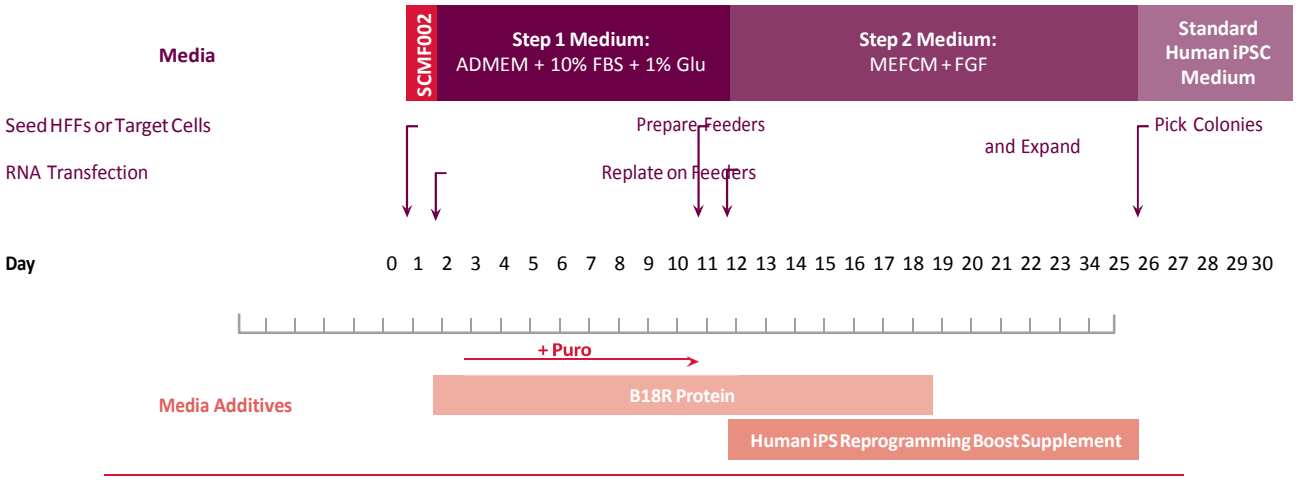

Figure 3.

Timeline showing points at which Simplicon™ reprogramming steps were performed.

Generation of human iPSCs using Simplicon™ RNA Technology

4 x 10^5 human foreskin fioblasts (HFFs, Cat. No. SCC058) were plated in each well of a 6-well plate in low serum fioblast medium (Cat. No. SCMF002) and allowed to attach overnight. HFFs were pretreated with B18R growth factor (Cat. No. GF153, 200 ng/mL) for 2 h at 37 ˚C and 5% CO2 . HFFs were then transfected with 1µg of Simplicon™ VEE-OKS-iG and B18r RNA in 2.5 µLof Lipofectamine® 2000 transfection reagent diluted with Opti-MEM™ medium (Life Technologies) following the manufacturer's protocol. The mixture of Simplicon™ RNA and transfection reagent was incubated at 37 ˚C, 5% CO2 for 3 h. Following transfection with RNA, medium was exchanged with 2 mL/well of ADMEM medium containing 10% fetal bovine serum (FBS), 1% Gluta-MAX™ supplement and B18R protein (200 ng/mL).

Starting the day after transfection, cells were fed daily with ADMEM with 10% FBS, 1% GlutaMAX™ supplement, B18R protein and 0.5 µg/mL puromycin for a total of 10 days. 30-60% cell death was observed from day 4-5 and puromycin-resistant cells started to grow back at day 7-9 after puromycin selection.

At day 10, approximately 5 x 10^4 to 1 x 10^5 reprogrammed cells were replated on fresh EmbryoMax® Primary Mouse Embryo Fibroblasts (PMEF, Cat. No. PMEF-CF) in MEF-con- ditioned medium containing B18R protein (200 ng/mL) supplemented with small molecules contained in the Human iPS Reprogramming Boost Supplement II (Cat. No. SCM094). Cell morphology was monitored daily, and small iPS cell colonies started to appear around day 15-16. At day 20, reprogrammed cells were transitioned to standard human embryonic stem cell medium without B18R protein and colonies were selected based on colony morphology and expanded for future experiments.

Results

Transfection efficiency using Simplicon™ RNA Reprogramming Technology

HFFs were transfected with either a control RNA replicon encoding green fluorescent protein (GFP) alone (Figure 4A) or with the Simplicon™ Reprogramming RNA (Figure 4B), and analyzed the following day to determine transfection efficiency. A high percentage of cells in Figure 4A showed GFP signal; transfection of HFFs using Simplicon™ Reprogramming RNA resulted in roughly 5-10% Oct-4 expression one day after transfection (green, Figure 4B).

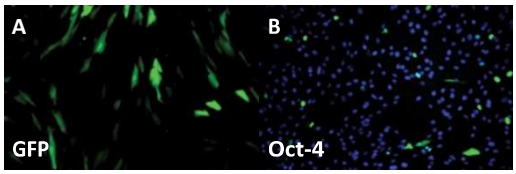

Figure 4.

Fluorescent micrographs of HFFs transfected with a GFP RNA replicon control (A) or Simplicon™ Reprogramming RNA (B) and analyzed the following day to estimate transfection efficiency. The green signal in (B) reflects OCT-4 expression.

Reprogramming kinetics

To determine the kinetics of reprogramming, Simplicon™ transfected HFFs were analyzed at four time points by brightfield microscopy (Figure 5). Colonies of human iPSCs emerged between days 17-21 after a single transfection at day 1.

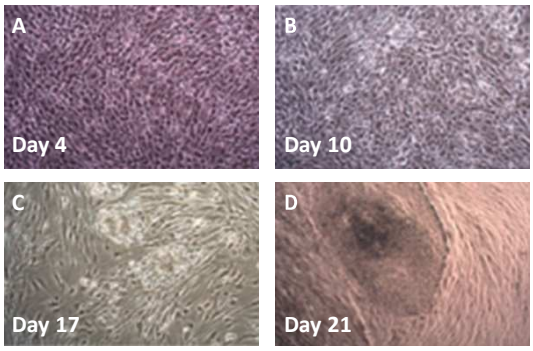

Figure 5.

Brightfield microscopy shows HFFs being reprogrammed over a 21 day time period using a single transfection of

Simplicon™ Reprogramming RNA. After 10 days of puromycin selection and replating on fresh PMEF cells, colonies started to emerge at day 17-21.

Characterization of human iPSCs

Simplicon™ reprogrammed fibroblasts were assayed for alkaline phosphatase activity, a pluripotency marker, 28 days after Simplicon™ transfection (Figure 6). Both HFF and BJ fibroblasts showed high expression levels of alkaline phosphatase activity, with the HFF cells showing the most intense signal.

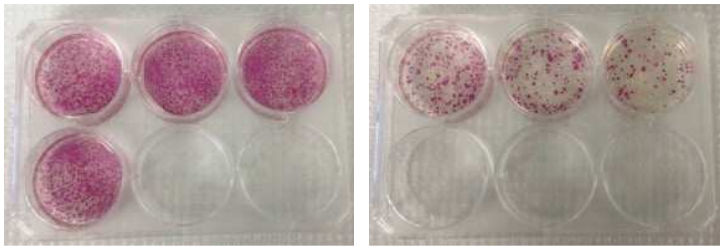

Figure 6.

Alkaline phosphatase staining of day 28 reprogrammed fibroblasts (HFF and BJ) generated using Simplicon™ RNA Reprogramming Technology.

Lot-to-lot reproducibility

To compare the performance of different manufacturing lots of the synthetic Simplicon™ RNA strands, we used four different lots to transfect HFFs (10,000 cells per transfection) and then counted the number of hiPSC colonies that appeared 17-21 days later.
Calculating reprogramming efficiency as the number of reprogrammed colonies divided by the number of original cells, we determined that all the manufacturing lots displayed similar reprogramming efficiencies, ranging from 0.5% to 1%.

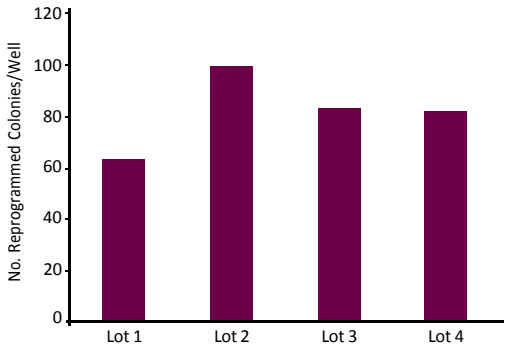

Figure 7.

Similar reprogramming efficiencies ranging from 0.6%-1% were generated using different manufacturing lots of Simplicon™ RNA.

Discussion

We have described a unique, synthetic, polycistronic RNA-based technology that enables efficient reprogramming of human somatic cells into induced pluripotent stem cells using a single transfection event. The Simplicon™ RNA reprogramming technology does not rely on lentiviral, retroviral or RNA-based viruses and is a step toward a more defined system for iPS cell generation. This RNA-based reprogramming approach has broad applicability for the efficient generation of hiPSCs for use in disease cell-modeling studies, transdifferentiation studies, and eventual human cell therapy/regenerative medicine applications.

References

1. Yamanaka S., et al. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 Aug 25;126(4):663-76.
2. Ding, S, et al. Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell. 2010 Dec 3;7(6):651-5.
3. Dowdy, SF, et al. Efficient generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell. 2013 Aug 1;13(2):246-54.
4. Yoshioka, N, et al. Enhanced generation of iPSCs from older adult human cells by a synthetic five-factor self-replicative RNA. PLoS One. 2017 Jul 27;12(7)

Ordering Information

Description	Catalogue No.
Simplicon™ Reprogramming RNA (OKSG)	SCR549
Simplicon™ RNA Reprogramming Kit (OKSG)	SCR550
TagRFP Simplicon™ RNA Kit	SCR712
Human OKSG-cMyc Tag GFP Simplicon RNA	SCR714
TagGFP2 Simplicon™ RNA	SCR713
B18R, Human Recombinant Carrier-Free	GF153
Human iPS Reprogramming Boost Supplement II	SCM094
EmbryoMax® Primary Mouse Embryo Fibroblasts, Strain CF1, Mytomycin C treated, passage 3	PMEF-CF
FibroGRO™ Xeno-Free Human Foreskin Fibroblasts	SCC058
FibroGRO™-LS Complete Media Kit for Culture of Human Fibroblasts	SCMF002
PluriSTEM™ Human ES/iPS Medium	SCM130
PluriSTEM-XF™ Human ES/iPS Medium	SCM132
PluriSTEM™ Dispase-II Solution	SCM133
PluriSTEM-XF™ Recombinant Vitronectin	CC130
Alkaline Phosphatase Detection Kit	SCR004
Fibroblast Growth Factor basic, Human Recombinant	GF003
HumanKine® Thermostable bFGF, Human Recombinant	GF446-50ug
Anti-Oct-4 Antibody, clone 10H11.2	MAB4401
Anti-SOX-2 Antibody, clone 10H9.1	MAB4423
Anti-TRA-1-60 Antibody, clone TRA-1-60	MAB4360
Anti-TRA-1-81 Antibody, clone TRA-1-81	MAB4381
Anti-NANOG Antibody, clone 7F7.1	MABD24

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