Application Note

Enhanced Reprogramming of Human Somatic Cells using Human STEMCCA Polycistronic Lentivirus and Human iPS Cell Boost Supplement

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Abstract

The ability to "reprogram" differentiated adult cells to a state that resembles embryonic stem cells has created wide-ranging opportunities for development of relevant in vitro disease models and patient-specific cell replenishment therapies.

Initial efforts to generate human induced pluripotent stem cells (iPS cells) required simultaneous co-infection of cells with four separate retroviral expression vectors (Oct-4, Klf4, Sox-2 and c-Myc). Each vector carried one transcription factor, which resulted in a high number of genomic integrations. Single polycistronic lentiviral vectors, such as those in EMD Millipore's STEMCCA lentivirus reprogramming kits, can improve efficiency and reduce the number of viral integrations. In one report, high percentages of disease-specific human iPS clones were isolated which possessed only a single viral integrant2. Despite these advances, reprogramming human somatic cells remains a highly inefficient and time-consuming process. Small molecules targeting specific signaling pathways have been shown to enhance reprogramming and/or replace the transcription factors required for reprogramming5-7. Here, a cocktail of small molecules was identified that, when used in conjunction with the STEMCCA kits, further increased reprogramming efficiency, decreased time required to establish full reprogramming, and maintained the desired iPS cell morphology and pluripotency.

Introduction

We have previously developed mouse STEMCCA lentivirus kits that have been validated for the generation of both mouse and human iPS cells from mouse embryonic fibroblasts (MEFs) and human foreskin fibroblasts (HFFs), respectively. However, a high multiplicity of infection (MOI = 200) was required for reprogramming human somatic cells. The recently launched human STEMCCA kits replaced mouse genes with human transcription factors, yielding similar numbers of colonies but at a dramatically reduced MOI (MOI = 20). Both human and mouse STEMCCA lentivirus kits are available in constitutive and Cre/LoxP-regulated formats for higher reprogramming efficiency of normal and diseased post-natal somatic cells2-4.

Although lenti- and retro-virus based reprogramming remain the most efficient methods to deliver exogenous reprogramming factors into the host cell genome, human iPS cell generation is still slow (around 4 weeks) and inefficient (0.01-0.1% efficiency), resulting in mixed populations of partial and full reprogrammed colonies. Substantial effort and progress have been made to generate iPSCs with fewer or no exogenous genetic manipulations – for example, by introduction of chemical compounds that can functionally replace reprogramming transcription factors and/or enhance efficiency and kinetics of reprogramming5-7. Human keratinocytes8 and mouse fibroblasts9 have now been reprogrammed to iPSCs with a single gene, Oct4, and a cocktail of small molecules.

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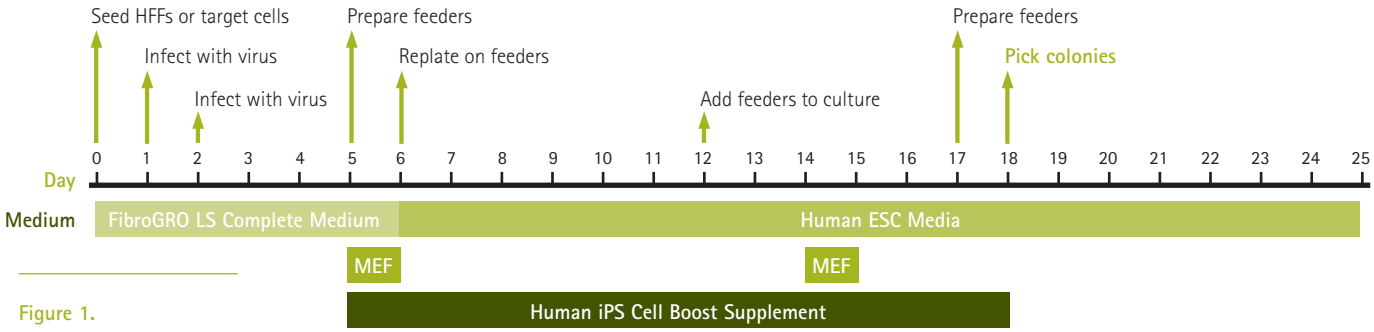


Figure 1.
Time course schematic of reprogramming human somatic cells using STEMCCA polycistronic lentivirus kits combined with Human iPS Cell Boost Supplement.

Here, various combinations of small molecules involved in TGFa, Wnt, and MAPK signaling pathways along with epigenetic modifiers were screened fortheir effects on (1) increasing the ratio of fully reprogrammed SSEA4+TRA-1-60+ Hoechstdim iPS cells versus reprogramming intermediates; (2) increasing colony numbers and (3) reducing the time to establishment of full reprogrammed iPS cell colonies.

From this screen, a small molecule boost supplement was identified which in combination with the STEMCCA lentivirus kits fulfilled the three aforementioned criteria. The small molecule boost supplement was validated on feeder-based (KOSR) and serum-free, feeder-free culture systems and was furthermore shown to be highly effective in reprogramming different primary fibroblast cell lines.

Materials and Methods

The reprogramming protocol followed is outlined in Figure 1. FibroGRO™ Xeno-free Human Foreskin Fibroblasts (HFFs, Cat No. SCC058) were seeded at a density of 10,000 cells/well and transduced with the Human STEMCCA Constitutive Polycistronic Lentivirus Reprogramming Kit (Cat No. SCR544). Cells were replated onto irradiated MEFs in the presence or absence of a panel of small molecules at day 6, using the human ESC medium of choice (either KOSR-based media on inactive MEFs (Cat No. PMEF-CF) or on Matrigel™-coated or Geltrex™- coated plates if using mTeSR® or StemPRO® media, respectively).

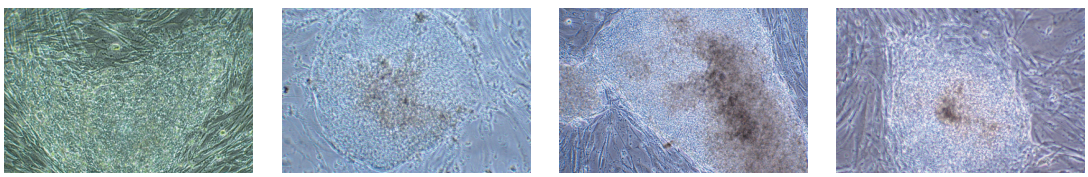
The medium was changed every other day and colonies were picked when they reached several hundred cells in size. A total of 25 small molecule boost cocktails (proprietary formulations) were tested and several treatments were able to give rise to large flat 2D colonies that displayed morphological features similar to hESCs and could be easily passaged.

Colonies were stained with anti-Tra-1-60 (Cat. No. FCMAB115F) and anti-SSEA-4 (Cat. No. MAB4304). Tra-1-60 is a marker that is expressed at a later stage of reprogramming10. Tra-1-60 and SSEA-4 double positive staining has been suggested to be a valid marker for full reprogrammed iPSCs11.

Results

HFF were transduced with STEMCCA lentivirus, replated onto irradiated MEFs in the presence or absence of a panel of small molecules at day 6, and evaluated for number of colonies generated, morphology, ease of passaging, and days to passage (Figure 2, 3). Chemically treated human iPSCs possessed fast proliferation kinetics; early passages from P0 to P3 were shortened to 5-6 days per passage period - a timeframe that is similar to the proliferation rate of normal hESC (Figure 3). Treatment 2 was selected on the basis that it significantly improved both the quality of colonies formed and the efficiency of reprogramming. Treatment 2 is herein referred to as Human iPS Cell Boost.

Figure 2.
Small molecule combinations were screened for their effects on colony morphology, number of colonies generated, ease of passaging and relative proliferation rate as measured by days to passaging. Treatment 2 was selected for further characterization. Treatment 2 is referred to as Human iPS Cell Boost Supplement.



	Untreated	Treatment 1	Treatment 2	Treatment 3
Morphology:	3D	Flat 2D	Flat 2D	Flat 2D
# Colonies:	1x	3.6x	2.6x	0.5x
Passageability:	Difficult	Easy	Easy	Easy
Days to Passage:	10-12 days	5-6 days	5-6 days	5-6 days

In the presence of the Human iPS Cell Boost Supplement, the number of colonies formed increased 3-fold (Figure 4A) when used in combination with the mouse STEMCCA lentivirus kits (Cat. Nos. SCR530, SCR531) and 15-fold when used in combination with the human STEMCCA lentivirus kits (Cat. Nos. SCR544, SCR545). Colonies stained positive for both human ESC markers, SSEA-4 (Figure 4F) and TRA-1-60 (Figure 4G). TRA-1-60+ colonies were not observed in the untreated control cultures (Figure 4D).

Human iPS colonies generated in the presence of Human iPS Cell Boost Supplement could be readily expanded for multiple passages (over 30 passages). Human iPS cells displayed the morphology characteristic of human ESCs, had a normal karyotype and stained positive for pluripotent markers (Figure 5).

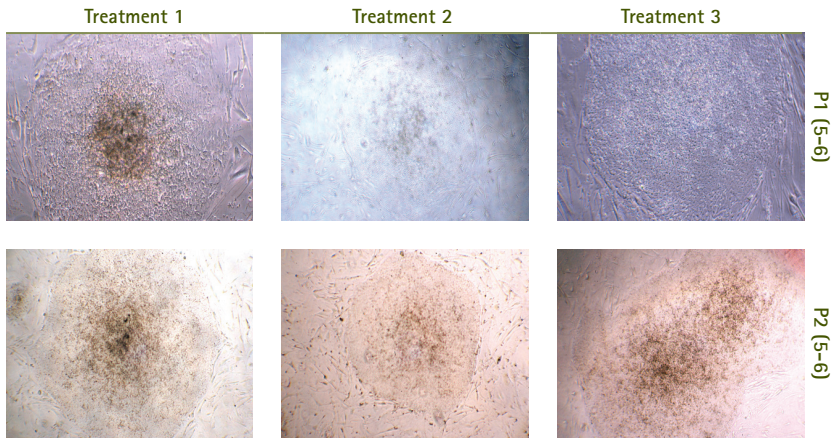


Figure 3.

Proliferation kinetics similar to human ESC were obtained for all three chemical treatments starting at p1 (5-7 days till passaging) versus from p3 for untreated control (data not shown).

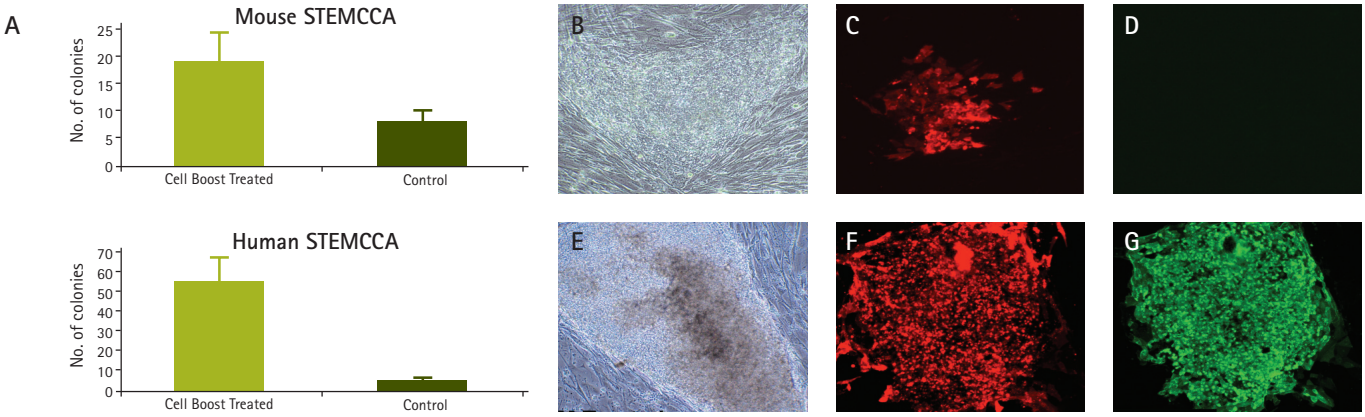


Figure 4.

Addition of Human iPS Cell Boost Supplement to a polycistronic lentivirus-based reprogramming regime (STEMCCA) dramatically increased the efficiency of colony formation (A) and shortened the time to establishment of full reprogrammed human iPS clones (E, F, G). Human iPS Cell Boost Supplement enhanced colony formation by 2-3 fold when used in combination with the mouse STEMCCA lentivirus kits (SCR530, SCR531) and 15-fold when used in combination with the human STEMCCA lentivirus kits (SCR544, SCR545) (A). Four independent experiments (MOI = 200) were performed using mouse STEMCCA lentivirus kit while two experiments were performed (MOI = 10) using human STEMCCA lentivirus kits. Reprogramming without chemical treatment was used as a control for all experiments. p0 human iPS colonies generated from FibroGRO Xeno-free Human Foreskin Fibroblasts (Cat. No. SCC058) reprogrammed with mouse STEMCCA lentivirus (SCR530) in the presence of the Human iPS Cell Boost Supplement exhibited larger colony sizes, a flat 2D morphology (E), and are SSEA-4- positive (F) and TRA-1-60-positive (G). This is in marked contrast to untreated control where the colonies are smaller, are 3D in morphology (B) and are SSEA-4 positive (C) but TRA-1-60-negative (D) at similar timepoints.

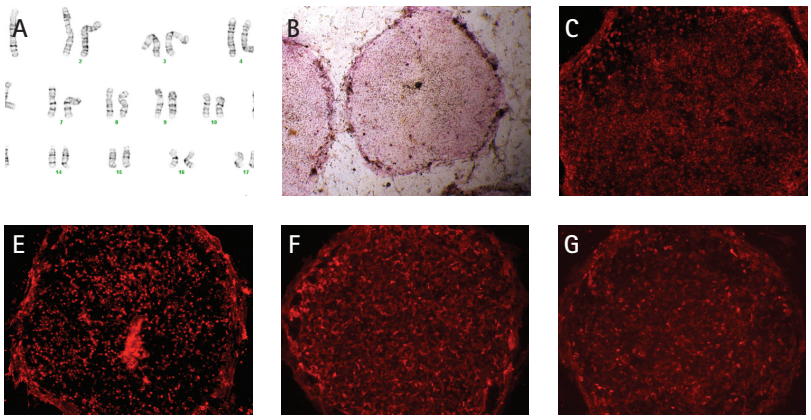
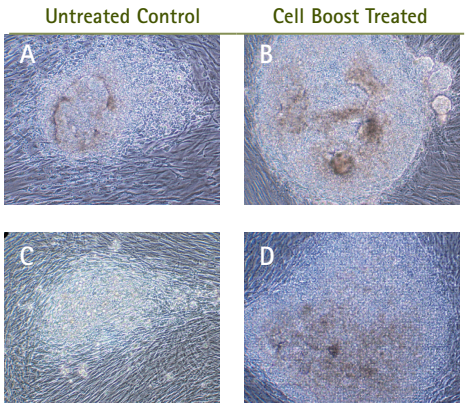


Figure 5.

Human iPS cells generated using the Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Kit (SCR545) in combination with Human iPS Cell Boost Supplement possessed an apparently normal karyotype (A) and expressed the appropriate human pluripotent markers, alkaline phosphatase (B), SSEA-4 (C), Oct-4 (D), SSEA-3 (E), TRA-1-60 (F), and TRA-1-81 (G). Cytogenic analysis was performed on twenty G-banded metaphase cells from p9 human iPS cells. All twenty cells demonstrated an apparently normal male karyotype. No abnormal cells were detected (A, Cell Line Genetics).

The versatility of the Human iPS Cell Boost Supplement was further demonstrated by enhanced reprogramming of multiple primary human fibroblast cell lines including HFF and BJ (ATCC) (Figure 7) in either KOSR feeder-based or serum-free, feeder-free culture systems (mTeSR and StemPRO) (Figure 6).

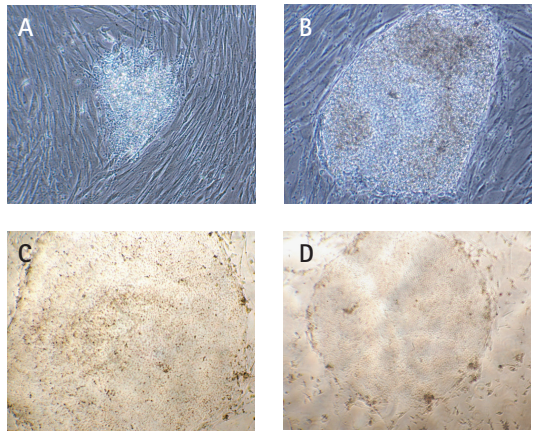


E

Feeder/serum free culture	No. of cells/well re-plated in feeder-free culture	Chemical Treatment	Colony#
mTeSR	5 x 10^4	+	70
		-	6
	10^5	+	76
		-	22
StemPro	5 x 10^4	+	23
		-	9
	10^5	+	15
		-	1

Figure 6.

Human iPS Cell Boost Supplement improves reprogramming efficiency in serum-free, feeder-free based culture systems and increased colony size relative to untreated controls (compare B, D to A, C). Human iPS colonies were generated using mouse STEMCCA lentivirus kit in the absence or presence of the Human iPS Cell Boost Supplement. Cells were cultured on either Geltrex-coated plates in StemPRO medium (A, B) or Matrigel-coated plates in mTeSR medium (C, D). Day 23 fully reprogrammed hiPS colonies were isolated and continually passaged in mTeSR conditions exhibited typical hESC morphology (F).



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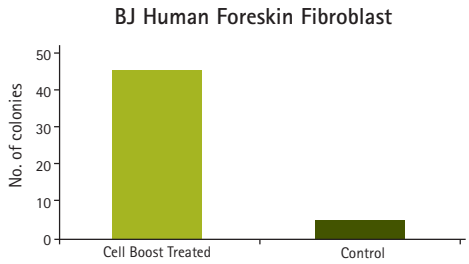

Figure 7.

Day 12 human iPS colonies generated from BJ neonatal foreskin fibroblasts using mouse STEMCCA lentivirus kit in combination with Human iPS Cell Boost Supplement exhibited a 10-fold increase in colony numbers (E) and displayed increased colony size (B) relative to untreated control (A). Human iPS colonies picked at day 21 and passaged on MEFs exhibited similar flat 2D morphology (C, D) and similar proliferation kinetics as human ES cells (data not shown).

Conclusion

In summary, we have established a robust reprogramming method that is amenable to efficient reprogramming of different fibroblasts readily available from human donors. By using small molecules that modulate key signaling pathways and epigenetic modifiers, we could dramatically improve the quality and quantity of human iPS colonies generated.

This novel method increased the number of human iPS colonies by 2-3 fold when the mouse STEMCCA lentivirus kit was used and up to 15-fold when the human STEMCCA lentivirus kit was used. The colonies formed possessed the distinctive flat 2D morphology that are more reminiscent of human embryonic stem cells and could be easily passaged in contrast to untreated control that exhibited 3D morphology and were more difficult to isolate. Furthermore, the time to establishment of fully reprogrammed human iPS colonies that are SSEA4+TRA-1-60+Hoechstdim was significantly shortened by 50%. Reprogramming with the small molecule boost supplement was further validated on multiple human fibroblast cell lines in feeder-free and serum-free culture systems. In summary, the use of the STEMCCA polycistronic lentivirus in combination with the small molecule boost supplement provides a convenient solution for enhanced reprogramming efficiency.

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