**TITLE:**

Direct Lineage Reprogramming of Adult Mouse Fibroblast to Erythroid Progenitors

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**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 bodies1. 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 neural2, hematopoietic3-5, hepatic6 and nephrotic7, progenitor cells. For developmental biologists, DLR has become an important tool for interrogating aspects of lineage commitment and terminal differentiation processes8,9. 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 expression10. 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. 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 decrease11.

* 1. 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.
  2. 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.
  3. 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.
  4. 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. 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).
  2. 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.
  3. Transfer tail fragments in medium to a gelatin-coated dish and incubate at 37 °C in 5% CO2 and 4% O2­, 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. 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.
  2. 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 system10.

## 2. Retrovirus Production

* 1. Seed retroviral packaging cells at approximately 2.5 × 104 cells/cm2 (2.0 × 106 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% CO2.
  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.
  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.
  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.

* 1. 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. 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

* 1. Seed the tail tip fibroblasts at 1 × 104 cells/cm2 on 0.1% gelatin pre-coated dishes in FEX medium and incubate at 37 °C for 24 h.
  2. The following day, prepare a transduction mixture as follows.
     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%).
     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. 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% CO2 and 4% O2).
  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).
  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.
  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 development12,13 (**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 8iEPs 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, respectively10. 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 inducederythroid 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 expression10. 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 cells14 **(Figure 2F)**. Neither red nor non-red colonies show significantly increased expression of typical megakaryocyte genes15. 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 ≤ 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 ≤ 0.0005; \*\*\*\*p ≤ 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 ≤ 0.001; \*\*\*\*p ≤ 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 ≤ 0.05; \*\*p ≤ 0.005. \*\*\*p ≤ 0.0005; \*\*\*\*p ≤ 0.0001 by two-way ANOVA. **(F)** Relative expression of selected genes known for their expression in erythroid (top), megakaryocyte (middle), and macrophage (bottom) cellsin 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 ≤ 0.05; \*\*p ≤ 0.005. \*\*\*p ≤ 0.0005; \*\*\*\*p ≤ 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 iEPs10. 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 species10. 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 adult10,14. Interestingly, addition of Gata2 and Runx1 to the four-factor cocktail and thrombopoietin in the medium biases the reprogramming process towards the megakaryocytic lineage16.

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:**

We thank Evelyn Wang and Gregory Hyde (Whitehead Institute, Cambridge, MA) for cloning and Harvey Lodish (Whitehead Institute) for providing many of the plasmids used for generating the retroviral library. We thank Kavitha Siva and Sofie Singbrant (Department of Molecular Medicine and Gene Therapy, Lund University), Göran Karlsson and Shamit Soneji (Department of Molecular Hematology, Lund University) for their roles in description of iEP production. We would also like to acknowledge and thank Julian Pulecio (Centre of Regenerative Medicine, Barcelona Biomedical Research Park), Violeta Rayon-Estrada (The Rockefeller University, New York), Carl Walkley (St. Vincent’s Institute of Medical Research and Department of Medicine, St Vincent’s Hospital, University of Melbourne), Ángel Raya (Catalan Institution for Research and Advanced Studies, Barcelona), and Vijay G. Sankaran (Broad Institute of the Massachusetts Institute of Technology and Harvard, Cambridge) for their previous contributions to this work. This work was supported by the Ragnar Söderberg Foundation (to J.F.); the Swedish Research Council (to J.F.); Stiftelsen Olle Engkvist Byggmästare (to J.F.); the Swedish Foundation for Strategic Research (to J.F.); Åke Wiberg’s Foundation (to J.F.); a Marie Curie integration grant (to J.F.).

**DISCLOSURES:**

The authors have no conflicts of interest to report.

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