Introduction

Stem cells have attracted a great deal of attention and have excellent potential in a variety of clinical applications. Human embryonic stem cells (hESCs) are capable of indefinite self-renewal and can differentiate into all somatic cell types, meaning that they are widely considered to have tremendous potential in medical research. However, on account of the ethical and legislative hurdles associated with their use (e.g., restricted numbers of embryos permitted to be used for research), a great deal of work has been devoted toward developing functional replacements for hESCs without the ethical constraints associated with the use of human embryos.

In 2006, it was demonstrated that mouse fibroblasts could be reprogrammed by retroviral overexpression of four transcription factors to an embryonic-like state. These reprogrammed somatic cells were termed induced pluripotent stem cells (iPSCs). The following year, it was shown for the first time that iPSCs could be generated from human fibroblasts using the same four factors – POU5F1 (a.k.a. OCT3/4), SOX2, KLF4 and MYC – via retrovirus-mediated transfection. Since this first method of generating human iPSCs (hiPSCs), there have been several noted experimental successes leading to the development of new reprogramming techniques for a variety of somatic cells in an effort to improve the derivation efficiency of hiPSCs.

Non-integrative and non-viral reprogramming methods can be very important for generating hiPSCs destined for human therapeutic applications. Synthetic mRNA delivery has generated iPSCs with a high level of efficiency and does not have the associated insertion mutagenesis that can result in tumor formation. However, the process is technically demanding, requiring very specific reagents, and places a significant burden on available time due to the required daily mRNA transfections. A near non-integrative approach to reprogramming is the use of episomal vectors. This non-viral method transfects cells using an episomal vector derived from the Epstein Barr virus, but without the viral packaging. Successful reprogramming via episomal vector-mediated transfection has been demonstrated using Lonza’s Nucleofector Technology. Lonza has developed its own protocol for reprogramming using peripheral blood mononucleated cells (PBMCs), which are combined with the L7™ hPSC Culture System for subsequent feeder- and xeno-free culture of the generated hiPSCs.

In this study, we evaluated the Lonza protocol for a certain individual – referred to as individual #418 – for which various reprogramming attempts had failed thus far. Previously, we successfully generated hiPSCs from primary cells of numerous individuals using retro- or Sendai virus-mediated methods. However, in the case of individual #418, these approaches failed to yield colonies following transduction attempts with both dermal fibroblasts from several biopsies and epithelial cells from urine samples. Using the Lonza L7™ hiPSC Reprogramming and hPSC
Culture System, we have generated hiPSCs from PBMCs of individual #418. The data presented here demonstrate that the Lonza protocol can be used for the generation of high quality hiPSCs, capable of maintaining their differentiation potential. Stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs).

**Materials and Methods**

**Reprogramming of PBMCs**

PBMCs were cultured over 6–8 days in a priming medium (provided by Lonza) that supports differentiation of the mixed PBMC population toward erythroblasts. After this priming phase, 1 million cells/sample were co-transfected with 3 µg of the vector cocktail (provided by Lonza) using the P3 4D-Nucleofector™ Kit (Lonza, cat. no. V4XP-3012) and program ED-115. Transfected cells were plated on 6-well plates coated with L7™ hPSC Matrix (Lonza, cat. no. FP-5020) and cultured in an optimized recovery medium containing a reprogramming enhancer (both provided by Lonza) for 2 days. At day 2, post transfection, the recovery medium was mixed 1:1 with fresh L7™ hPSC Medium (Lonza, cat. no. FP-5007) before replacing it completely at day 4 with L7™ hPSC Medium. Typically, iPSC colonies appear at days 9–10, post transfection, with some colonies being large enough for picking from day 12 onwards.

**Culture of hiPSCs**

After transfer into L7™ hPSC Medium, the medium was replaced every other day with 2 ml freshly supplemented media until colonies were large enough to subculture. The initial colonies were manually passaged (P1) into separate 12-well plates coated with L7™ hPSC Matrix (Lonza, cat. no. FP-5020) and containing supplemented L7™ hPSC Medium. Cells were cultured in a humidified 37° C incubator under normoxic conditions (20.9% O₂, 5% CO₂). To subculture colonies during expansion at P3 and later passages, L7™ hPSC Passaging Solution (Lonza, cat. no. FP-5013) was used according to the product instructions. Some cells (P2) were also manually passaged onto radiation-inactivated mouse embryonic fibroblast (MEF) feeders and continuously cultured in DMEM/F12 media with L-glutamine containing 20% KnockOut™ Serum Replacement, 100 µM non-essential amino acids, 100 µM β-mercaptoethanol (all from Life Technologies, Carlsbad, CA), and 12 ng/ml FGF2 (Stemgent, Cambridge, MA).

**Embryoid Body Formation**

For differentiation into each germ layer by embryoid body (EB) formation, we used our previously reported protocol. Pluripotent cell colonies were harvested and cultured in ultra-low attachment plates with FGF-deficient DMEM/F12 medium with L-glutamine containing 20% KnockOut™ Serum Replacement, 100 µM non-essential amino acids, and 100 µM β-mercaptoethanol (all from Life Technologies, Carlsbad, CA) for 7 days. On day 8, EBs were transferred onto gelatin-coated coverslips and cultured in the same medium for an additional 7 days. EBs were then fixed, permeabilized and incubated with antibodies against biomarkers relevant to the three germ layers.

**Melanocyte Differentiation**

To differentiate hiPSCs into melanocytic derivatives, we used a directed differentiation protocol previously developed by our group. In brief, hiPSC colonies were harvested and cultured in suspension as cell aggregates for 7 days in bFGF-deficient DMEM/F12 medium with L-glutamine containing 20% KnockOut™ Serum Replacement, 100 µM non-essential amino acids, and 100 µM β-mercaptoethanol (bFGF-deficient hESC medium; all components from Life Technologies, Carlsbad, CA). Cell aggregates were then plated onto plates coated with PBS containing 10 µg/ml human fibronectin (BD Biosciences, San Jose, CA) and cultured in the MelDiff medium which consists of MelM basal medium (ScienCell Research Laboratories, Carlsbad, CA), 4 ng/ml bFGF (Stemgent, San Diego, CA), 20 µM cholera toxin (Enzo Life Sciences, Plymouth Meeting, PA), 50 ng/ml human Wnt3a (R&D Systems, Minneapolis, MN), 50 ng/ml human stem cell factor (SCF; R&D Systems, Minneapolis, MN), 0.1 µM endothelin-3 (Sigma-Aldrich, St. Louis, MO), 100 µM L-ascorbic acid (Sigma-Aldrich, St. Louis, MO), 1x melanocyte growth supplement (ScienCell Research Laboratories, Carlsbad, CA) and 1x insulin-transferin-selenium solution (Life Technologies, Carlsbad, CA) for 7 additional days. On day 15, the cells were further propagated and matured by culturing them in MelDiff medium containing 1 µM α-MSH (Sigma-Aldrich, St. Louis, MO) for 14 more days. At the end of differentiation (around day 30), cells were maintained in MelM medium (ScienCell Research Laboratories, Carlsbad, CA).

**Fluorescence Staining**

For staining hiPSCs, EBs and melanocyte differentiation derivatives, cells were rinsed with PBS and then fixed in PBS containing 4% paraformaldehyde for 25 minutes, permeabilized in PBS containing 0.2% Triton X-100 and incubated with antibodies against specific biomarkers. The primary antibodies used here were purchased from Cell Signaling (P0U5F1), Milipore (NANOG and SMA), R&D Systems (SSEA4 and SOX17), Covance (TUBB3), Thermo Scientific (MITF), and Santa Cruz (MART-1/Melan-A).
Results and Discussion

Prior investigations attempting to reprogram either dermal fibroblasts or epithelial cells from individual #418, using traditional virus-mediated transductions (Sendai virus and retrovirus), were unsuccessful.

The Lonza L7™ hiPSC Reprogramming and hPSC Culture System was proved a useful tool in overcoming certain reprogramming hurdles during the generation of hiPSCs for individual #418. The system yielded hiPSC-like colonies 6 days after transfection using Nucleofector™ Technology. These hiPSC-like cells displayed typical morphologies of hPSCs in both feeder and feeder-free culture (Figure 2). These cells were positive of many biomarkers for cellular pluripotency including NANOG, POU5F1 and SSEA4 (Figure 3). In addition, the hiPSCs (PBMC418iPS1506 cells) generated from individual #418 PBMCs can be differentiated into cell types belonging to all three germ layers (Figure 4) and also successfully differentiated into melanocytes (Figure 5).

Figure 2  Reprogramming PBMCs isolated from the blood sample of individual #418. 7 days after Nucleofection (D15), small but iPSC-like cell colonies (indicated by an arrowhead) can be observed. PBMC418iPS1506 hiPSCs showed typical morphologies of hPSCs in feeder and feeder-free culture.

Figure 3  PBMC418iPS1506 cells were positively stained with biomarkers (NANOG, POU5F1 and SSEA4) for cellular pluripotency.

Figure 4  Differentiation of PBMC418iPS1506 cells into cell types relevant to three germ layer lineages through embryoid body formation. Distinct cell types were clearly differentiated as demonstrated by germ layer-specific gene expression: TUBB3 – ectoderm, SMA – mesoderm, SOX17 – endoderm.

Figure 5  Directed differentiation of PBMC418iPS1506 hiPSCs into melanocytic cells. The differentiated derivatives (PBMC418iPS1506_Mel Diff) expressed melanocytic biomarkers MITF and MART-1.
Conclusion

Here we have shown that the system devised by Lonza effectively reprogrammed PBMCs via episomal vector-mediated transfection, leading to the generation of viable hiPSCs. Pluripotency of the hiPSCs was maintained as demonstrated by subsequent non-directed differentiation (EB formation) and directed differentiation (melanocyte differentiation). Regenerative medicine and the reprogramming of somatic cells are research fields still very much in their infancy, and the generation and culture of consistently robust and high quality hiPSCs represents an ongoing challenge to stem cell biologists. However, human pluripotent stem cells show great promise in numerous research and clinical applications, and thus new developments in technology continue to drive the research forward. For example, our recent work has shown that functional melanocytes can be successfully differentiated from transgene-free hiPSCs\(^{10}\), providing a reliable protocol for generating unlimited numbers of melanocytes that could be used for modeling physio-pathological development of melanocytes and for providing material for transplantation.

hiPSCs open up multiple opportunities for the development of novel therapies through the exploration of disease mechanisms and novel therapeutic targets, as well as the development of drug screening platforms. The Lonza L7™ hiPSC Reprogramming and hPSC Culture System represents a reliable and robust tool for the generation of hiPSCs, which successfully overcame the “reprogramming-resistance” challenge from a specific case (individual #418) presented in our study. The L7™ hiPSC Reprogramming and hPSC Culture System is likely to be a complete and inclusive approach for hiPSC-generation that streamlines the process of somatic cell reprogramming.

References