**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

This manuscript by Ilsley et al. describes a protocol to reprogram somatic cells (in this specific protocol mice tail-derived fibroblasts) into erythroid precursors using a specific set of transcriptional regulators. The basis of the protocol is described in a cell report paper by the same corresponding author in 2016 in cell reports (Capellera-Garcia et al., Cell Rep. 2016). The protocol is written in a straight forward and easy to follow manner, thus facilitating reproducibility in other laboratories. I have one major point and several minor points which I have addressed below.

Major Concerns:

Do be a bit blunt, if this protocol is to be used in the research field or to produce erythroid cells for transfusion purposes as the authors expressed, this protocol should generate significant amounts of erythroid precursors. However, the authors do not provide any yield or absolute number count after reprogramming from a set number of fibroblasts (colony forming units are not sufficient). In the protocol at line 158-162 there is room to provide these numbers and I would strongly suggest to include these numbers.

Our intention is to use direct reprogramming for studying developmental process and not for generating RBCs for transfusion purposes. We removed a sentence suggesting this.

Related to this important yield question, but less important to the actual protocol, if the erythroid cells can be expanded for multiple days to yield large numbers of erythroid cells this could substitute for low initial reprogramming yield. In other words, a growth curve of the induced erythroid cells in SFEM, epo/SCF/dex would be highly informative.

We now included data showing reprogramming efficiency of around 1% can be achieved. We also explain iEPs have a poor proliferation and enucleation capacity similar to the primitive erythroid progenitors they resemble.

Minor Concerns:

Abstract:   
Abstract line 36, "We utilize…." Is a weird sentence, I know what the authors are trying to say but it should be rewritten as it makes no sense now.

We have re-written this sentence.

Introduction:   
Line 46 agreed, this is maybe a bit of a petty remark but: "in human bodies" should change to "from human bodies", as in human bodies, bacteria are the most pronounced cells concerning quantity.

Changed to ‘..of human bodies’

Line 62: quote the original paper

Done

Protocol:   
Line 2: what do the authors mean with pure DMEM (nothing added?), then plain DMEM (or just DMEM or DMEM not supplemented with etc etc) would be better.

The word ‘pure’ has been removed.

Line 101 point 12: after a few days is a bit vague.

Changed to ‘After 5 – 7 days’

Line 136: kick frozen in liquid nitrogen or just frozen?

Minor change of wording

I am not sure how qualitative the protocol is allowed to be, but phrases like "more efficient if fresh viral supernatant is used" are a bit challenging with respect to reproducibility.

We agree with the reviewer that qualitative information should be avoided in protocol steps. However, we believe that this is important information at this point in the protocol, though we do not have quantitative data for this statement. As discussed below in response to a comment by reviewer 3, the expression vectors have no reporter or selection gene, titration of the virus is near impossible. One can check that the cells are expressing each gene by qPCR but this impractical to perform for every experiment.

Line 143: the "volume" of viral supernatant plus polybrene needs to be diluted into FEX?

This step has been re-worded to clear up instructions.

Discussion:  
Line 231. Add reference 9.

Done.

Line 232. "function". Delete function as I did not see any assays on the function of erythroid cells, oxygen binding assays, deformability, expression of structural proteins, absolute haemoglobinisation etc etc compared to control erythroid cell grown from adult or cord blood.

Replaced ‘function’ with ‘Colony-forming ability’ as we have thoroughly described this.

Do the authors continue to grow the cells in the SFEM medium supplemented with the growth factors as indicated in lines 152-155? If so, did the authors take into consideration that the low number of reticulocytes may be due to the continuous presence of SCF and the glucocorticoid agonist dexamethasone, as glucocorticoid receptor activation and SCF have been shown to cause strong inhibition of erythroid differentiation? Interestingly, this mix should thus result in expansion of the erythroid cells (major remark on yield).

Yes, we made several unsuccessful attempts at culturing the iEPs in conditions reported to induce terminal maturation in erythroid progenitors, such as DMSO. Unfortunately, the cells do not survive long enough to draw conclusions. This could be due to the cells entering terminal erythroid differentiation and exhausting their lifespan, even without enucleating.

However, since the cells actually never induce c-kit expression, SCF could not prevent differentiation as the signals will not be induced. Improvement of the reprogramming with additional factors could potentially lead to c-kit expression and this would assist in the expansion of iEPs leading to a better yield. We have added some discussion related to this topic.

The authors, on the other hand indicate, that colony efficiency is low and spontaneous differentiation is observed, questioning whether the "term bona erythroid progenitor" should actually be down termed to "erythroid progenitor like cells".

The term ‘*bona fide* erythroid progenitors’ is used in reference to FL and BM samples that the iEP cells are compared to. We conclude that the iEPs ‘resemble’ *bona fide* erythroid progenitors by comparing them to FL and BM cells. However, to avoid confusion, an instance of using ‘*bona fide’* has been removed.

Representative results:

Line 170: "progenitor" needs to be "progenitors"

Fixed. Now line 216.

**Reviewer #2:**

Manuscript Summary:

This manuscript describe the method to generate mouse erythroid progenitors from mouse adult fibroblasts using overexpression of four transcription factors: Gata1, Tal1, Lmo2 and cMyc.

Comments:  
1. Mice. Please succinctly describe the Epor-cre mice, as this is a critical strain for this JOVE manuscript.

A new description has been added at the beginning of the representative results section of the manuscript.

2. Why do the mice have to be between 6 and 8 weeks of age? This should be explained to the reader.

The older the age of the mice, the lower is their reprogramming ability. This was observed throughout experimentation and has also been reported by others (reviewed in Mahmoudi et al, 2012 Aging and reprogramming: a two-way street). Information and reference added in lines 103-104

3. Point 12, 'after a few days...' Please clearly indicate the number of days required.

Changed to ‘After 5 – 7 days’

4. Retrovirus production and Phoenix cells. Are these made available to the research community?

Phoenix-GP cells are available commercially and have been added to material list.

cDNA vectors were generated in-lab but are available upon request. Will be made available through a repository such as Addgene.

5. Discussion: switch between primitive and definitive erythropoiesis. For the treatment of hemoglobinopathies, the switch involved is the fetal to adult definitive. The authors may want to consider tweaking their sentence.

This sentence and discussion point has been altered for understandability.

6. Is there any potential implications for the study of human erythropoiesis?

This has now been further discussed in the last paragraph of the discussion section.

**Reviewer #3:**

Manuscript Summary:

In this manuscript, the authors present a method for converting murine tail tip fibroblasts into erythroid progenitors via transcription factors reprogramming. The protocol is clearly described and is interesting for a large readership. A few additions and amendments could still improve the manuscript and the ability of investigators to reproduce it in their own laboratories further increasing the impact of its publication.

Major Concerns:

1. It is important to provide the readers with a source for all the reagents utilized to guarantee reproducibility. For example, are the exact plasmids expressing the transcription factors available? From where? Addgene? Would the authors provide MEFs from their reporter mouse line? Is this mouse line available in a repository?

The use of the erythroid lineage tracking mouse is not a vital part of the procedure which can be carried out in fibroblasts from any mouse strain. We use this strain to assist in visualization of commitment the erythroid lineage. We have reworded some parts to make this clear in the protocol.

We will be making the plasmids available through Addgene.

2. There is no indication of the robustness of the process. It would be important to provide a quantification of the extent of reprogramming over a large number of experiments (10 for example). It would give the reader a better idea of the efficiency expected.

We have included data from a new experiment showing efficiency of up to 1%.

3. It is not clear how the quality of the virus production was controlled. It is probably critical that each batch of viruses is tittered (or the expression of the transcription factors normalized) to warrant optimal levels of expression of each transcription factor and reproducible results.

Since the expression vectors have no reporter or selection gene, titration of the virus is near impossible. One can check that the cells are expressing each gene by qPCR but this impractical to perform for every experiment. Using fresh viral supernatant is usually better than using frozen virus which would be required to titer the viruses. While this gives some batch variation, other factors such as the health of the fibroblast gives much greater variation.

4. The suggestion that the generation of more red colonies at day 8 than at day 5 could be explained by silencing of the expression vector is worrisome. If the reprogramming is really successful, endogenous proteins should be expressed and the reprogramming should not rely anymore on expression of exogenous proteins.

We believe this is a result of successful reprogramming and indeed successful induction of the endogenous *primitive* erythroid program, which means cells will differentiate and not proliferate. It is likely the fact that we fail to induce reprogramming to *definitive* proliferating and enucleating progenitors that explains that the cells despite SCF and dexamethasone in the medium differentiate instead of proliferate.

The reviewer points out that silencing should not affect the reprogramming ability since the endogenous expression of each factor should be induced. To address this, we have included expression data of both the exogenous and endogenous genes (Figure 2C) and discussed this in lines 253-257.

What are the white colonies? The ones in figure 1K might be macrophage cells. It would be important to characterize further these colonies.

The white (or non-red) colonies appear to be macrophage-like cells as the reviewer suggested. In response to this comment, we have added a new figure (Figure 2E-F) and section investigating the identity of the non-red iEP colonies, using our previously published microarray data, reanalyzed for this purpose. This can be seen in lines 280-285 of the revised manuscript.

5. It is striking that reprograming is observed after only 5 days. In general it is a much slower transition. How did the authors control for the possibility to contamination of MEFs by hematopoietic progenitors? Are red or white colonies observed when the experiment is performed with empty vectors? Are the MEFs FACS sorted?

The reprogramming to iEP is remarkable fast. Usually when TTF cultures are first generated, we perform a depletion of hematopoietic cells using MACS magnetic bead separation. This was inadvertently left out of the original submission but has now been added at lines 141-143.

6. It is not clear if the MEFs are directly reprogrammed to erythroid cells. Did the authors try to examine if multipotent progenitors are first generated? Do the reprogramming transit through a hemogenic endothelium stage.

The lack of early progenitor expression at any time and the fact that Ter119+ cell emerge already after 6 days strongly suggest the cells do not transition through haemogenic endothelium but potentially through an intermediate progenitor. We have added some data to the revised manuscript that investigates this concern. See lines 234-238 of revised manuscript.

Minor Concerns:

1. The reprogramming is performed under hypoxic conditions. What are these conditions? Low oxygen incubators or hypoxic chamber? What is the O2 levels? It would be important to indicate if this is critical as not all the laboratories have the ability to perform cultures in hypoxic conditions.

Oxygen levels are indicated on line 134 and 190. The incubation is performed in a low oxygen (or hypoxic) incubator. I would argue that this can be assumed by the reader if no special equipment is specified by the author.

We have also included some data supporting that reprogramming is improved in hypoxic conditions but not completely necessary (see lines 309-311.)

2. The authors indicate that unfortunately very few enucleated reticulocytes are observed. Is there a way to improve enucleation?

We have attempted culturing iEPs in several conditions reported to induce terminal maturation in erythroid progenitors. We couldn’t draw many conclusions because cells do not survive for long. See lines 391-400 in which we discuss this.

3. I would suggest to add Batta et al. (25466247) to the references describing reprograming to hematopoietic cells (reference 3 and 4) for the following reasons. Nobody has been able to reproduce the Szabo et al. study and the hematopoietic potential generated in Pereira et al. is very limited.

Reference included.

4. It would be interesting to further discuss how this protocol differs (efficacy, timing…) from others that also support the generation of red blood cells.

As far as we are aware, our protocol is currently the only protocol that generate red blood cells from other somatic cell fates. However, we have included some discussion about some other cell culture systems used to study erythropoiesis in vitro.

**Reviewer #4:**

In the manuscript titled "Direct Lineage Reprogramming of Adult Mouse Fibroblast to Erythroid Progenitors" Ilsley et al. set out to provide a more in depth description of the methodology used by Capellera-Garcia et al. Stem Cell Reports 2016 in deriving induced erythroid progenitors (iEPs)using Gata1, Tal1, Lmo2, and c-Myc from mouse fibroblasts.

In the manuscript essentially 3 procedures are described: derivation of tail tip fibroblasts, retrovirus production and transduction of fibroblasts. All these procedures are already nicely illustrated in published JoVe papers.

The figure with representative images presented here also don't add anything new to the literature as they are a simple copy of already published pictures (see Capellera-Garcia et al. Stem Cell Reports 2016 Figure 1 and 2).

Therefore I think this manuscript in its current form, won't be particularly helpful to the scientific community.

We have added some new and some reanalyzed data to the manuscript. We now believe that it provides new information that cannot be understood from the original publication.