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Impact of Passaging Method on iPSC Quality During Early Clonal Establishment

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ABSTRACT

Sendai viral-based reprogramming has emerged as the gold standard for the generation of induced pluripotent stem cells (iPSC) from diverse somatic cell sources including hard-to-manipulate blood-derived cells. As iPSC progress towards translational application, there is a need to establish best practices that ensure consistent quality. This requires robust and streamlined methods for clonal establishment of iPSC that are viable, pluripotent, and foot-print free.
In this study, iPSC were generated from both fibroblasts and blood-derived cells using the Sendai-virus based CytoTune 2.0 Reprogramming kit in Essential 8 media system. At least 5 clones respectively from each reprogramming were isolated and subjected to either traditional manual passaging or bulk passaging with EDTA. Cell survival and morphology for each clone was monitored up to passage 10. Additionally, RNA was collected at alternate passages to monitor viral clearance. Clones passaged manually showed better survival and morphology compared to bulk passaged clones. Further, majority of clones were footprint-free, albeit the manual passaged clones had faster rate of viral clearance. On average, manually passaged clones also had better survival and morphology during clonal expansion. These results indicate manual passaging to be a better method for early clonal establishment. Given the tedious nature of this method, alternate approaches are being currently explored that are less labor-intensive that produce high quality iPSC clones. This is important for rapid, large-scale generation of iPSC from diverse sources.

INTRODUCTION

Embryonic stem cells (ESCs) are suitable to treat various diseases, but the ethical considerations make their use a potential challenge (1). Another way to avoid these issues is to generate cells that show a pluripotent stem cell–like behavior identical to embryonic stem cells from patient's own cells. Such cells identified as induced pluripotent stem cells (iPSC) possess characteristics of giving rise to numerous diverse cell types like embryonic stem cells (ESC), and to be derived from various somatic cell types.
The process of iPSC generation via somatic cell reprogramming starts with the introduction of four transcription elements, Oct3/4, Sox2, Klf4, and c-Myc, also known as the similar fundamental elements of pluripotency in other undifferentiated cells that are capable to give rise to other cells like ESC (2). Since this process was first reported by Yamanaka in 2006 (1), various methods have been used to deliver transcription factors into somatic cells to initiate the reprogramming process. Traditional methods rely on retrovirus or lentivirus that integrates into the host's genome, thus making them not ideal for clinical use (3). Nonintegrational methods such as episomal vectors, RNA based Sendai virus and synthetic RNA are appealing alternatives. The Sendai virus based reprogramming method is particularly appealing for its ability to transduce a wide variety of cells under different conditions for successful generation of iPSC.
After somatic cells transduction, Sendai virus backbone is diluted out with cell proliferation. Since foot-print free iPSC are critical to the quality of the stem cell banks and to their downstream applications, it is essential to establish methods that ensure complete viral clearance and better cell survival during early clonal establishment of iPSC.
Induced pluripotent stem cells were generated from fibroblasts using Sendai virus based reprogramming kits Cytotune®-iPS 2.0. More than five of the best emerging induced pluripotent stem cells clones were selected and expanded using clonal versus bulk expansion methods to monitor cell survival and morphology. Samples were collected at every second passage from the second to the tenth passage for total RNA extraction. The presence of Sendai virus vectors backbone was monitored relative to a house keeping gene with RT-qPCR using TaqMan Reagents. Greater cells survival and faster viral clearance was observed by the tenth passage with most clones expanded clonally using manual manipulation compared to the bulk passaged using non-enzymatic method. Thus, methods supporting faster viral clearance and better cells survival are critical for high quality iPSC generation that are suitable for use in downstream translational applications.

MATERIALS AND METHODS

Cyropreserved Gibco human dermal adult fibroblasts (cat#C0135C) were thawed and seeded in fibroblast medium in T-75 treated culture flask in accordance with fibroblast culture protocol.

Reprogramming Fibroblasts Using Cytotune 2.0
Human dermal adult fibroblasts were reprogrammed using Invitrogen™ CytoTune® -iPS 2.0 Sendai Reprogramming Kits. The virus was removed one day after transduction and transduced cells were replated onto vitronectin (VTN) coated plates in two seeding densities of 5.0×10^3 cells/cm^2 and 7.5×10^3 cells/cm^2, and fed with Essential 8 Medium every day until Day 21.

iPSC Expansion and sample Collection
At least five of the best emerging clones at day 21 were picked and expanded using manual passaging method for clonal expansion and EDTA for bulk expansion. Samples were collected from both expansion methods using TRIzol® Reagents and stored at -80 °C until use.

RNA Preparation
RNA was extracted from sample using cholroform phase separation technique. Any residual DNA present in the RNA sample was removed with Deoxyribonuclease I, Amplification Grade (Cat# 18068-015). The concentration of the total RNA was measured using Thermo Fisher Scientific Nano Drop, and its integrity was assessed with 2% agarose gel electrophoresis.

cDNA Synthesis and SeV backbone Quantification with Rt-qPCR
cDNA was synthesized from 1ug of DNAse treated RNA using High-Capacity cDNA Reverse Transcription Kit (Part # 4368814).
RNA extracted from samples was measured for the presence of Sendai virus vector backbone in a real time PCR reactions for Sendai virus relative to β actin (housekeeping gene) using TaqMan® gene expression assays. The cycle threshold (C_T) values obtained fro RT-qPCR were analyzed and the Relative quantification (RQ) was determined and plotted using excel file.

RESULTS

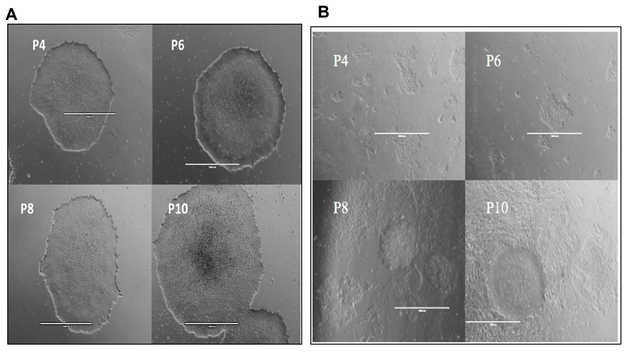
Table 1. Clones passaged manually show better survival compared to bulk passaged clones

CytoTune 2.0 E8/VTN		Passaging Method															
		Manual									EDTA						
Users	Clones	P2	P3	P4	P5	P6	P7	P8	P9	P10	P2	P3	P4	P5	P6	P7	P8	P9	P10
User 1 (HDFa 286)	6																		
	7																		
	8																		
	9																		
	10																		
User 2 (HDFa 286)	1																		
	2																		
	3																		
	4																		
	5																		
User 3 (HDFa 193)	1																		
	2																		
	3																		
	4																		
	5																		

< 20% differentiation
> 40% differentiation
Repicked from previous passage
Culture crashed/lost
Sample not collected

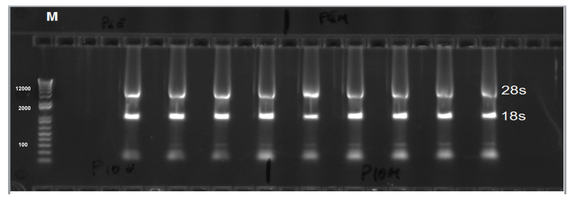
Comparison of both passaging methods used for CytoTune 2.0 derived iPSC early clonal establishment. Cells were grown on Essential 8 (E8) media system. Green represents clones with less than 20% differentiation; while yellow symbolizes clones with more than 40% differentiation. Lost/crashed culture is marked with red color. Clones repicked from previous manually passaged culture are shown in orange. Samples not collected are represented in gray. HDFa 286 and HDFa 193 represent the cell lines. User 1 was new with less than one year experience, user 2 was more proficient with more than one year experience, and user 3 is an expert with more than two years of experience.

Figure 1.iPSC clones passaged manually show less differentiated morphology than bulk passaged clones



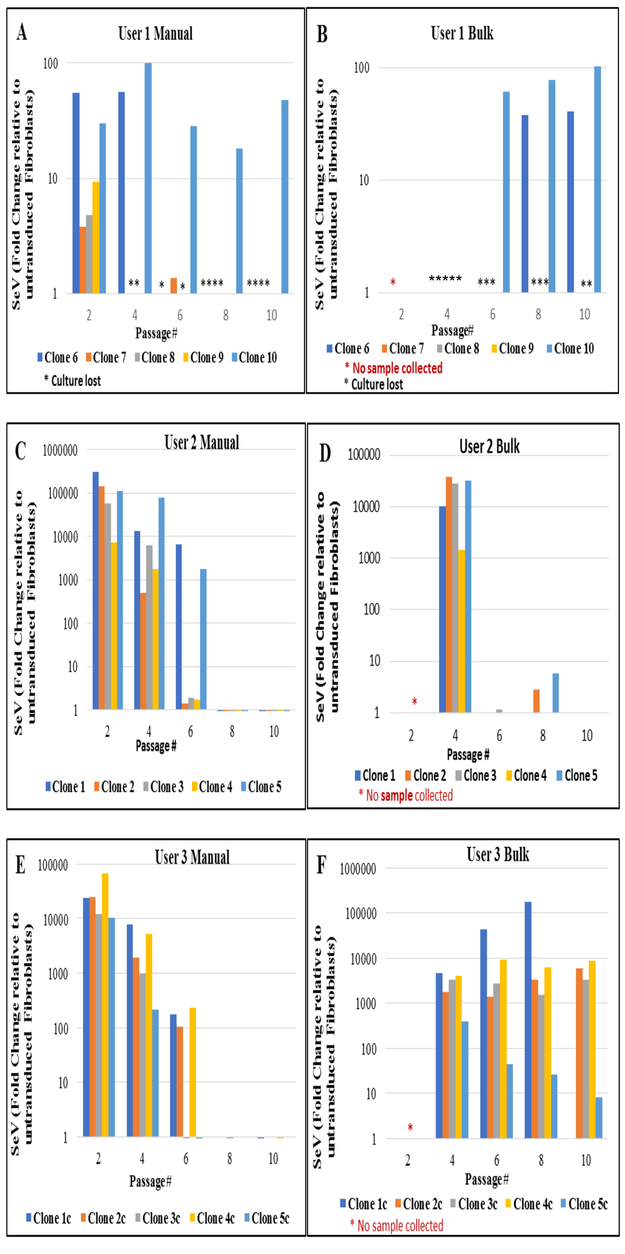
Visual image of the morphology of iPSC passaged using traditional manual expansion method (**A**) at P4, P6, P8, P10 showing compact colonies with well-defined edge, compared to of iPSC passaged using EDTA expansion method (**B**) at P4, P6, P8 and P10 where clones are more diffuse, lack defined edges, and appear along side large individual cells.

Figure 2. 2% agarose gel electrophoresis with 28S and 18S bands indicating high integrity of the RNA.



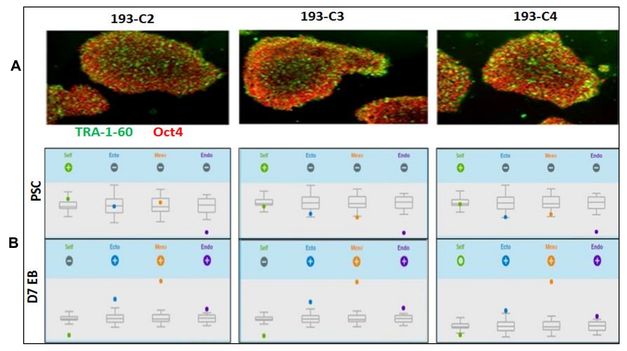
The integrity of the total RNA was assessed with 2% agarose Gel electrophoresis. The appearance of intact 28S and 18S bands with light smear of the different size mRNA transcripts indicate lack of RNA degradation. 28S and 18s are ribosomal RNA subunits used to assess the integrity of RNA. The marker lane is indicated by letter M.

Figure 3. Greater number of manually passaged clones are SeV free by passage 10 compared to bulk passaged clones.



qPCR results of RNA samples from 3 users. **A**, **C** and **E** represent the traditional manual method while **B**, **D** and **F** are EDTA passaging for bulk expansion. Each user collected samples from 5 clones in both manual and clonal expansion methods. User 1 shown in figure (**A** and **B**) experienced considerable loss of culture, leading to missing data points. The "Y" axis represents Sendai virus backbone fold change relative to untransduced fibroblasts serving as control, and the "X" axis is the passage number. Individual clone SeV footprint pattern is represented by each colored graph.

Figure 4. iPSC clones with residual SeV expressing PSC markers and trilineage differentiation .



Immunocytochemistry of iPSC with the presence of the residual SeV revealed the expression of the pluripotent stem cell marker TRA-1-60 in green and Oct4 in red (**A**). Embroid bodies were generated from clones with the presence of residual SeV and tested for gene expression at Day 7 using RT-qPCR. These clones were able to demonstrate their pluripotency in vitro by spontaneously differentiating into the three germ layers: ectoderm, mesoderm and endoderm (**B**).

CONCLUSIONS

Traditional manual passaging method for clonal expansion offers better cell survival rates and faster SeV clearance in emerging iPSC, compared to the EDTA technique used for bulk expansion.

Manual passaging is the method of choice for early clonal establishment, but is not practical for large scale expansion of iPSC. This demonstrate the need for consistent bulk expansion methods.

SeV clearance rate is clone dependent.

Experience level and good practice of aseptic techniques have a significant impact on cell survival, growth and differentiation.

iPSC clones with the presence of residual SeV were able to express pluripotent markers and to spontaneously differentiate into the three germ layers in vitro.

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