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A LIFE OF SCIENCE

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Dr. Michelle Kinahan
Science Editor
JoVE

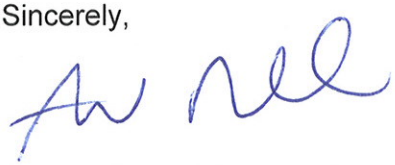
Manuscript title: " Efficient iPS Cell Generation from Blood Using Episomes and HDAC Inhibitors." MS # JoVE52009

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.

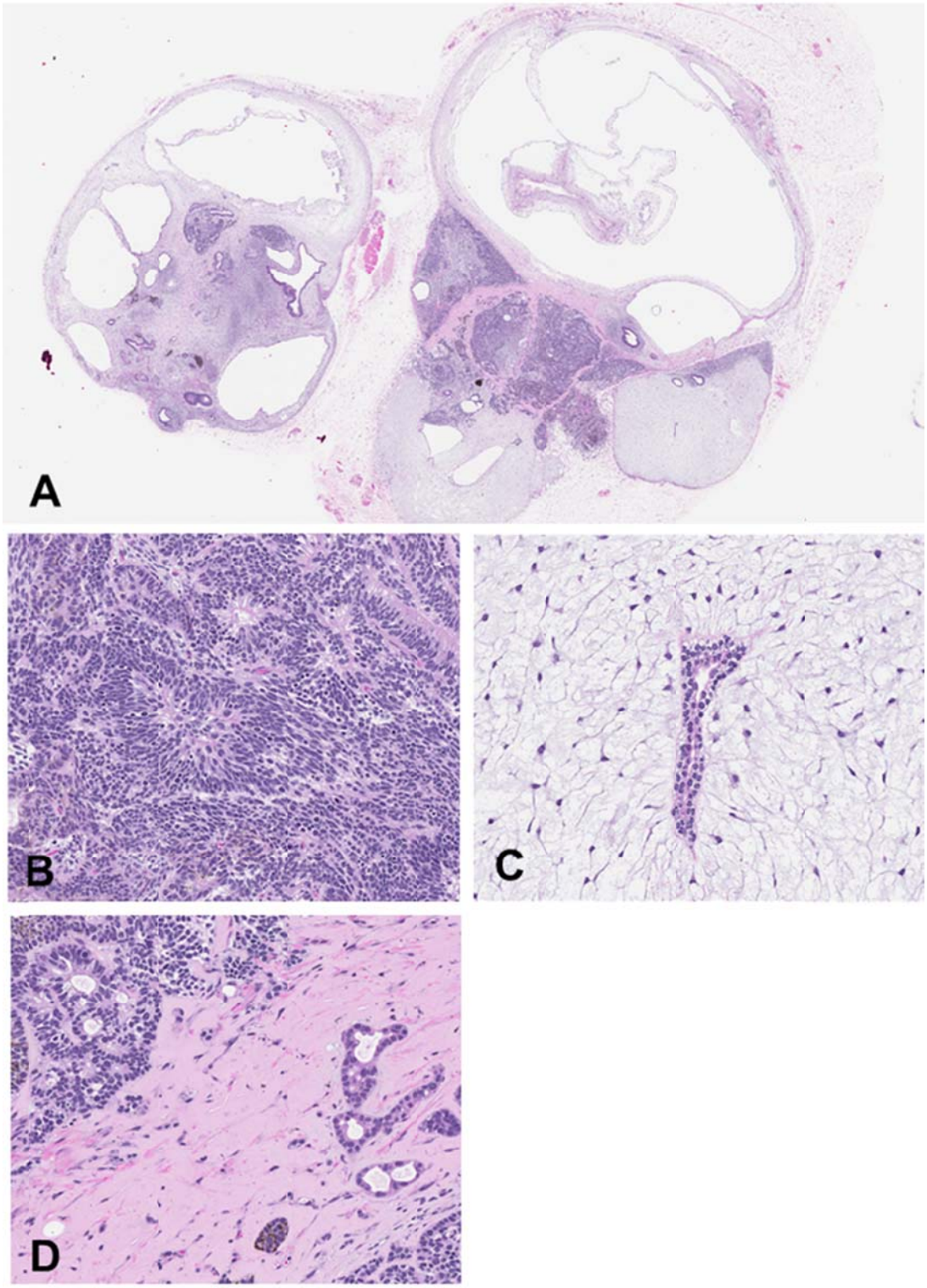
Reviewer #3:

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.