Lonza

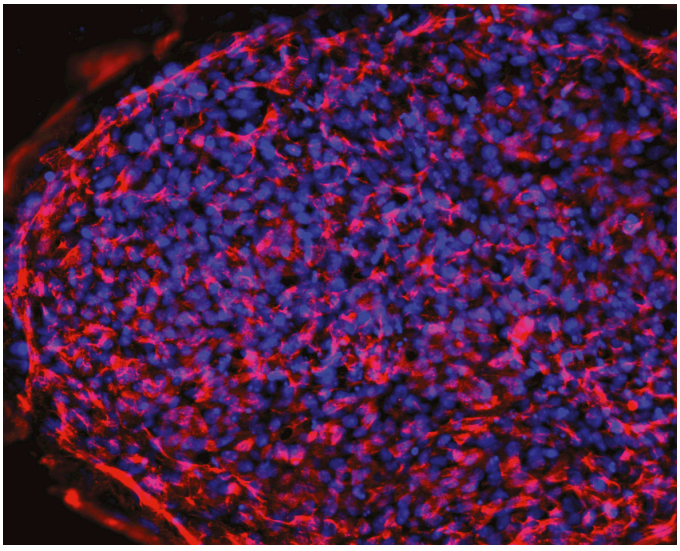
Generation of hiPSCs from Cord Blood Cells with the 4D-Nucleofector® System

by Inbar Friedrich Ben Nun, Xu Yuan, Patrick Walsh,
Amy Burkall, Don Paul Kovarcik and Thomas Fellner
Lonza Walkersville, Inc., Walkersville, MD, USA

In 2007, Dr. Shinya Yamanaka and colleagues at Kyoto University became the first to successfully convert adult human cells to an embryonic stem cell(ESC)-like state[1]. To generate the first human induced pluripotent stem cells (hiPSCs), Dr. Yamanaka's team first utilized viruses to deliver transcription factors necessary for reprogramming cells. These viruses integrate, thus altering the host genome and potentially leading to aberrant gene expression. Since then, efforts have been made to generate hiPSCs using integration-free methods. Here, we successfully deliver non-viral vectors encoding the transcription factors by using Lonza's 4D-Nucleofector® System into cord blood CD34+ cells which results in the generation of hiPSC colonies in less than 14 days.

Introduction

Pluripotent stem cells, such as hESCs or hiPSCs, have the ability to self-renew and generate any cell type in the human body. Therefore, they have the potential to produce an infinite quantity of cells for different applications, such as regenerative medicine, disease modeling and drug development. In many research laboratories, viruses are the preferred method used to deliver reprogramming factors into the cells. Although the process is robust and efficient, integration of exogenous viral DNA fragment



may result in altered gene expression. To avoid genome modifications, several technologies were developed. One of the most promising methods utilizes episomal plasmids to generate integration-free hiPSCs. Several laboratories have successfully reprogrammed multiple cell types using episomal vectors encoding the transcription factors[2, 3]. hiPSCs generated using this method eventually lose the episomal vectors after several passages, leaving no trace of exogenous DNA. Here, we describe a reliable method for the introduction of episomal plasmids by utilizing Lonza's 4D-Nucleofector® System ,resulting in the efficient generation of "zero footprint" hiPSCs.

Materials and Methods

Cell Culture

Cord blood CD34+ cells (Lonza, cat. no. 2C-101) were isolated from mononuclear cells using positive immunomagnetic selection. Cryopreserved cord blood CD34+ cells were thawed and grown in serum-free medium (SFM) containing 50% IMDM, 50% Ham's F12, 1x chemically defined lipid concentration, 1x Insulin-Transferrin-Selenium-X, 2 mM GlutaMAX™ I (Invitrogen) and 50 µg/mL ascorbic acid (Sigma). The medium is supplemented with cytokines: 100 ng/mL SCF, 100 ng/mL Flt3-ligand, 20 ng/mL TPO and 10 ng/mL IL-3 (PeproTech). CD34+ cells were cultured in SFM for 4–5 days before transfection with the 4D-Nucleofector® System (Lonza, cat. no. AAF-1001B, AAF-1001X).
hiPSCs were maintained on mytomycin C-treated mouse embryonic fibroblast (MEF) feeders (Millipore) in hESC medium containing Knockout-DMEM/F12, 20% Knockout serum replacement, 1x NEAA, 2 mM GlutaMAX™ I, 0.11 mM 2-mercaptoethanol, and 10 ng/mL basic fibroblast growth factor (bFGF) (Invitrogen). The cells were passaged with 1 mg/mL collagenase IV (Invitrogen).

Vectors

The episomal vectors pEB-C5 and pEB-Tg were used to generate hiPSCs2. The pEB-C5 plasmid encodes five mouse cDNAs, including *Oct3/4, Sox2, Klf4, c-Myc* and *Lin28*. The pEB-Tg plasmid encodes SV40 Large T antigen.

Nucleofection®

CD34+ cells were co-transfected with the two episomal vectors (pEB-C5 and pEB-Tg) by using the 4D-Nucleofector® System according to the manufacturer's instructions. We used 10^6 CD34+ cells per sample and suspended them in 100 µL P3 Primary Cell Nucleofector® Solution containing 8 µg pEB-C5 and 2 µg pEB-Tg. Cells were transfected with program EO117. After transfection, 0.5 mL warmed SFM supplemented with cytokines was added to the cells in the cuvette. The transfected cells were gently suspended in the cuvette and transferred into 1.5 mL pre-warmed SFM supplemented with cytokines in one well of a 12-well plate.

Generation of hiPSCs

Generation of hiPSCs from CD34+ cells was performed as previously described[3]. After co-transfection of pEB-C5 and pEB-Tg, the transfected cells were cultured in SFM supplemented with cytokines for 2 days. The cells were collected and spun down (200 xg, 5 minutes). We used MEF medium (DMEM, 10% FBS, 1x NEAA) to suspend the cells and plate them into one well of a 6-well plate pre-coated with MEF feeders and gelatin (Millipore). After 24 hours, MEF medium was replaced with hESC medium containing 0.25 mM sodium butyrate (NaB) (Stemgent). Medium was replaced every other day. One week later, cells were fed with MEF-conditioned hESC medium, supplemented with 10 ng/mL bFGF and NaB. Colonies were observed as early as 6 days after transfection.

Characterization of Lonza hiPSCs

We performed immunocytochemistry to test pluripotent stem cell-associated marker expression in Lonza hiPSCs. Cells were fixed with 4% paraformaldehyde, permeabilized and stained with anti-SSEA4 antibody (Milipore, cat. no. MAB4304), anti-TRA-1-60 antibody [Millipore, cat. no. MAB4360], anti-OCT3/4 antibody [Abcam, cat. no. 19857] and anti-NANOG antibody [R&D Systems, cat. no. AF1997]. Cells were tested for alkaline phosphatase activity using Alkaline Phosphatase Kit II (Stemgent, cat. no. 00-0055).

Flow cytometry was performed to study the surface markers of Lonza hiPSCs. The following antibodies were used in the flow cytometry analysis: mouse anti-human SSEA4-PE(BD, cat. no. 560128), mouse anti-human TRA-1-60-PE (BD, cat. no. 560193) and mouse anti-human TRA-1-81-PE (BD, cat. no. 560161).

Pluripotency was demonstrated by *in vitro* differentiation via embryoid body (EB) formation followed by immunocytochemistry. Undifferentiated hiPSCs were dissociated with 1mg/mL collagenase IV and cultured in suspension to form EBs. After culturing in suspension for 7 days in the presence of 20% FBS, EBs were plated onto a gelatin-coated plate for further differentiation. The differentiating cells were maintained in DMEM medium containing 20% FBS and stained for markers of the three germ layers with polyclonal rabbit anti-human alpha-1-fetoprotein (AFP) antibody (Dako, cat. no. A000829), anti-tubulin beta III monoclonal antibody (TUJ1) (Millipore, cat. no. MAB1637), and mouse anti-actin smooth muscle (SMA) antibody (Millipore, cat. no. CBL171).

To confirm the absence of the exogenous plasmid DNA in Lonza hiPSCs, PCR with three pairs of specific primers for the episomal vectors was performed using hiPSC genomic DNA. Genomic DNA was purified using DNeasy® Blood & Tissue Kit (Qiagen, cat. no. 69540Q). The primer sequences are in Table 1. The PCR program is: 95°C, 3 minutes; 95°C, 30 seconds; annealing 30 seconds; 72°C, 30 seconds; repeat step 2 to step 4 for 30 times; 72°C, 5 minutes; 4°C hold.

Primers	Sequences	Annealing Tempature
EBNA1-D	TTTAATACGATTGAGGGCGTCT	51.2°C
EBNA1-U	GGTTTTGAAGGATGCGATTAAG	51.2°C
Tg-F	GCCAGGTGGGTTAAAGGAGC	54.4°C
Tg-R	GGTACTTATAGTGGCTGGGCTGT	54.4°C
SK-F	CCATTAACGGCACACTGCCCCTGT	62.6°C
SK-R	AGGACGGGAGCAGAGCGTCGCTGA	62.6°C
Actin F	TGAAACAACATACAATTCCATCATGAAGTGTGAC	60°C
Actin R	AGGAGCGATAATCTTGATCTTCATGGTGCT	60°C

Table 1. PCR primer sequences.

Karyotyping analysis was performed on Lonza hiPSCs by Cell Line Genetics, Inc. (Madison, WI).

Results

Efficient Reprogramming of Human Cord Blood CD34+ Cells Using Episomal Vectors and the 4D-Nucleofector® System

Cryopreserved human cord blood CD34+ cells were thawed and cultured in SFM for 4–5 days before transfection with the episomal plasmids using the 4D-Nucleofector® System. CD34+ cells were reprogrammed as described in the Materials and Methods. After plating the transfected cells on the MEF feeders, small colonies could be identified as early as 6 days after transfection (Figure 1). On days 13–16, colonies exhibiting flat hESC-like morphology were manually picked and plated onto fresh MEF feeders for further expansion and characterization (Figure 1).

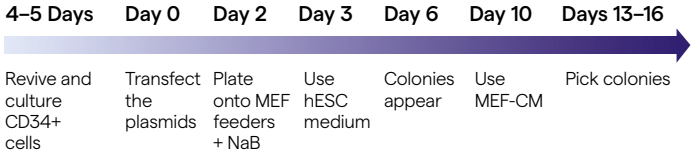


Figure 1.
Timeline of cord blood CD34+ cell reprogramming with two episomal vectors. Cord blood CD34+ cells were co-transfected with pEB-C5 and pEB-Tg using Lonza's 4D-Nucleofector® System. After plating onto MEF feeders, small colonies appeared on day 6. Some colonies were large enough for picking on day 13.

Lonza hiPSCs Are Capable of Self-Renewal and Exhibit hESC-like Characteristics

Select Lonza hiPSC colonies were successfully expanded and further characterized. They are similar to hESCs in their self-renewal ability and exhibit flat colony morphology (Figure 2A). These cells are positive for alkaline phosphatase activity (Figure 2B). More importantly, Lonza hiPSCs express the core regulators of pluripotency: OCT3/4 and NANOG (Figure 3). The activation of endogenous NANOG expression in generated hiPSCs is a critical milestone indicating successful reprogramming of CD34+ cells. Furthermore, both flow cytometry analysis and immunocytochemistry demonstrated the presence of typical hESC-associated surface markers SSEA4, TRA-1-60 and TRA-1-81 (Figure 3 and Table 2).

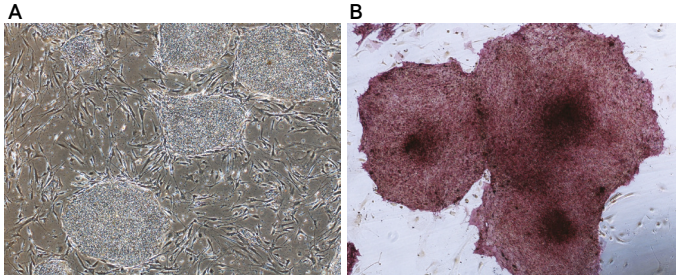


Figure 2.
Lonza hiPSCs show typical hESC-like colony morphology (A) and express alkaline phosphatase activity (B).

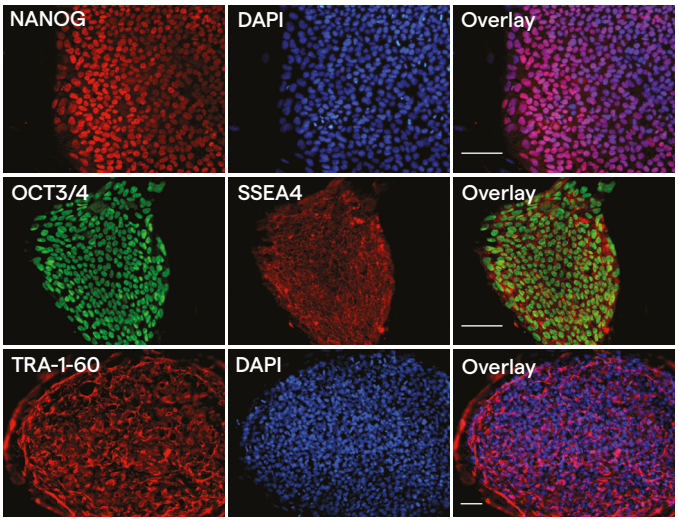


Figure 3.
Lonza hiPSCs express transcription factors NANOG and OCT3/4, and exhibit surface markers SSEA4 and TRA-1-60. Cells were fixed and stained for NANOG (top, red), OCT3/4 (middle, green), SSEA4 (middle, red) and TRA-1-60 (bottom, red). DAPI: blue; Scale bar 100 µm.

Surface Markers	SSEA4	TRA-1-60	TRA-1-81
hiPSC line 1	90%	85%	85%
hiPSC line 2	96%	94%	92%
hiPSC line 3	97%	92%	91%
hiPSC line 4	95%	91%	89%

Table 2.
Flow cytometry analysis reveals the presence of surface markers SSEA4, TRA-1-60 and TRA-1-81 in Lonza hiPSCs. Flow cytometry analysis was performed on four hiPSC lines generated in two different experiments. Live cells were dissociated and stained with PE-conjugated antibodies for SSEA4, TRA-1-60 and TRA-1-81. PE-conjugated mouse IgG3 and IgM were used to set up the corresponding controls. For each line, >80% of cell population is positive for the three surface markers tested.

Lonza hiPSCs Can Differentiate into Cells Representing Three Germ Layers

Pluripotency was demonstrated through *in vitro* differentiation. After 14 days of differentiation in the presence of FBS, differentiated cells were tested for the expression of neuron-specific beta-tubulin III, alpha-fetoprotein and smooth muscle actin. These proteins are expressed in representative cell types of the three germ layers. As shown in Figure 4, Lonza hiPSCs are able to differentiate into neurons (ectoderm), alpha-fetoprotein positive cells (endoderm) and smooth muscle cells (mesoderm), thus displaying differentiation potential similar to hESCs.

Lonza hiPSCs Maintain Normal Karyotype and Contain No Trace of Exogenous Episomal DNA

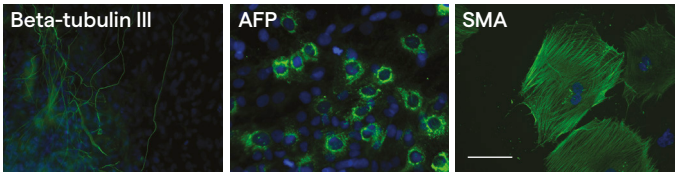


Figure 4.
Lonza hiPSCs can differentiate into cells of all three germ layers. We show differentiated cells derived from Lonza hiPSCs through EB formation. From left to right: Beta-tubulin III positive neurons (green), AFP positive cells (green) and SMA positive cells (green). DAPI: blue; Scale bar 100 µm.

We cultured Lonza hiPSCs on MEF feeders and enzymatically passaged those cells using collagenase IV. Karyotype analysis (Cell Line Genetics, Inc.) was performed on four hiPSC lines at passages 7, 16 and 18. All four hiPSC lines have a normal karyotype and maintain genome stability. Figure 5 shows a representative picture of a normal hiPSC karyotype.

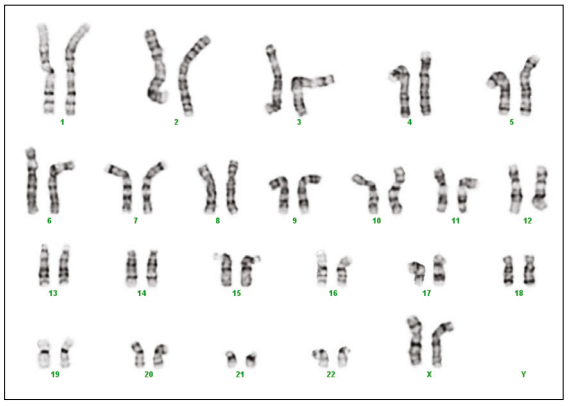


Figure 5.
Lonza hiPSCs maintain a normal karyotype. Shown: Normal karyotype of one hiPSC line at passage 7, generated from a female donor.

The episomal vectors initially delivered into the CD34+ cells did not integrate into the cell genome and were gradually lost after serial passaging. Three pairs of PCR primers targeting elements of the episomal plasmids were designed to detect the presence of episomal DNA in the hiPSC genome. Genomic DNA (gDNA) was prepared from both Lonza hiPSCs and the transfected CD34+ cells, the latter serving as a positive control. We collected gDNA from different hiPSC lines of different passages (Passages 5–7). PCR analysis failed to amplify any episomal DNA fragments from hiPSC gDNA, confirming the absence of the episomal vectors in established hiPSC lines. However, the same primers did amplify episomal DNA fragments from the transfected CD34+ cells (Figure 6).

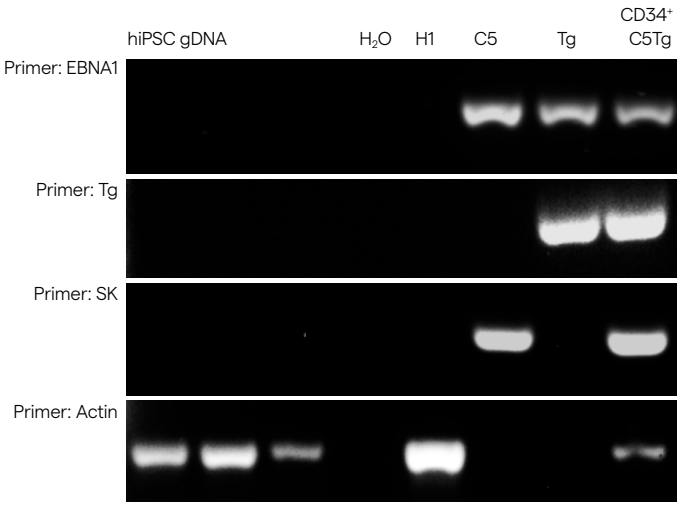


Figure 6.
Absence of episomal vector DNA in Lonza hiPSCs. Genomic DNA was purified from three hiPSC lines and the pEB-C5/Tg-transfected CD34+ cells were collected 2 days after transfection. PCR amplification was performed using three pairs of primers (EBNA1, Tg and SK) targeting episomal vectors and actin primers as a control. No DNA fragments were amplified from the gDNA of hiPSCs, confirming the loss of episomal plasmids. PCR templates from left to right: hiPSC gDNA; H_2O is the PCR negative control without DNA template; gDNA purified from hESC line H1; pEB-C5; pEB-Tg; gDNA purified from CD34+ cells co-transfected with pEB-C5 and Tg.

Conclusion

We successfully reprogrammed cord blood CD34+ cells into hiPSCs by delivering episomal vectors expressing five transcription factors and SV40 Large T antigen. The two episomal vectors were delivered using Lonza's 4D-Nucleofector® System. The Lonza hiPSCs share similar characteristics with hESCs, including self-renewal ability, hESC-associated surface marker expression, alkaline phosphatase activity, and key pluripotency transcription factor expression. These lines maintain a normal karyotype and can efficiently differentiate into cells of all three germ layers. Notably, Lonza hiPSCs show no trace of exogenous DNA integration, confirming that cells were reprogrammed with a "zero footprint" technology. Recently, using the same technology, we were able to generate hiPSCs under feeder-independent conditions with a defined medium and matrix.

References

1. Takahashi, K. and S. Yamanaka, Induction of pluripotent stem cells from mouse embry-onic and adult fibroblast cultures by defined factors. Cell, 2006; 126(4); 663–676.
2. Chou, B.K., *et al*., Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res., 2011; 21(3); 518–529.
3. Mack, A., *et al*., Generation of induced pluripotent stem cells from CD34+ cells across blood drawn from multiple donors with non-integrating episomal vectors. PlosOne, 2011; 6(11).

Contact Us

North America
Customer Service: + 1 800 638 8174 (toll free)
order.us@lonza.com
Scientific Support: + 1 800 521 0390 (toll free)
scientific.support@lonza.com

Europe
Customer Service: + 32 87 321 611
order.europe@lonza.com
Scientific Support: + 32 87 321 611
scientific.support.eu@lonza.com

International
Contact your local Lonza Distributor
Customer Service: + 1 301 898 7025
Fax: + 1 301 845 8291
scientific.support@lonza.com

Lonza Walkersville, Inc. – Walkersville, MD 21793

CD-SP033 10/23

bioscience.lonza.com
lonza.com/ipsc