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Efficient iPS Cell Generation from Blood Using Episomes and HDAC Inhibitors

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Abstract:	This manuscript illustrates a protocol for efficiently creating integration-free human induced pluripotent stem cells (iPSCs) from peripheral blood using episomal plasmids and histone deacetylase (HDAC) inhibitors. The advantages of this approach include: (1) the use of a minimal amount of peripheral blood as a source material; (2) non-integrating reprogramming vectors; (3) a cost-effective method for generating vector-free iPSCs; (4) a single transfection; and (5) the use of small molecules to facilitate epigenetic reprogramming. Briefly, peripheral blood mononuclear cells (PBMCs) are isolated from routine phlebotomy samples and then cultured in defined growth factors to yield a highly proliferative erythrocyte progenitor cell population that is remarkably amenable to reprogramming. Non-integrating, non-transmissible episomal plasmids expressing OCT4, SOX2, KLF4, MYCL, LIN28A, and a p53 short hairpin (sh)RNA are introduced into the derived erythroblasts via a single nucleofection. Co-transfection of an episome that expresses enhanced green fluorescent protein (eGFP) allows for easy identification of transfected cells. A separate replication-deficient plasmid expressing Epstein-Barr nuclear antigen 1 (EBNA1) is also added to the reaction mixture for increased expression of episomal proteins. Transfected cells are then plated onto a layer of irradiated mouse embryonic fibroblasts (iMEFs) for continued reprogramming. As soon as iPSC-like colonies appear at about twelve days after nucleofection, HDAC inhibitors are added to the medium to facilitate epigenetic remodeling. We have found that the inclusion of HDAC inhibitors routinely increases the generation of fully reprogrammed iPSC colonies by 2 fold. Once iPSC colonies exhibit typical human embryonic stem cell (hESC) morphology, they are gently transferred to individual

	iMEF-coated tissue culture plates for continued growth and expansion.
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To the JoVE Editorial Board:

Induced pluripotent stem cells (iPSC) hold tremendous promise for use in regenerative medicine, disease modeling, and gene therapy applications. However, the reprogramming process is still inefficient, and robust protocols are needed-- especially when working with precious patient samples. Here, we describe our protocol for efficient iPSC generation in a manuscript entitled **"Efficient iPS Cell Generation from Blood Using Episomes and HDAC Inhibitors."** The advantages of our protocol include: (1) the use of a minimal amount of peripheral blood; (2) non-integrating reprogramming vectors; (3) a cost-effective method for generating vector-free iPSCs; (4) a single transfection; and (5) the use of small molecules to facilitate epigenetic reprogramming. We have used this protocol successfully to efficiently generate iPSC for disease modeling experiments from individuals as old as 87 years of age. We believe that sharing our protocol for iPSC generation using the JoVE format will be very useful for the scientific community.

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- Hubbard, Jesse J: conception and design of protocol, performed experiments, wrote and edited the manuscript;
- Sullivan, Spencer K: conception and design of protocol, editing of manuscript;
- Mills, Jason A: substantial contribution to conception and design of protocol;
- Hayes, Brian: substantial contribution to conception and design of protocol, performed experiments, editing of manuscript;
- Torok-Storb, Beverly J: writing and editing of manuscript, provided research support;
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TITLE: Efficient iPS Cell Generation from Blood Using Episomes and HDAC Inhibitors

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KEYWORDS: *Induced pluripotent stem cells, iPSC, iPSC generation, human, HDAC inhibitors, histone deacetylase inhibitors, reprogramming, episomes, integration-free*

SHORT ABSTRACT:
Here we describe a protocol for generating human induced pluripotent stem cells from peripheral blood using an episome-based reprogramming strategy and histone deacetylase inhibitors.

LONG ABSTRACT:
This manuscript illustrates a protocol for efficiently creating integration-free human induced pluripotent stem cells (iPSCs) from peripheral blood using episomal plasmids and histone deacetylase (HDAC) inhibitors. The advantages of this approach include: (1) the use of a minimal amount of peripheral blood as a source material; (2) non-integrating reprogramming vectors; (3) a cost-effective method for generating vector-free iPSCs; (4) a single transfection; and (5) the use of small molecules to facilitate epigenetic reprogramming. Briefly, peripheral blood mononuclear cells (PBMCs) are isolated from routine phlebotomy samples and then cultured in defined growth factors to yield a highly proliferative erythrocyte progenitor cell population that is remarkably amenable to reprogramming. Non-integrating, non-transmissible episomal plasmids expressing *OCT4*, *SOX2*, *KLF4*, *MYCL*, *LIN28A*, and a p53 short hairpin (sh)RNA are

introduced into the derived erythroblasts via a single nucleofection. Co-transfection of an episome that expresses enhanced green fluorescent protein (eGFP) allows for easy identification of transfected cells. A separate replication-deficient plasmid expressing Epstein-Barr nuclear antigen 1 (*EBNA1*) is also added to the reaction mixture for increased expression of episomal proteins. Transfected cells are then plated onto a layer of irradiated mouse embryonic fibroblasts (iMEFs) for continued reprogramming. As soon as iPSC-like colonies appear at about twelve days after nucleofection, HDAC inhibitors are added to the medium to facilitate epigenetic remodeling. We have found that the inclusion of HDAC inhibitors routinely increases the generation of fully reprogrammed iPSC colonies by 2 fold. Once iPSC colonies exhibit typical human embryonic stem cell (hESC) morphology, they are gently transferred to individual iMEF-coated tissue culture plates for continued growth and expansion.

INTRODUCTION:

iPSCs are derived from somatic tissues via ectopic expression of a minimal set of pluripotency genes. This technique was initially demonstrated by retroviral transduction of human fibroblasts with *OCT4*, *SOX2, KLF4,* and *cMYC*, which are highly expressed in the pluripotent state[1]. These transiently expressed "reprogramming factors" alter the target cell's epigenetic landscape and gene expression profile analogous to human embryonic stem cells[2]. Once created, iPSCs can potentially be differentiated into any tissue type for further investigation. Thus, they hold promise for use in regenerative medicine, disease modeling, and gene therapy applications. However, disrupting the genome with integrating viruses has the potential to alter endogenous gene expression, influence cellular phenotype, and ultimately bias scientific results. Furthermore, random viral integrations can lead to deleterious cellular effects, including the possibility of malignant transformation[3] or re-expression of the oncogenic transgenes[4]. Future clinical applications will require non-integrating iPSC generation.

Episomes, which are extra chromosomal circular DNA molecules, offer a strategy to generate cost-effective, integration-free iPSCs[5]. The combination of episomal vectors shown in Table 1 express the reprogramming factors *OCT4, SOX2, KLF4, MYCL*, and *LIN28A*. The *pCXLE_hOCT3/4-shp53-F* plasmid also contains a p53 shRNA for temporary suppression of *TP53* to enhance cellular reprogramming[6]. The replication-deficient *pCXWB-EBNA1* vector promotes amplification of reprogramming factors and increased reprogramming efficiency by providing a transient increase in *EBNA1* expression[7]. The pCXLE_EGFP plasmid can be added to the nucleofection mixture for the purpose of determining the transfection efficiency or for cell-sorting applications. With the exception of p*CXWB-EBNA1*, the episomal plasmids used in this protocol contain the Epstein-Barr virus origin of viral replication and *EBNA1* gene, which mediate replication and partition of the episome during division of the host cell[8]. The episomes are spontaneously lost with successive iPSC expansion[7]. Subcloning and characterization of iPSCs with episomal vector loss, which can be inferred from loss of eGFP expression, can lead to completely integration-free iPSCs for future clinical applications.

Inherent to the process of iPSC generation is suppression of lineage-specific genes and re-activation of pluripotency-associated genes. Regulation of gene expression occurs at multiple levels within the nucleus, including modifications to DNA and chromatin to allow transcription

factors, regulatory DNA elements, and RNA polymerase access to target genes. Remodeling of the epigenetic landscape via global chromatin modifications is a key component to re-expression of the pluripotency genetic program. A specific chromatin modification that is important in regulation of gene expression is acetylation of histones at particular lysine residues, which allows access to target genes through decreased tension of the histone-DNA coil. HDAC inhibitors are small molecules that have been shown to enhance iPSC reprogramming and hESC self-renewal[9,10], likely due to supporting the acetylated state[11]. The protocol described below, adapted from a prior publication using integrating lentiviruses[12], provides a step-by-step method for optimized iPSC generation from peripheral blood using episomes and HDAC inhibitors. The HDAC inhibitor concentrations used here are half of those described by Ware, et al[9], and have routinely led to a 2 fold increase in fully reprogrammed iPSC colonies over standard episomal reprogramming protocols without HDAC inhibitors. This level of reprogramming is on par with the efficiency we observe with lentiviral methods. Using this protocol, we have efficiently generated iPSC from individuals as old as 87 years of age.

PROTOCOL:

Written informed consent, as approved by the Institutional Review Boards of the Fred Hutchinson Cancer Research Center and the Children's Hospital of Philadelphia, was obtained from patients before collecting peripheral blood samples. All institutional guidelines were observed. All animal experiments including MEF generation and teratoma formation were approved by the Institutional Animal Care and Use Committee.

1. Ficoll Separation of PBMCS and Expansion of Erythroblasts – Day 0

1.1) Dilute peripheral blood 1:1 with Dulbecco's Phosphate Buffered Saline (DPBS). In a 15 mL round-bottom polystyrene tube, carefully layer 7 mL of diluted blood onto 3 mL of room temperature Ficoll-Hypaque.

1.2) Centrifuge the sample at 400 x g for 30 minutes.

1.3) The PBMCs will have settled to the interface between the plasma and the Ficoll-Hypaque, seen as a cloudy white layer. Using a sterile transfer pipette, collect the PBMCs into one 15 mL conical tube. Bring the volume to 10 mL using DPBS.

1.4) Using a hemacytometer, count the PBMCs.

1.5) Pipette 2×10^6 PBMCs into a separate 15 mL conical tube and centrifuge at 300 xg for 5 minutes. Spin down the remaining cells and freeze at a concentration of 2×10^6 PBMCs/mL in 90% fetal bovine serum (FBS)/10% dimethyl sulfoxide (DMSO).

1.6) Prepare Expansion Medium: QBSF-60 serum-free medium (protect from light) + penicillin/streptomycin (1%) + ascorbic acid (50 ng/mL) + stem cell factor (SCF, 50 ng/mL) +

interleukin 3 (IL-3, 10 ng/mL) + erythropoietin (EPO, 2 U/mL) + insulin-like growth factor 1 (IGF-1, 40 ng/mL) + dexamethasone (1 µM; protect from light; thaw a fresh aliquot each media change).

1.7) Resuspend 2 × 10^6 PBMCs in 2 mL of freshly made Expansion Medium and place into one well of a 12-well tissue culture plate and incubate in a 37°C humidified incubator with an atmosphere of 5% O_2, 5% CO_2, and 90% N_2.

1.8) On Day 3 and Day 6, replace media.

1.8.1) Collect the cells and wash the well with 2 mL of QBSF-60 to collect any remaining cells.

1.8.2) Centrifuge the cell suspension at 300 xg for 5 minutes and aspirate the supernatant.

1.8.3) Resuspend the cells in 2 mL of new Expansion Medium and return them to the same 12-well plate.

2. Reprogramming Cells by Nucleofection – Day 9

2.1) Pipette the reprogramming plasmids into a sterile 1.5 mL Eppendorf tube (Table 1).

2.2) Mix the nucleofection solution with Supplement (supplied by manufacturer) and pipette into a sterile 1.5 mL Eppendorf tube.

2.3) Pipette 2 mL of Expansion Medium into one well of a 12-well plate and place in a humidified 37°C incubator (5% O_2, 5% CO_2, and 90% N_2) for equilibration prior to adding cells.

2.4) Collect the cultured cells and wash the well with 2 mL of QBSF-60 to collect remaining cells.

2.5) Centrifuge the cells at 300 x g for 5 minutes and aspirate the supernatant.

2.6) Wash the cells by resuspending in 5 mL of DPBS and centrifuging at 300 xg for 5 minutes.

2.7) Aspirate the supernatant.

2.8) Resuspend the cell pellet in 100 µL of nucleofection solution + Supplement, taking care to avoid generating bubbles.

2.9) Pipette the cell suspension into the tube containing the reprogramming plasmids, pipette up and down once, and pipette the sample into the bottom of the nucleofection cuvette.

2.10) Place the cuvette into the nucleofection machine and run Program T-019.

2.11) Transfer 100 µL of the pre-warmed Expansion Medium (from the equilibrating plate in Step 2.3) into the nucleofection cuvette.

2.12) Immediately collect the entire sample and deposit into the well of pre-warmed Expansion Medium. Avoid collecting the white, floating dead cell debris.

2.13) Place the plate in a humidified 37°C incubator (5% O_2, 5% CO_2, and 90% N_2) for growth.

3. Plating Feeder Cells – Day 10 OR 11

3.1) On Day 10, plate feeder cells.

3.1.1) Coat one 6-well tissue culture plate with gelatin.

3.1.1.1) Pipette 1 mL of 0.1% gelatin in Hank's Buffered Saline Solution (HBSS) into each well of the plate.

3.1.1.2) Place the plate in a humidified 37°C incubator for at least 5 minutes.

3.1.1.3) Immediately before use, aspirate the gelatin solution from the plate.

3.1.2) Thaw one vial of 2×10^6 mouse embryonic fibroblasts irradiated at 3000 cGy into 10 mL of pre-warmed MEF Media (Dulbecco's Modified Eagle Medium (DMEM) + FBS (10%) + penicillin/streptomycin (1%)).

3.1.3) Centrifuge the cells at 300 xg for 5 minutes and aspirate the supernatant. Resuspend the cells in 12 mL of MEF Media and pipette 2 mL into each well of the gelatin-coated 6-well plate. Place the plate in a humidified 37°C incubator (5% O_2, 5% CO_2, and 90% N_2) until Day 12.

4. Plating Cells on Feeder Cells and Changing Medium – Day 12

4.1) Collect the nucleofected cells into one 15 mL conical tube and collect any remaining cells by washing the well with 2 mL of QBSF-60. Centrifuge the sample at 300 xg for 5 minutes.

4.2) Prepare Reprogramming Medium: Iscove's Modified Dulbecco's Medium (IMDM) + FBS (10%) + non-animal L-glutamine (1 mM) + non-essential amino acids (NEAA, 1%) + penicillin/streptomycin (1%) + β-mercaptoethanol (0.1 mM) + basic fibroblast growth factor (bFGF, added fresh at feeding, 10 ng/mL) + ascorbic acid (added fresh at feeding, 50 µg/mL).

4.3) Aspirate the supernatant and resuspend the cells in 12 mL of Reprogramming Medium. Pipette 2 mL per well of the cell suspension into the 6-well plate of feeder cells (as prepared in Section 3). Centrifuge the plate at 500 rpm for 30 minutes at room temperature.

4.4) Place the plate in a humidified 37°C incubator (5% O_2, 5% CO_2, and 90% N_2) for growth.

Change the Reprogramming Medium every other day until iPSC colonies begin appearing (1-2 weeks).

4.5) Prepare hESC Medium: DMEM/F12 1:1 + Knockout Serum Replacement (20%) + penicillin/streptomycin (1%) + NEAA (1%) + non-animal L-glutamine (1 mM) + sodium pyruvate (1%) + sodium bicarbonate (7.5%) + β-mercaptoethanol (0.1 mM) + bFGF (added fresh at feeding, 10 ng/mL)

4.6) Prepare HDAC inhibitors: sodium butyrate (added fresh at feeding, 100 µM), suberanilohydroxamic acid (SAHA, added fresh at feeding, 200 nM). Once iPSC colonies appear, begin feeding the cells with hESC Medium + HDAC inhibitors, changing medium every day.
Note: Sodium butyrate is a small molecule that is adsorbed to plastic tubes, therefore store it in a borosilicate glass tube at 4°C.

5. Isolating iPSC Clones

5.1) Once iPSC colonies are large enough to be isolated (20-25 cells), pick them with a P20 pipette.

5.2) Prepare HDAC inhibitors: sodium butyrate (added fresh at feeding, 100 µM), SAHA (added fresh at feeding, 200 nM)

5.3) Place the isolated colonies onto iMEFs in individual 35 mm dishes containing hESC Medium + HDAC inhibitors + Cloning & Recovery Supplement.

5.4) The day after picking the colonies, change the medium to hESC Medium + HDAC inhibitors.

5.5) Change the medium every day until iPSC colonies are ready for passaging.

5.6) Remove HDAC inhibitors from the medium once colonies stabilize at passage 2-3.

REPRESENTATIVE RESULTS:
Three days after nucleofection and before plating the nucleofected cells onto iMEFs, the efficiency of successful nucleofection should be estimated by fluorescence microscopy for eGFP. Figure 1 shows a typical nucleofection experiment with approximately 5-10% of the total cell population expressing eGFP.

Reprogrammed iPSC colonies will begin to appear approximately two weeks after nucleofection. The colonies are generally circular with well-defined borders and may be identified by characteristic hESC morphology including (1) small, tightly packed cells with a high nuclear-cytoplasmic ratio and (2) visible nucleoli (Figure 2, Bright field). Incompletely reprogrammed cells will either deteriorate or form heterogeneous cell masses that are easily distinguished from true iPSC colonies.

Of the representative lines that we have chosen to characterize completely, all express the standard pluripotency markers (Figure 2), reactivate endogenous pluripotency genes (Figure 2), and form teratomas when injected into NOD-SCID mice.

Figure Legends:

Figure 1: Nucleofected Cells. Stimulated PBMCs three days after nucleofection with plasmids containing *OCT4, SOX2, KLF4, MYCL*, *EBNA1*, p53 shRNA, and eGFP (Scale bar is 100 μm).

Figure 2: IPSC Characterization. IPSC generated using this method exhibit hESC-like morphology when grown on iMEF (Bright field), test positive for alkaline phosphatase activity (AP), and are positive by immunohistochemistry for pluripotency markers TRA-1-60, TRA-1-81, and SSEA4. Additionally, endogenous expression of the pluripotency genes Oct4 (O), Sox2 (S), Klf4 (K), and cMyc (M) is detectable by RT-PCR. (All scale bars are 100 μm).

Table 1: Optimal Ratio of Reprogramming Plasmids[6]. These plasmids are available to order through Addgene.

DISCUSSION:

For successful iPSC generation when using this protocol, there are several important caveats that should be considered. During the erythroblast expansion stage, the media change schedule should be strictly followed, as deviations may lead to inefficient stimulation of the target progenitor cell population and a lower efficiency of iPSC generation. It is important to make new expansion medium with fresh dexamethasone with each media change; and the QBSF-60 base medium and dexamethasone should be protected from light during storage. During nucleofection, the DPBS wash is critical to remove excess solutes from the cell suspension that may cause the electric current from the nucleofector to arc, resulting in widespread cell death. Approximately 10 days after nucleofection, the plate should be examined daily for iPSC colony appearance. Once several small colonies are present, the Reprogramming Medium should be changed to hESC Medium (with HDAC inhibitors) as prolonged exposure to serum may induce spontaneous differentiation.

Depending on laboratory set-up and available resources, modifications to the protocol may be considered. We prefer a low-O_2 incubator because hypoxic conditions have been shown to increase the efficiency of iPSC generation[13]; however, we have had success generating iPSCs from peripheral blood using normoxic conditions with 5% CO_2. Therefore, a standard humidified CO_2 incubator can also be used. The MEFs used in this protocol may be purchased from a life science vendor. However, due to commercial batch variability, we prefer to generate iMEFs from CF-1 mice following a published protocol for MEF isolation, culture, and irradiation[14]. The inclusion of HDAC inhibitors is a favorable aspect of this protocol as epigenetic remodeling is a crucial aspect of cellular reprogramming[9,15]. HDAC inhibitors may be omitted, however the number of fully reprogrammed iPSC colonies will be reduced. Lastly, we have generated iPSCs using this method with a bone marrow sample with no additional modifications needed.

In summary, this protocol uses a plasmid-based reprogramming system that avoids some of the drawbacks of iPSC generation with integrating vectors, including random insertions into the host genome and safety concerns regarding the handling of recombinant virus. Additionally, small-scale virus production and characterization is relatively labor intensive when compared to plasmid generation. Reprogramming efficiency can also vary significantly due to batch variability inherent to virus production, while one large-scale production of quality plasmids can lead to consistent iPSC generation over time. Combined with HDAC inhibitors, this protocol offers an efficient method to generate integration-free and footprint-free iPSCs for stem cell research.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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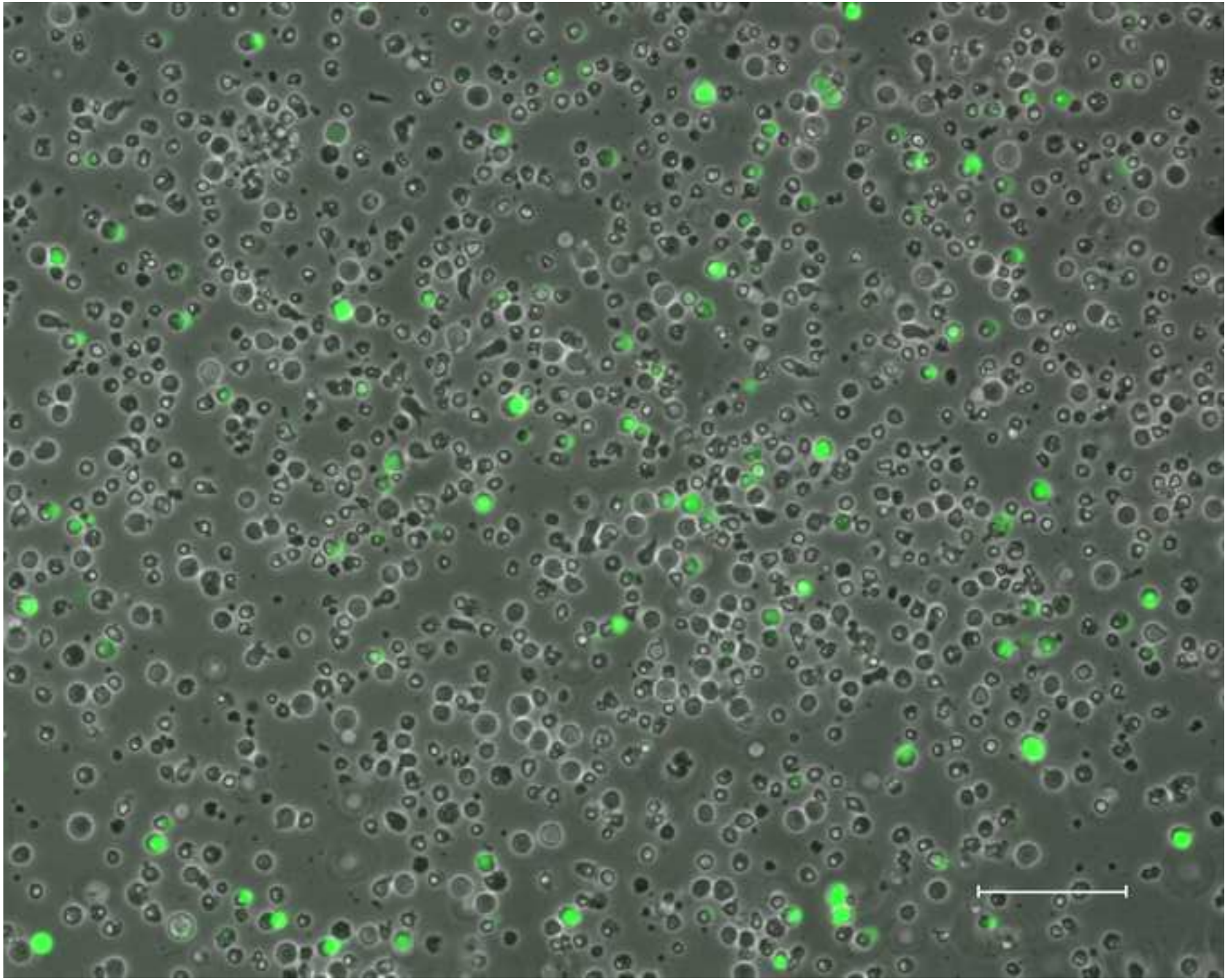

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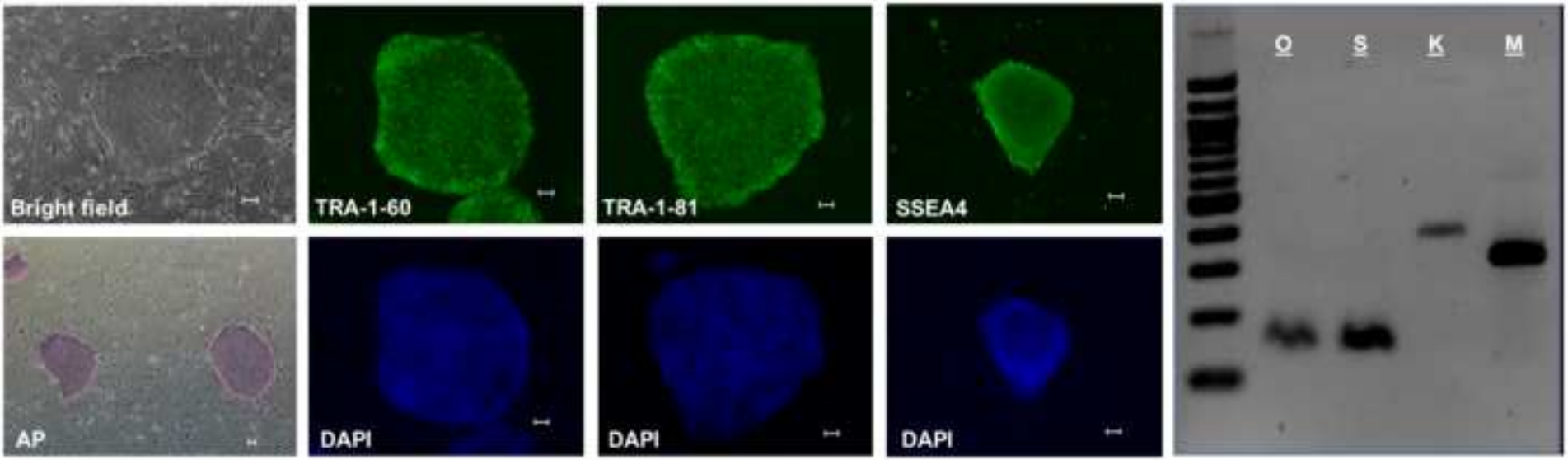

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Click here to download Table: Table 1.xlsx

Episome	Mass per reaction (ng)
pCXLE-hOct3/4-shp53-F	830
pCXLE-hSK	830
pCXLE-hUL	830
pCXLE-EGFP	500
pCXWB-EBNA1	500

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number
Ficoll-Paque PLUS	Fisher Scientific	45-001-750
DPBS	Life Technologies	14190-250
RPMI 1640	Life Technologies	11875-093
fetal bovine serum (FBS)	Life Technologies	10437028
dimethyl sulfoxide (DMSO)	Sigma-Aldrich	154938
QBSF-60 serum-free medium	Fisher Scientific	50-983-234
penicillin/streptomycin	Life Technologies	15140122
Cell Line Nucleofector Kit V	Lonza	VCA-1003
2% gelatin solution	Sigma-Aldrich	G1393
Hank's Balanced Saline Solution (HBSS)	Life Technologies	14175-103
Dulbecco's Modified Eagle Medium (DMEM)	Life Technologies	11965-092
Iscove's Modified Dulbecco's Medium (IMDM)	Life Technologies	12440-061
L-glutamine, 200 mM	Life Technologies	25030-081
non-essential amino acids (NEAA), 100X	Life Technologies	11140-050
DMEM/Ham's F12, 1:1	Fisher Scientific	SH30023.02
KnockOut Serum Replacement	Life Technologies	10828-028
sodium pyruvate, 100 mM	Life Technologies	11360-070
sodium bicarbonate, 7.5%	Life Technologies	25080094
basic fibroblast growth factor (bFGF)	Life Technologies	PHG0263
sodium butyrate	Sigma-Aldrich	B5887-1G
hES Cell Cloning & Recovery Supplement	Stemgent	01-0014-500
ESC-qualified BD matrigel	BD Biosciences	35-4277
StemSpan SFEM	STEMCELL Technologies	9650
ascorbic acid, powdered	Sigma-Aldrich	A4403-100MG
recombinant human stem cell factor (SCF)	R & D Systems	255-SC-010
recombinant human interleukin 3 (IL-3)	R & D Systems	203-IL-010
erythropoietin (EPO)	R & D Systems	287-TC-500
recombinant human insulin-like growth factor 1 (IGF-1)	R & D Systems	291-G1-200
β-mercaptoethanol	Sigma-Aldrich	M7522
dexamethasone	Sigma-Aldrich	D4902-25MG

SAHA (vorinostat)	Cayman Chemical	149647-78-9
12 well tissue culture plate	Fisher Scientific	08-772-29
15 mL conical tube	Sarstedt	62553002
1.5 mL Eppendorf tube	Fisher Scientific	05-408-129
6 well tissue culture plate	Fisher Scientific	08-772-1B
35 mm tisue culture plates	BD Biosciences	353001
10 mL disposable serological pipettes	Fisher Scientific	13-675-20
5 mL disposable serological pipettes	Fisher Scientific	13-675-22
2 mL disposable serological pipettes	Fisher Scientific	13-675-17
20 µL pipette tips, barrier tips	Genessee	24-404
glass Pasteur pipettes	Fisher Scientific	13-678-20D
pipette aid	Fisher Scientific	13-681-15
pCXLE-hOCT3/4-shp53-F	Addgene	27077
pCXLE-hSK	Addgene	27078
pCXLE-hUL	Addgene	27080
pCXLE-EGFP	Addgene	27082
pCXWB-EBNA1	Addgene	37624

Comments/Description
Store at 4°C. Warm to room temperature before use.
Store at 4°C.
Store at 4°C.
Store at -20°C until needed. Thaw, aliquot, store at 4°C.
Store at room temperature.
Store at 4°C.
Store at 4°C.
Store at 4°C.
Store at 4°C, liquify in 37°C water bath before use.
Store at 4°C.
Store at 4°C.
Store at 4°C.
Aliquot, freeze at -20°C.
Store at 4°C, away from light.
Store at 4°C.
Aliquot, freeze at -20°C.
Store at 4°C.
Store at 4°C.
Make 10 mg/mL stock in DPBS, aliquot, freeze at -20°C. Once thawed, store at 4°C.
Make 2000X stock (400 mM) in DPBS, aliquot, freeze at -20°C. Once thawed, store at 4°C.
Store at -20°C until needed. Once thawed, store at 4°C.
Thaw overnight on ice at 4°C, aliquot into pre-chilled tubes using pre-chilled pipette tips. Store at -20°C until needed. Thaw at 4°C, use immedi
Aliquot, freeze at -20°C.
Make 5 mg/mL stock in DPBS, sterile filter, store at 4°C.
Make 100 µg/mL stock in SFEM, aliquot, freeze at -20°C. Once thawed, store at 4°C.
Make 100 µg/mL stock in SFEM, aliquot, freeze at -20°C. Once thawed, store at 4°C.
Make 1000 U/mL stock in SFEM, aliquot, freeze at -20°C. Once thawed, store at 4°C.
Make 100 µg/mL stock in SFEM, aliquot, freeze at -20°C. Once thawed, store at 4°C.
Make 1000X stock (100 mM) in DPBS.
Make 50X stock (50 µM) in DPBS, sterile filter, store at 4°C.

Make 2000X stock (400 mM) in DPBS, aliquot, freeze at -20°C. Once thawed, store at 4°C.

ately.

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Author(s): Hubbard JJ, Sullivan SK, Mills JA, Hayes BJ, Torok-Storb BJ, Ramakrishnan A

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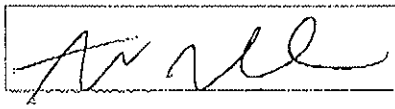
Signature:	 Date: 01/30/14

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April 7th, 2014

Dr. Michelle Kinahan
Science Editor
JoVE

Manuscript title: " Efficient iPS Cell Generation from Blood Using Episomes and HDAC Inhibitors." MS # JoVE52009

Dear Dr. Kinahan:

Please find attached, our revised manuscript to be considered for publication in *JoVE*, the Journal of Visualized Experiments. First, we would like to thank you for obtaining comments from reviewers, as we found their opinions to be helpful. We have expanded both the introduction and discussion to add more detail and clarity about how this protocol is distinguished from previous iPSC generation protocols. Specifically, in response to reviewer 1, we have added statements comparing the efficiency of this iPSC generation protocol compared to episomal reprogramming without HDAC inhibitors and lentiviral mediated reprogramming. Additionally we have eliminated confusing terminology, as requested by reviewer 2, and made the nomenclature similar to previous articles, while indicating in the text the reasons for deviating from published methods. We feel that the characterization requested by reviewer 3 for every line we have generated using this protocol is beyond the budget of the current study and the scope of this manuscript. Never the less we have included a representative example from one of our lines. We hope that we have addressed each of the comments and criticisms, point-by-point responses follow. We have formatted this letter in the following manner: reviewers' comments are italicized, followed by our responses. We hope that you will find the revised manuscript acceptable for publication.

Thank you for your consideration of this manuscript.

Sincerely,

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Editorial comments:

** Please add a scale bar to each of your figures and state the size of the scale bar in the figure legend.*

Scale bars have been added to Figures 1 and 2. The sizes of the scale bars have been listed in the respective legends.

** Please expand your discussion section to focus more on the details of the protocol and cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

The Discussion section has been expanded to more completely include the required components including comparisons to existing protocols and alternative cell sources and equipment.

Reviewers' comments:

Reviewer #1:

Major Concerns:
The only major concern is that there is no measure of efficiency of the process included - especially useful would be an efficiency comparison to a standard lentiviral approach.

The concentration of the HDAC inhibitors when combined together were 50% of those that were described in Ware et al. We have routinely observed a 2 fold increase in fully reprogrammed iPSC colonies over standard episomal reprogramming protocols when HDAC inhibitors are added. While we have not compared every type of retroviral reprogramming vector, we have found this protocol to be as efficient, if not better than a single lentiviral reprogramming cassette.

Minor Concerns:
page 3 - top paragraph - where reference 9 is cited, it should also include reference 11.

Azuara, et al. is now referenced.

protocol 1.3 - from where within the tube are the PBMC collected

We have included the text “The PBMCs will have settled to the interface between the plasma and the Ficoll-Hypaque, seen as a cloudy white layer.”

page 4 - Protocol 2.3 - "hypoxic incubator" is an odd way to describe a 5% O2, 5(?)%CO2, 90% N2 atmosphere - it would be better to say that 37C, humidified incubator had an atmosphere of ..... and from then on in to refer it simply as "incubator" (i.e. in 2.13

We have changed all references to incubators to include the information that we utilize a humidified 37°C incubator with an atmosphere of 5% O2, 5% CO2, and 90% N2.

page 6 - Protocol 4.10 - it should be mentioned that sodium butyrate is a very small molecule that is lost within an Eppendorf tube - and so the stock should be stored in a borosilicate glass tube at 4C.

We have included the text “Sodium butyrate is a small molecule that is adsorbed to plastic tubes, so it should be stored in a borosilicate glass tube at 4°C.”

Figure 2 should include a size bar for reference ("40x magnification" is not as accurate)

Scale bars have been added to Figures 1 and 2. The sizes of the scale bars have been listed in the respective legends.

page 7 - Discussion - 2nd paragraph - (approximately 25-20 cells) should read (approximately 20-25 cells)

We have changed the text to reflect that approximately 20-25 cells comprise colonies.

Additional Comments to Authors:
Overall, this was clearly written and will be easy to follow. Possibly referring to MEF generation through a reference could be less overwhelming for someone completely new to the field, since MEF preparation is only a side technique and many new to the field will choose to buy in pre-generated MEF's

We have added a reference (Abbondanzo, et al.) regarding the generation of iMEFs and have included the information that some investigators prefer to purchase MEFs from a commercial vendor.

Reviewer #2:

Major Concerns:
Several technical differences appear between the protocol presented and the basic protocols used to reprogram human adult cells. These can be explained by the necessary adaptations needed when working on non-adherent, low proliferating cells, but should be addressed and explained, even briefly in the accompanying text.

1. Hypoxia
The authors culture the cells in a hypoxic environment (5%O2) (2.3, 2.13, 4.7). Even though there have been reports showing that hypoxia increases reprogramming efficiency (A7), I am not aware that any of the papers cited by the authors suggest using hypoxia (5%O2, 5%CO2) as a standard reprogramming condition. As most laboratories are not equipped with hypoxic incubators, I suggest that the authors clarify this point in the discussion, and if the authors have experience of using their protocol in normoxic conditions (20%O2, 5%CO2) it should be mentioned.

We prefer to routinely generate iPSC in 5% O2, 5% CO2, and 90% N2, for maximal efficiency but have added text to the discussion to indicate that we have had success, albeit less efficient, generating iPSC in a standard humidified 5% CO2 incubator.

2. Cell Culture media
In agreement with the previous report by Sommer et al., once plated on MEFs, the cells are cultured for one to two weeks in a medium they call 'IPSC medium', which contains

FBS, bFGF and Ascorbic Acid, changed every 2 days. As this medium cannot support IPSC self renewal, to avoid confusion I would suggest not calling this medium "IPSC medium" but instead 'reprogramming medium'. Indeed, in the original report cited by the authors (A1) this medium is called 'MEF medium containing 10 ng/ml bFGF and 50 µg/ml Ascorbic Acid'.

We have modified the text to “Reprogramming Medium.”

As from step 4.11 could you confirm that the hESC medium is :
1. changed every other day, and not daily ?

We have corrected this error.

2. [Medium is] composed of only 15% KOSR instead of 20% ?

In Protocol Section 4.9, an error in the composition of the hESC medium was changed from 15% KOSR to 20%.

3. HDAC inhibitors
In the paper cited by the authors (Ware et al.,) Na-Butyrate is used at 200-300nM but SAHA (Vorinostat) is used at 400nM. It would be useful to add, in the introduction, a short explanation of the experience of the authors in determining the optimal concentrations of HDACi, and notably to mention what results could be expected without HDACi.

The concentration of the HDAC inhibitors when combined together were 50% of those reported in Ware et al.

Minor Concerns:
1. Legends
In the legends 'episome' should be replaced with 'plasmid' (Legend Fig 1, Legend Table 1).

‘Episome’ was changed to ‘plasmid’ in the figure and table legends.

2. Addgene Ref

Addgene plasmids, including their reference numbers, have been added to the Materials Table.

3. MEF
Could the authors precise the source of the MEFs used, and in particular the mouse strain used to generate these MEFs ?
Our own experience shows that CD1 or CF1 mice strains are better sources for feeders used in cell reprogramming than C57BL/6,
and this information would be relevant to users wishing to reproduce the protocol.

We have added a reference (Abbondanzo, et al.) regarding the generation of iMEFs and have included the information that some investigators prefer to purchase MEFs from a commercial vendor.

4. Insertional mutagenesis
In the introduction the authors state, quite truly, that « random viral integrations can lead to deleterious cellular effects, including the possibility of malignant transformation », and cite the following paper

Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. Nature 448, 313-7 (2007).

In this paper Okita show that malignant transformation is not due to insertional oncogenesis, but rather to the re-expression of the transgene.

I suggest the following :
« random viral integrations can lead to deleterious cellular effects, including the possibility of malignant transformation (ref PMID 16544975) or re-expression of the oncogenic transgenes (ref Okita et al)»

The suggested reference (Okita, et al.) regarding malignant transformations arising from viral methods of iPSC generation has been added in the first paragraph of the Introduction.

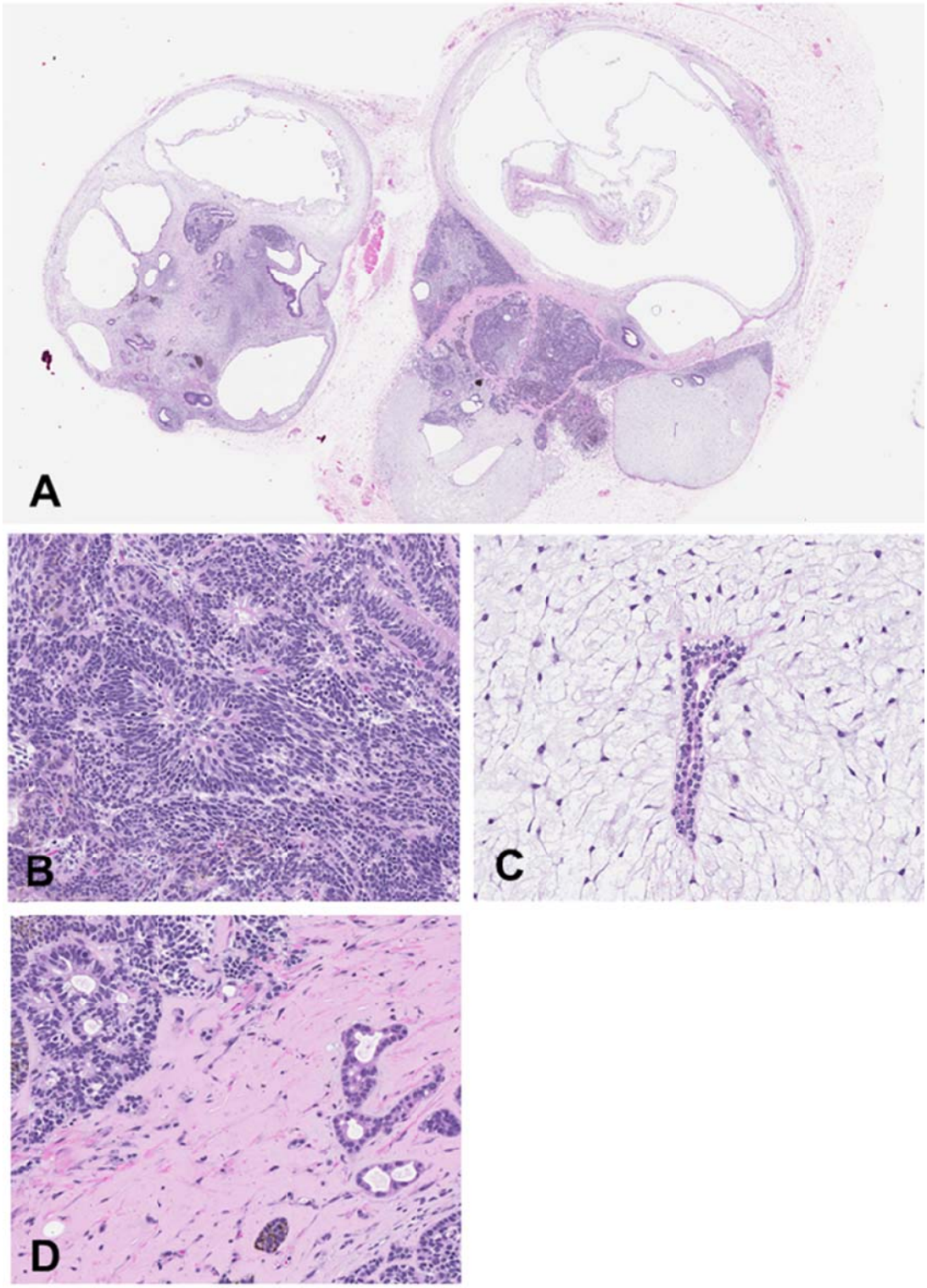
Reviewer #3:

Major Concerns:
1.More experiments are required to characterize iPSCs. For instance, IF or QPCR of pluripotency markers (OCT3/4, SOX2, NANOG, TRA-1-81, TRA-1-60) may be needed.

We have added immunofluorescence data for TRA-1-60, TRA-1-81, and SSEA-4. We have also added alkaline phosphatase histochemistry as well as PCR amplification of the endogenous *OCT4*, *SOX2*, *CMYC*, and *KLF4*.

2.Teratoma test in vivo is also needed.

We have performed teratoma tests on at least six iPSC lines generated in our lab. We have not done this for every line due primarily to cost. However, every line we generated that has been positive for the pluripotency markers shown in figure 2 has formed a teratoma *in vivo* when tested. We are confident the most recent lines will as well.



Teratoma formed from an iPSC line generated in our laboratory. A cross-sectional image shows a diverse group of tissues formed within the teratoma (Box A), while higher magnification images depict tissues indicative of the three developmental germ layers: primitive neuronal (ectoderm, Box B), glandular (endoderm, Box C), and skeletal muscle (mesoderm, Box D) tissues are present.

3.The methylation level of OCT4 promoter should be tested.

We believe re-expression of endogenous pluripotency genes such as OCT4 are a surrogate for this and this PCR data is included in Figure 2.