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Direct Lineage Reprogramming of Adult Mouse Fibroblast to Erythroid Progenitors

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Corresponding Author:	Johan Flygare Lunds Universitet Lund, SWEDEN
Corresponding Author's Institution:	Lunds Universitet
Corresponding Author E-Mail:	johan.flygare@med.lu.se
Order of Authors:	Melissa Ilsley
	Sandra Capellera-Garcia
	Kishori Dhulipala
	Alban Johansson
	Johan Flygare
Additional Information:	
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Faculty of Medicine

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Ronald Myers, PhD.
Science Editor JoVE

Johan Flygare, MD, PhD.
Assistant professor.
Dept. Molecular Medicine and Gene Therapy,
Lund Stem Cell Center
Lund University, Lund Sweden

Dear Dr. Ronald Myers,

I am pleased to resubmit the original research article entitled "Direct Lineage Reprogramming of Adult Mouse Fibroblast to Erythroid Progenitors" by Melissa Ilsley and colleagues for further consideration for publication in the *Journal of Visualized Experiments*. We thank the reviewers for their comments on our submitted manuscript. We have endeavored to address all concerns brought forward by the reviewers and yourself. In response to these comments, we have made a number of additions to the manuscript. One example is addition of microarray data comparing gene expression of successfully reprogrammed hemoglobinized cells with non-hemoglobinized cells, which suggest the non-hemoglobinized cells are macrophages.

Regarding major concerns Reviewer #1 and #2 express concern our method can not generate enough erythrocytes for transfusion and that we need to demonstrate how efficiency and yield from the reprogramming. We first would like to clarify that we propose the method described in this paper is first and foremost useful for studying aspects of erythroid development, which certainly could lead to discoveries enabling industrial production of erythrocytes. Direct reprogramming *per se* is likely not a scalable method for generating erythrocytes for transfusion and we therefore removed a sentence suggesting this from the discussion. We also agree with reviewers that information about robustness and yield is valuable to the manuscript and has attempted to address both of these concerns by providing details reprogramming ability of cells from set numbers of fibroblasts and included some further discussion about this concern.

Two other major concerns from reviewers are that reprogrammed cells proliferate poorly and have low enucleation efficiency. We now explain this is because cells are reprogrammed to primitive erythroid progenitors which are known to hardly proliferate *in vitro* and do not enucleate. We have corrected the grammatical issues pointed out in the manuscript and accompanying figures.

Finally, to simplify the process, I have attached a second document outlining our responses to all other comments.

Thank you for your consideration,

Sincerely,

Johan Flygare

Postal address: BMC A12 *Office:* +46 46 462220687, *Cell* +46 727395959 *E-mail:* johan.flygare@med.lu.se

TITLE:

Direct Lineage Reprogramming of Adult Mouse Fibroblast to Erythroid Progenitors

AUTHORS AND AFFILIATIONS:

Melissa Ilsley[1], Sandra Capellera-Garcia[1], Kishori Dhulipala[1], Alban Johansson[1], Johan Flygare[1]

[1]Department of Molecular Medicine and Gene Therapy, Lund University, Sweden

Corresponding Author:

Johan Flygare

johan.flygare@med.lu.se

Tel: +46 46 222 06 87

Email Addresses of Co-authors:

Melissa Ilsley (melissa.ilsley@med.lu.se)

Sandra Capellera-Garcia (sandra.capellera_garcia@med.lu.se)

Kishori Dhulipala (kishori.dhulipala@med.lu.se)

Alban Johansson (nat12aj2@student.lu.se)

KEYWORDS:

Direct lineage reprogramming; Erythropoiesis; Transcription factors; Developmental biology; Gata1; Tal1; Lmo2; c-Myc.

SUMMARY:

Here we present our protocol for producing induced erythroid progenitors (iEPs) from mouse adult fibroblasts using transcription factor-driven direct lineage reprogramming (DLR).

ABSTRACT:

Erythroid cell commitment and differentiation proceed through activation of a lineage-restricted transcriptional network orchestrated by a group of cell fate determining and maturing factors. We previously set out to define the minimal set of factors necessary for instructing red blood cell development using direct lineage reprogramming of fibroblasts into induced erythroid progenitors/precursors (iEPs). We showed that overexpression of *Gata1*, *Tal1*, *Lmo2*, and *c-Myc* (GTLM) can rapidly convert murine and human fibroblasts directly to iEPs that resemble *bona fide* erythroid cells in terms of morphology, phenotype, and gene expression. We intend that iEPs will provide an invaluable tool to study erythropoiesis and cell fate regulation. Here we describe the stepwise process of converting murine tail tip fibroblasts into iEPs *via* transcription factor-driven direct lineage reprogramming (DLR). In this example, we perform the reprogramming in fibroblasts from erythroid lineage-tracing mice that express the yellow fluorescent protein (YFP) under the control of the erythropoietin receptor gene (EpoR) promoter, enabling visualization of erythroid cell fate induction upon reprogramming. Following this protocol, fibroblasts can be reprogrammed into iEPs within five to eight days.

While improvements can still be made to the process, we show that GTLM-mediated reprogramming is a rapid and direct process, yielding cells with properties of *bona fide* erythroid progenitor and precursor cells.

INTRODUCTION:

Red blood cells (RBCs) are essential in all vertebrates and make up 84% of all cells of human bodies[1]. From embryonic to adult life, our health is highly dependent on exact regulation of RBC homeostasis. The ongoing production of mature RBCs throughout development into adulthood is known as erythropoiesis. A major challenge in erythropoiesis research is to define the master regulators that orchestrate RBC development and the switch between primitive and definitive erythropoiesis. Direct lineage reprogramming of erythroid progenitors presents an opportunity to further understand erythroid development *in vivo*.

Direct lineage reprogramming (DLR), also known as transdifferentiation, is the process of reprogramming one cell type directly into another, bypassing pluripotent and multipotent progenitor stages. DLR has thus far been used to produce numerous cell types including neural[2], hematopoietic[3-5], hepatic[6] and nephrotic[7], progenitor cells. For developmental biologists, DLR has become an important tool for interrogating aspects of lineage commitment and terminal differentiation processes[8,9]. DLR can complement and partially replace *in vivo* studies for understanding mechanisms of cell fate determining factors during development. The DLR protocol for reprogramming to erythroid progenitors described in this paper provides the field a complimentary method for developmental studies of erythropoiesis.

We have previously demonstrated that overexpression of a four-factor cocktail, *GATA1*, *TAL1*, *LMO2,* and *c-MYC (GTLM)*, is sufficient to reprogram both murine and human fibroblasts directly to induced erythroid progenitors (iEPs)[10]. The GTLM-reprogrammed erythroid cells greatly resemble *bona fide* primitive erythroid progenitors in terms of morphology, phenotype, and gene expression[10]. Thus iEPs have limited proliferation capacity and mature to nucleated erythrocytes similar to those transiently produced in the early embryo before onset of definitive erythropoiesis. By making changes in the reprogramming conditions (*e.g.,* point mutations in reprogramming factors or addition of other factors), one can understand how this leads to changes in erythroid development and differentiation. We have for example shown that addition of *Klf1* or *Myb* to the GTLM cocktail changes the globin expression pattern from predominantly embryonic (primitive) to mainly adult (definitive). This finding corroborates the validity of using DLR as a tool for defining developmental factors in erythropoiesis.

Here, we outline the process of generating iEPs from mouse tail tip fibroblasts (TTF). In our representative results, we performed the reprogramming on fibroblasts from the erythroid lineage-tracing mice (*Epor*-Cre *R26*-eYFP) which express the yellow fluorescent protein (eYFP) from the *Rosa26* locus in all cells that have expressed the erythropoietin receptor, allowing easy visualization of commitment to the erythroid lineage. Using this method, YFP positive (EpoR+) cells are present as early as five days after transduction. This protocol, therefore, offers a quick and robust technique for the generation of erythroid progenitors *in vitro*.

PROTOCOL:

1. Establishment and Maintenance of Primary Mouse Tail Tip Fibroblast Cultures

1.1 Prepare gelatin-coated dishes (recommend a 10 cm dish for one tail) by covering the surface with 0.1% gelatin and incubating the dishes for approximately 20 min at 37 °C. Aspirate the gelatin solution from the dish and allow it to dry for at least 2 h.

1.2 Euthanize the mice by cervical dislocation. Remove the tail with scissors, cutting at the base of the tail. Put the tail in Dulbecco's phosphate-buffered saline (DPBS) with 2% fetal bovine serum (FBS) until ready to use.

Note: For the best results, tails should be taken from mice around 6 to 8 weeks of age. Tails can be taken from mice older than 8 weeks of age, however, as the mice age, the proliferation capability of the fibroblasts and the efficiency of reprogramming decrease[11].

1.3 Perform all subsequent steps of this protocol in a tissue culture hood under sterile conditions. Prepare a diluted trypsin solution of 0.02% trypsin-EDTA in DPBS and add 5 mL into an uncoated 10 cm dish.

1.4 Wash the tail, first in 70% ethanol, then in DPBS. In a dish, place the tail flat and use forceps to hold it in place. Make an incision on the tail along its longitudinal axis from the base to the tip.

1.5 Grasp the tail with one pair of forceps and hold it vertically. Using a second pair of forceps, grip the skin beside the incision at the base of the tail and peel it back. Do this at both sides of the incision until the skin can be peeled off by pulling downwards toward the tip of the tail.

1.6 Hold the peeled tail with forceps over the dish containing the trypsin solution and cut the tail into approximately 1 cm long pieces. With the tail pieces in the trypsin solution, use scissors to fragment the pieces into smaller pieces. Incubate the tail pieces in the trypsin solution at 37 °C for 10 min.

Note: The smaller the pieces are preferred so as to provide each piece a high surface area to volume ratio.

1.7 Quench the trypsin using 2 volumes of fibroblast expansion (FEX) medium (high-glucose Dulbecco Modified Eagle Medium (DMEM) with 15% FBS, 2 mM L-glutamine, Non-essential amino acids (NEAA) and 100 U/mL Penicillin/Streptomycin).

1.8 Collect entire contents of the dish into a 50 mL tube and centrifuge at 350 × g for 5 min at 4 °C. Aspirate the supernatant and resuspend the tail fragments in 10 mL of fresh FEX medium.

1.9 Transfer tail fragments in medium to a gelatin-coated dish and incubate at 37 °C in 5% CO_2 and 4% O_2, adding fresh FEX medium every 2 days.

Note: After five to seven days, tail fragments have attached at the bottom of the dish and fibroblasts can be seen moving away from them.

1.10 Once clusters of fibroblasts are spotted, gently shake the dish to dislodge tail pieces and aspirate the medium and all the bone fragments leaving the fibroblasts attached to the plate. Add new FEX medium and culture the fibroblasts until confluent.

1.11 To ensure no contamination of fibroblasts by hematopoietic progenitors, dissociate the cells from the plate using 1X trypsin-EDTA for 5 mins and collect cells. Deplete for cells expressing hematopoietic markers (CD117, CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119) using a magnetic separation system[10].

2. Retrovirus Production

2.1 Seed retroviral packaging cells at approximately 2.5×10^4 cells/cm^2 (2.0×10^6 cells for a 10 cm dish) on a tissue culture-treated (by vacuum-gas plasma) dish and culture overnight in high-glucose DMEM with 10% FBS, 10 mM Sodium Pyruvate, and 100 U/mL Penicillin/Streptomycin at 37 °C and 5% CO_2.

2.2 On the following morning, change the medium to DMEM with no additives using half the volume used to culture overnight (5 mL for a 10 cm dish). In the afternoon, check that the cells are 70–80% confluent and begin the transfection.

2.3 For each reprogramming factor, ***Gata1*, *Tal1*, *Lmo2*, and *c-Myc*,** prepare a 2:1 mixture of the expression vector (pMX) and helper vector (containing gag and pol genes). For a 10 cm dish of fibroblasts, use 6 µg of expression vector and 3 µg of helper vector in a final volume of 100 µL in DMEM medium.

2.4 For each reprogramming factor, prepare 300 µL of room temperature (RT) DMEM in a sterile polystyrene tube and carefully add 27 µL of commercial transfection reagent.

Note: The transfection reagent should be brought to RT before use and must be added directly into the medium as the electrostatic properties of the compound can make it stick to the plastic wall of the tube.

2.5 Add the plasmid mix into the transfection reagent-containing tube, vortex briefly and incubate the transfection reagent-DNA mixture for 15 min at RT. Briefly vortex the mixture and add it dropwise to the retroviral packaging cells so that the transfection reagent-DNA mix is evenly spread over the culture and incubate at 37 °C overnight.

2.6 24 h after transfection, change the medium to DMEM with 20% FBS and 100 U/mL Penicillin/Streptomycin. 48 h after transfection, collect the supernatant and filter it through a 0.22 µm pore-size syringe filter.

Note: Viral supernatants can be frozen to -80 °C and kept until required, although transduction is more efficient if fresh viral supernatant is used.

3. GTLM transduction and iEP harvest

3.1 Seed the tail tip fibroblasts at 1 × 10^4 cells/cm^2 on 0.1% gelatin pre-coated dishes in FEX medium and incubate at 37 °C for 24 h.

3.2 The following day, prepare a transduction mixture as follows.

3.2.1 Add 1 volume of viral supernatant for each reprogramming factor supplemented with 4 µg/mL of retroviral infection reagent (40%) to 6 volumes of FEX medium (60%).

3.2.2 For a 10 cm dish of fibroblasts, add 1 mL of each viral supernatant (4 × viruses = 4 mL) supplemented with 4 µg/mL of retroviral infection reagent to 6 mL of FEX medium, giving a total of 10 mL transduction mixture.

3.3 Aspirate FEX medium from the fibroblast culture and replace it with the transduction mixture. Incubate the transduction for 4 h at 37 °C in hypoxic conditions (5% CO_2 and 4% O_2).

3.4 Aspirate the transduction mixture and replace it with fresh reprogramming medium (Serum-free Expansion medium (SFEM), 100 U/mL Penicillin/Streptomycin, 100 ng/mL murine Stem Cell Factor (mSCF), 10 ng/mL murine interleukin-3 (IL3), 2 U/mL human recombinant Erythropoietin (hrEPO), and 100 nM Dexamethasone).

3.5 Incubate for 8 days at 37 °C in hypoxic conditions, adding fresh reprogramming medium every 2 days. After five to eight days, successful reprogramming will yield clusters of cells that have detached from the plate.

3.6 To collect reprogrammed cells for analysis, gently pipette up and down to harvest them directly from the dish. To harvest untransduced fibroblasts for comparison, dissociate the cells from the plate using 1X trypsin-EDTA and collect.

REPRESENTATIVE RESULTS:

Here we present a reproducible protocol for the production of iEPs from adult fibroblasts using transcription factor-driven DLR. We evaluate the cell reprogramming using flow cytometry, colony-forming assays, and gene expression analysis. In order to assist in the visualization of the conversion to erythroid cell fate, we performed the reprogramming on fibroblasts from the erythroid lineage-tracing mice (*Epor*-Cre *R26*-eYFP) which express the yellow fluorescent protein (eYFP) from the *Rosa26* locus in all cells that express the erythropoietin receptor (*Epor*, Cre knocked into one allele of the endogenous *Epor* locus) transcript at any stage of their development[12,13] (**Figure 1A)**. After successful reprogramming of induced erythroid progenitors, YFP positive (EpoR+) cells are observable as early as five days after transduction. By day 5 of reprogramming, cells become round, lift from the surface of the plate and begin forming clusters.

Day 5-iEPs display an erythroid precursor-like morphology, that feature a characteristic central nucleus, coarse chromatin, and blue cytoplasm after May-Grünwald-Giemsa staining **(Figure 1C)**. Hemoglobinization of some cells is evident by positive benzidine staining and a mildly red appearance when pelleted **(Figures 1D-E)**. In addition, a small fraction of day 5-iEPs co-express YFP and the erythroid-specific surface marker Ter119 **(Figure 1F)**. By day 8, large YFP+ clusters can be seen. Day 8 iEPs present a more differentiated erythroid phenotype than Day 5 iEPs; they are significantly smaller, have accumulated more hemoglobin, and also show significantly upregulated expression of Ter119 **(Figures 1B–1G)**. Cyto-spins of day 8-iEPs reveal erythroid-like morphology, while very few enucleated reticulocytes are observed **(Figure 1H)**.

Gene expression analysis by quantitative polymerase chain reaction (qPCR) of bulk iEPs collected at day 8 shows that they have almost shutdown expression of fibroblast genes and have upregulated many erythroid genes including globin genes, predominantly expressing embryonic types **(Figure 1I)**. To assess whether the reprogrammed cells transition through multipotent progenitors, we performed a time course and analyzed the expression of hematopoietic markers throughout reprogramming. We previously reported that erythroid precursor output was highest on day 6, followed by day 8, with 10.5% ± 4.6% and 6.6% ± 0.5% of live YFP+ cells co-expressing CD71 and Ter119, respectively[10]. There is also a population of CD41 positive cells before the appearance of Ter119 positive cells. Neither c-kit nor CD45 markers, which are usually expressed in hematopoietic progenitor and downregulated in erythropoiesis, are expressed at any point in the reprogramming **(Figure 1J)**. These data suggest that the cells do not go through a multipotent hematopoietic progenitor or earlier stage, though do perhaps transition through a megakaryocyte-erythroid progenitor.

A reliable way to assess the efficiency of the reprogramming is to perform BFU-E colony-forming assays on the reprogrammed cells. *In vitro* differentiation capacity of day 5- and day 8-iEPs was assayed in methylcellulose supplemented with human erythropoietin, murine stem cell factor, and dexamethasone. After 8 days, iEPs formed two types of colonies: distinctly red (red iEP) and not-visibly red (non-red iEP). While cells from red colonies displayed erythroblast morphology, cells from non-red colonies did not resemble erythroid cells, and were irregular, had large deep blue and granular cytoplasm (**Figure 2A)**.

Of the day 5-iEPs, approximately 1 in 1,000 formed red colonies, while only approximately 1:10,000 Day 8-iEPs formed red colonies, with a much higher ratio of non-red colonies formed **(Figure 2B)**. This reduction in colony-forming ability between day 5- and day 8-iEPs could be explained by iEPs undergoing differentiation from days 5–8 and the GTLM expression vectors suffering silencing over time. qPCR analysis confirmed that expression of the exogenous transcript decreases significantly from days 5 to 8. As expected in successful reprogramming, expression levels of endogenous *Gata1*, *Tal1*, *Lmo2*, and *c-Myc* are induced, however, endogenous Lmo2 expression is only very lowly expressed **(Figure 2C)**.

A point of note, and a limitation of the assay, is that non-red colonies containing macrophage-like cells outnumber the red hemoglobinized iEP colonies formed by reprogrammed fibroblasts in colony-forming assays. Colony-forming ability to create red iEP colonies can be improved by

changing the stoichiometric ratios of GTLM factors. Transducing the cells with double the amount of *Tal1* and *Lmo2* expression vectors led to a slight increase in the ratio of red colonies over non-red colonies, while extra *Gata1* led to a significant increase in the formation of red colonies and nearly a complete elimination of non-red colonies supporting the important role for these factors in erythropoiesis **(Figure 2D)**. Interestingly, increasing the expression of *c-Myc* drastically increases the production of non-red colonies despite being indispensable for reprogramming. To obtain pure induced erythroid progenitors for downstream analysis, colonies can be isolated from the colony-forming assays. We performed gene expression analysis in the form of microarray on red and non-red colonies from Day 5-iEPs and colonies from 14.5 days post coitus (dpc) fetal liver (FL) and adult bone marrow (BM) for comparison. The microarray data are accessible through NCBI's Gene Expression Omnibus (GEO): GSE73344. We have previously shown that red-iEP colonies resemble the *bona fide* erythroid colonies (FL and BM) by gene expression[10]. Examination of the expression of *Gata1*, *Tal1*, *Lmo2,* and *c-Myc* in each colony shows that while the red colonies show similar levels of expression of all four factors to FL and BM, non-red colonies have lower expression of all factors, especially *Gata1* **(Figure 2E)**. To understand what the non-red colonies containing macrophage-like cells are, we surveyed the expression of cell type-specific genes in each type of colony from the microarray data. As previously reported, the red colonies express many erythroid-specific genes, similar to that of FL and BM. After revisiting the data, it is clear that the non-red colonies show high expression of a number of genes characteristic of macrophage cells[14] **(Figure 2F)**. Neither red nor non-red colonies show significantly increased expression of typical megakaryocyte genes[15]. Together, these data suggest that the non-red colonies more closely resemble macrophages than erythrocytes. Since non-red colonies are never observed when one of the GTLM factors are removed these macrophage-like cells seem to be formed when expression levels of one or more of the reprogramming factors *Gata1*, *Tal1*, *Lmo2* are lower than required for reprogramming to iEPs. Together, this suggests that the expression levels of all four GTLM factors are important in TTF to iEP reprogramming and that heterogeneity is explained by incomplete reprogramming of individual cells, which potentially can be corrected by adjusting stoichiometric ratios.

To assess efficiency and robustness of our protocol, we tested the ability of set numbers of fibroblasts to produce visible clusters of iEP cells after 5 days when cultured in 384-well plates. We determined that out of 24 transductions of 20, 30, 40, and 50 TTFs, the number of wells with at least one iEP cluster was 3 (0.6% of plates fibroblasts), 15 (2.0% of plates fibroblasts), 15 (1.6% of plates fibroblasts), and 13 (1.1% of plates fibroblasts), respectively. This suggests that GLTM iEP reprogramming is a robust and reliable process that can reach an efficiency of 1%.

Other factors that can affect efficiency of reprogramming include the passage number of the fibroblasts and the culture conditions. We compared the reprogramming efficiency of TTFs that had been passaged three times or nine times prior to transduction. TTFs that had been passaged nine times (P9) showed a dramatic reduction in the ability to produce clusters of iEPs **(Figure 3A)**. Interestingly, inclusion of serum in the reprogramming media completely blocks reprogramming, replacing serum with erythroid cytokines is necessary to allow the transduced factors to drive the new cell fate without compounding signals from the serum. Removal of the serum and the expression of new factors within the cell is likely to be stressful on the cells. Indeed, blocking p53

activation greatly increased the number of iEPs generated. This effect can also be seen in reprogramming of p53 knockout fibroblasts **(Figure 3B-D).** Inhibition of p53 signaling leads to more cell survival and better reprogramming and is one validated method for improving yield. Finally, reprogramming in hypoxic conditions is favorable but not vital for iEP production. However, transduced TTFs cultured in normoxia are much slower to reprogram and iEP clusters are observed after 10 days instead of five to eight days **(Figure 3E)**.

FIGURE AND TABLE LEGENDS:

Figure 1: Forced expression of *Gata1, Tal1, Lmo2*, and *c-Myc* reprograms murine adult fibroblasts into erythroid progenitors which exhibit properties of *bona fide* erythroid cells. (A) Experimental design for transcription factor-mediated reprogramming of erythroid reporter (Epor-Cre R26-eYFP) tail tip fibroblasts (TTF) to EpoR+ reprogrammed cells. **(B–F)** Time course of iEP generation of untransduced TTFs (day 0) and bulk GTLM-transduced TTFs on days 5 and 8 (representative of n = 2–3). Transdifferentiation was evaluated by **(B)** live-cell, bright-field images of single wells (scale bar, 50 µm); **(C)** May-Grünwald's -Giemsa staining cyto-spin (scale bar, 20 µm); **(D)** Benzidine/Giemsa staining cyto-spin (scale bar, 20 µm); **(E)** macroscopic inspection of cell pellets; and **(F)** representative flow cytometry plots showing YFP/ Ter119 expression. **(G)** Cell diameter of iEPs harvested on days 5 and 8 measured from several cyto-spin slides, showing a decrease in cell size on day 8. Data are presented as mean ± SD (n = 21–25); ****$p \leq 0.0001$ by unpaired *t*-test. **(H)** Representative high-resolution benzidine/Giemsa images of GTLM-transduced TTFs on day 8. Scale bar, 5 µm. **(I)** Relative mRNA expression of relevant fibroblast- and erythroid-specific genes including globin genes in untransduced TTFs (gray columns) *versus* bulk GTLM-transduced TTFs (red columns) on day 8, determined by qPCR. Data are presented as mean ± SD (n = 4–6 for iEPs, n = 2 for untransduced TTFs). **(J)** Graph showing summary of time-course flow cytometry analysis of untransduced TTF (day 0) and bulk GTLM-transduced TTF harvested at day 2, 4, 6 and 8 showing YFP, CD45, CD71 and Ter119 expression (n = 3). Data are presented as mean ± SD. Figure is adapted from Capellera-Garcia S, *et al*.[10].

Figure 2: GTLM iEPs produce red and non-red colonies in BFU-E colony assays.
(A) Representative bright-field and May-Grünwald-Giemsa cytospin images of iEP-derived red and non-red colonies. Scale bars, 50 µm (colony images) and 10 µm (cyto-spin images). **(B)** Colony counts generated from plated untransduced TTFs, bulk day 5 iEPs and bulk day 8. Data are presented as mean ± SD (n = 3); ***$p \leq 0.0005$; ****$p \leq 0.0001$ by two-way ANOVA. **(C)** Relative mRNA expression of *Gata1*, *Tal1*, *Lmo2,* and *c-Myc* in untransduced TTF and bulk GTLM-transduced TTF harvested at day 5 and day 8, determined by qPCR. Primers were designed so that endogenous expression could be distinguished from total expression. Data are presented as mean ± SD (n = 4–6 for iEPs, n = 2 for untransduced TTF). **(D)** Colony counts generated from plated untransduced TTFs, and bulk day 5 iEPs generated by doubling the ratio of each of the GTLM factors. Data are presented as mean ± SD (n = 3); **$p \leq 0.001$; ****$p \leq 0.0001$ by two-way ANOVA. **(E)** Relative expression of *Gata1*, *Tal1*, *Lmo2,* and *c-Myc* in untransduced fibroblasts and picked colonies generated from GTLM-transduced iEPs (red and non-red), 14.5 dpc fetal liver and adult bone marrow, determined by microarray. Data are presented as mean ± SD; *$p \leq 0.05$; **$p \leq 0.005$. ***$p \leq 0.0005$; ****$p \leq 0.0001$ by two-way ANOVA. **(F)** Relative expression of selected

genes known for their expression in erythroid (top), megakaryocyte (middle), and macrophage (bottom) cells in untransduced fibroblasts and picked colonies generated from GTLM-transduced iEPs (red and non-red), 14.5dpc fetal liver and adult bone marrow, determined by microarray. Data are presented as mean ± SD; *$p \leq 0.05$; **$p \leq 0.005$. ***$p \leq 0.0005$; ****$p \leq 0.0001$ by two-way ANOVA. Figure is adapted from Capellera-Garcia S, *et al.*[10].

Figure 3: GTLM reprogramming is a robust and reliable process. (A) Graph of the number of clusters observed from 10,000 TTFs and representative pictures on day 5 of GTLM reprogramming of tail tip fibroblasts (TTF) that have undergone three passages (P3) or nine passages (P9) prior to reprogramming. Data are presented as mean ± SD (n = 6); scale bars are 50 µm. **(B–D)** Representative pictures of iEP clusters 6 days after **(B)** GTLM reprogramming of tail tip fibroblasts; **(C)** GTLM reprogramming of TTFs with addition of p53DN expression vector; **(D)** GTLM reprogramming of p53 knockout TTFs (scale bars = 50 µm). **(E)** Representative pictures of iEP clusters after 10 days of GTLM reprogramming performed at normoxic conditions (scale bar = 50 µm).

DISCUSSION:

Overexpression of a four-factor cocktail, ***GATA1***, ***TAL1***, ***LMO2,*** and ***c-MYC (GTLM)***, is sufficient to reprogram murine and human fibroblasts directly to iEPs[10]. The reprogrammed erythroid cells greatly resembled *bona fide* erythroid progenitors in terms of morphology, phenotype, gene expression, and colony-forming ability. This finding corroborates the rationale of using direct reprogramming as a tool for defining developmental factors in hematopoiesis. To support the validity of this method, iEPs can also be generated using GTLM induction of mouse embryonic fibroblasts and human foreskin fibroblasts, showing that it works for fibroblasts of different origins and across species[10]. In this report, we induce reprogramming of fibroblasts from the erythroid lineage-tracing mouse as a tool to visualize the conversion to an erythroid cell. Use of this mouse is useful but not critical to the protocol. We routinely perform reprogramming using multiple types of fibroblast from various mouse strains and identify iEPs by cell surface marker expression of Ter119 and CD71.

Our current method generates iEPs that exhibit a primitive erythroid phenotype as opposed to a definitive adult phenotype. One major difference between these phases of development is the expression of different globin genes. However, we have shown that addition of *Klf1* and *Myb* to the GTLM cocktail changes iEPs' globin expression pattern from predominantly embryonic to mainly adult[10,14]. Interestingly, addition of Gata2 and Runx1 to the four-factor cocktail and thrombopoietin in the medium biases the reprogramming process towards the megakaryocytic lineage[16].

The GTLM-induced iEPs produce erythroid progenitors with a primitive gene expression signature and a limited proliferative capacity. Furthermore, the iEPs produce very few enucleated erythrocytes. The poor erythroid progenitor expansion is explained by the failure to reprogram to kit+ definitive erythroid cells, which could potentially be achieved with addition of other factors inducing direct reprogramming to iEPs with a definitive gene expression program.

Our data also suggest poor enucleation efficiency is explained by the fact GTLM-induced iEPs generate precursors with a primitive, rather than a definitive gene expression program. Several attempts at optimizing culture conditions were attempted without improved enucleation. Ongoing attempts to increase both progenitor proliferation and enucleation efficiency are therefore focused at identifying missing factors for inducing reprogramming to definitive iEPs.

For the optimal production of red iEP colonies, it is vital that cells are transduced with all four vectors and GTL genes are expressed at high levels. Unfortunately the currently most efficient method does not allow prior control of vector titers. An attempt to improve the efficiency using bicistronic lentiviral vectors was not successful, possibly due to issues with inferior stoichiometry or insufficient expression levels. While work is ongoing to improve reprogramming vectors, the most efficient method remains using combinations of freshly produced vector supernatant with the previously described retroviral vectors expressing only one factor and no selection marker gene.

GTLM reprogramming to iEPs is the only reported protocol able to produce erythroid progenitor-like cells from a committed somatic cell. As a research tool the DLR iEP method therefore has several advantages over other erythroid cell model systems such as the erythroid cell lines HiDEPs/HuDEP cells (PMID: 23533656)[17]. While HiDEPs/HuDEP cells are more convenient tools for large scale erythrocyte production and evaluating gene function during terminal erythropoiesis, the unique advantage of GTLM reprogramming to iEPs is the ability to directly study of the core transcriptional programs determining erythroid cell fate. GTLM reprogramming provides an invaluable platform for studying both erythropoiesis in humans and mouse, for example, to study the switch between primitive and definitive erythropoiesis. Understanding this switch is of huge interest in the potential treatment of hemoglobinopathies, such as Sickle Cell Anemia, in which researchers strive to reverse the switch in adults to fetal hemoglobin to alleviate the disease.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no conflicts of interest to report.

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Table of Materials

Click here to download Table of Materials JoVE_Materials-2.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
DMEM without sodium pyruvate	GE Life Sciences	SH30022.01	Culturing media for PhGP cells
DMEM with sodium pyruvate	GE Life Sciences	SH30243.01	Culturing media for tail tip fibroblasts
StemSpan Serum Free Expansion Media (SFEM)	Stem Cell Technologies	9650	Reprogramming media
Fetal Bovine Serum HyClone	GE Life Sciences	SH30071.03HI	Growth factor
Penicillin/Streptomycin HyClone (100x)	Ge Life Sciences	SV30010	Antiboiotic
Non-Essential Amino Acids (100x)	Thermo Fisher	11140050	SNL media is suppplemented with this
Trypsin HyClone (1x)	GE Life Sciences	SH30042.01	Cell dissociation agent
Murine Stem Cell Factor (mSCF)	Peprotech	250-03	Added to reprogramming media
Recombinant Murine Il-3	Peprotech	213-13	Added to reprogramming media
human recombinant erythropoietin (hrEPO)	Peprotech	100-64	Added to reprogramming media
Dexamethasone	Sigma	50-02-2	Added to reprogramming media
Gelatin from porcine skin	Sigma	9000-70-8	Dissovled in dH2O and used for coating plates. Ensure sterility before use.
Blasticidin S hydrochloride	Sigma	3/9/3513	Selection antibiotic. Affects both pro- and eukaryotic cells
Dulbecco's Phosphate Buffered Saline (DPBS)	Ge Life Sciences	SH30850.03	Used for washing steps
Polybrene	Merck	TR-1003-G	Infection / Transfection Reagent
FuGENE HD Transfection Reagent	Promega	E2311	Transfection Reagent for PhGP cell line
Millex-GP Syringe Filter Unit 0.22 µm	Merck	SLGP033RS	Used for filtering virus supernatant
BD Emerald Hypodermic Syringe	Becton Dickinson	SKU: 307736	Used for filtering virus supernatant
100 mm Culture Dish	Corning	430167	Cell culture
6-well plate	Falcon	10799541	Cell culture
Jeweler Forceps #5	Sklar	66-7642	Used for handling small tail fragments
Sklarlite Iris Scissors	Sklar	23-1149	Used for cutting the tail into small pieces
Lineage Cell Depletion Kit, mouse	Miltenyi Biotec	130-090-858	For depletion of hematopoietic cells in fibroblast cultures
CD117 MicroBeads, mouse	Miltenyi Biotec	130-091-224	For depletion of hematopoietic cells in fibroblast cultures
PheonixGP cells	ATCC	CRL-3215	retroviral packaging cell line
EcoPAC vector (pCL-Eco)	Novus Biologicals	NBP2-29540	retroviral helper vector containing gag and pol genes
pMX-Gata1	Cloned in-lab		
pMX-Tal1	Cloned in-lab		
pMX-Lmo2	Cloned in-lab		
pMX-cMyc	Cloned in-lab		
CellSens Standard 1.6 software			Cytospin analysis software

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Author(s): Melissa Ilsley, Sandra Capellera-Garcia, Alban Johansson, Johan Flygare

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Department:	LABORATORY MEDICINE, LUND STEM CELL CENTER, DIV MOLECULAR MEDICINE & GENE THERAPY
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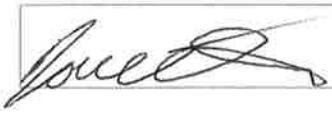
Signature:	 Date: 2018/05/15

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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:

<u>Abstract:</u>

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.

<u>Introduction:</u>

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

<u>Protocol:</u>

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.