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Hemogenic Reprogramming of Human Fibroblasts by Enforced Expression of Transcription Factors

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Dear Stephanie Weldon and Xiaoyan Cao,

We are re-submitting our manuscript entitled: **"Hemogenic Reprogramming of Human Fibroblasts by Enforced Expression of Transcription Factors"** to the Journal of Visualized experiments.

We were pleased to see that the reviewer's response was positive. In light of reviewers comments we have modified the text and included two supplementary figures to better support our protocol. In our response (appended to this revised submission) to the reviewer's comments we have addressed their concerns and include a list of the main changes to the manuscript. We are providing a word document with track changes to the main manuscript. We believe these revisions are adequate to address the reviewer concerns. We have also made the necessary revisions to bring the paper in line with the overall guidelines of Journal of Visualized experiments.

Sincerely,

Carlos-Filipe Pereira

TITLE:

Hemogenic Reprogramming of Human Fibroblasts by Enforced Expression of Transcription Factors

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KEYWORDS:

hemogenic reprogramming, fibroblast, transcription factors, hematopoiesis, hematopoietic stem and progenitor cells, endothelial-to-hematopoietic transition

SUMMARY:

This protocol demonstrates the induction of a hemogenic program in human dermal fibroblast by enforced expression of the transcription factors GATA2, GFI1B and FOS to generate hematopoietic stem and progenitor cells.

ABSTRACT:

The cellular and molecular mechanisms underlying specification of human hematopoietic stem cells (HSCs) remain elusive. Strategies to recapitulate human HSC emergence in vitro are required to overcome limitations in studying this complex developmental process. Here, we describe a protocol to generate hematopoietic stem and progenitor-like cells from human dermal fibroblasts through a direct cell reprogramming approach. These cells transit through a hemogenic intermediate cell-type, resembling the endothelial-to-hematopoietic transition (EHT) characteristic of HSC specification. Fibroblasts were reprogrammed to hemogenic cells via transduction with GATA2, GFI1B and FOS transcription factors. This combination of three factors induced morphological changes, expression of hemogenic and hematopoietic markers and dynamic EHT transcriptional programs. Reprogrammed cells generate hematopoietic progeny

and repopulate immunodeficient mice for three months. This protocol can be adapted towards mechanistic dissection of the human EHT process as exemplified here by defining GATA2 targets during the early phases of reprogramming. Human hemogenic reprogramming provides a simple and tractable approach to address the underlying mechanisms of hematopoietic reprogramming and to identify novel markers and regulators of human HSC emergence. In the future, faithful induction of hemogenic fate in fibroblasts may contribute for the production of patient-specific HSCs for autologous transplantation.

INTRODUCTION:

Definitive hematopoietic stem cells (HSCs) emerge in the aorta-gonad-mesonephros (AGM) region and placenta of embryos from endothelial precursors with hemogenic capacity, through an endothelial-to-hematopoietic transition (EHT)[1,2]. Hemogenic precursors (HPs) express both endothelial and hematopoietic markers, but their identification remains elusive, particularly in the human system. Despite being a relatively conserved process in mammals, HSC development still differs significantly between humans and mouse models[3,4]. Therefore, in vitro approaches to recapitulate human HSC development are needed.

Differentiation of pluripotent stem cells (PSCs) to HSCs, although promising, has met limited success over the past 20 years, mostly due to imperfect differentiation protocols, which result in primitive hematopoietic progenitors with poor engraftment ability[5–7]. Alternatively, direct cell reprogramming methodologies have been applied to generate hematopoietic stem and progenitor (HSPC)-like cells from multiple cell types, using transcription factors (TFs)[8,9]. In particular, the overexpression of three TFs, Gata2, Gfi1b and cFos, converted mouse embryonic fibroblasts into HSPCs through an HP intermediate with a defined phenotype (Prom1+Sca-1+CD34+CD45-)[10]. This process resembled the EHT that occurs in the embryo and placenta, during specification of definitive hematopoiesis. This phenotype enabled the identification and isolation of a population of HPs in the mouse placenta that after short-term culture and Notch activation generate serially transplantable HSCs[11].

So far, no phenotype has been established that distinguishes human HSCs from their precursors, but some molecules are known to be expressed in emerging HSCs. Integrin alpha 6 (ITGA6 or CD49f) is highly expressed in long-term repopulating HSCs, the most immature cells in the HSC compartment[12], and angiotensin-converting enzyme (ACE or CD143) is present in CD34 negative hematopoietic precursors in all embryonic blood-forming tissues[13].

Recently, we have demonstrated that human versions of the three TFs, GATA2, FOS and GFI1B reprogram human dermal fibroblasts (HDFs) into HPs with short-term engraftment capacity[14]. In the initial phase of reprogramming, GATA2 engages open chromatin and recruits GFI1B and FOS to repress fibroblast genes and activate endothelial and/or hematopoietic genes. Induced cells highly expressed CD49f and ACE, and contained a small percentage of cells expressing the HSPC marker CD34. The CD9 gene, which is expressed in HSCs[15] and important for HSC homing[16], was shown to be a direct target of GATA2 and among the most upregulated genes in reprogrammed cells[14]. CD9 may therefore constitute an additional marker for HPs of human definitive hematopoiesis.

In this protocol, we describe the generation of HSPC-like cells from human fibroblast through enforced expression of GATA2, GFI1B and FOS, as well as an adapted method for chromatin immunoprecipitation (ChIP)-sequencing (seq) analysis at the onset of reprogramming. TFs were encoded in a doxycycline (DOX)-inducible lentiviral vector (pFUW-tetO) that contains a tetracycline response element (TRE) and a minimal CMV promoter, and were transduced together with a constitutive vector containing the reverse tetracycline transactivator protein (pFUW-M2rtTA). When DOX (analog of tetracycline) is added after transduction, it binds to the rtTA protein which interacts with the TRE allowing TF transcription (Tet-On system). The procedure requires 25 days to complete. For ChIP-seq experiments, HDFs were transduced with flagged versions of GATA2 (pFUW-tetO-3xFLAG-GATA2) and GFI1B (pLV-tetO-HA-GFI1B), plus pFUW-tetO-FOS and TF binding sites were analyzed two days after DOX supplementation.

Ultimately, hemogenic reprogramming of human fibroblasts provides an in vitro tractable system to study the mechanisms underlying human developmental hematopoiesis and a potential source of patient-specific HSPCs for future clinical application.

PROTOCOL:

This protocol was preformed according to Lund University's human research ethics committee guidelines and should be done in accordance with each institutional guideline.

1. Reagent preparation

1.1. For Dulbecco's modified Eagle medium (DMEM)/20% fetal bovine serum (FBS), mix high glucose DMEM containing sodium pyruvate with 20% FBS, 1% penicillin-streptomycin (pen/strep), 1% L-glutamine, 1% non-essential amino acids and 10^{-4} M 2-mercaptoethanol.

1.2. For DMEM, mix high glucose DMEM containing sodium pyruvate with 10% FBS, 1% Pen/strep and 1% L-glutamine.

1.3. For hematopoietic medium, mix hematopoietic medium (**Table of Materials**) with 10^{-6} M hydrocortisone and 1% pen/strep.

1.4. Use phosphate-buffered saline (PBS) without calcium or magnesium.

2. Human dermal fibroblast isolation

NOTE: HDFs can be purchased from certified suppliers (**Table of Materials**). In this case, expand fibroblasts and use them directly in reprogramming experiments (section 4). Alternatively, HDFs can be isolated from donors. If fibroblasts are isolated from different donors, keep the samples separated from each other at all steps of the protocol. Label plates/wells and collection tubes with the identification number of the donors.

2.1. Obtain HDFs from 3 mm round skin punch biopsies performed by qualified physicians.

2.2. Coat three wells of a tissue-culture treated 6-well plate with 500 µL of 0.1% gelatin and incubate for 20 min at 37 °C.

2.3. Aspirate the remaining gelatin solution and add 750 µL of DMEM/20% FBS to each well. The entire surface of the well should be covered with medium.

2.4. Add 1.5 mL of DMEM/20% FBS to the inside surface of a sterile 100 mm petri dish lid and spread the drop with the aid of a 5 mL serological pipette.

2.5. Place the skin biopsy in the medium on the lid with sterilized forceps.

2.6. Dissect the skin biopsy into nine identical sections, using one sterilized scalpel to hold the biopsy in place and a second scalpel to cut.

2.7. Place three biopsy pieces per well using pointed forceps. Make sure the pieces attach to the bottom of the well.

2.8. Lay a 22 mm coverslip on top of the pieces and apply some pressure.

2.9. Incubate the plate at 37 °C, 5% CO_2, for a week. Check cells daily and add 200 µL of DMEM/20% FBS every 2 days to replace evaporated medium.

2.10. After one week, add up to 2 mL of DMEM/20% FBS and replace medium every 2−3 days.

2.11. Passage cells in a 1:4 ratio when wells are confluent (about 4−8 weeks).

2.11.1. Prepare 0.1% gelatin coated tissue-culture treated 6-well plates.

2.11.2. Aspirate medium from wells at 80% confluency and wash once with 1 mL of PBS.

2.11.3. Remove coverslip with sterile forceps and place the coverslip into a new well of a 6-well plate, with the tissue side up.

NOTE: Cells that remained attached to the coverslip will also be harvested.

2.11.4. Add 500 µL of dissociation solution (**Table of Materials**) per well (including the wells with the coverslips) and incubate at 37 °C, 5% CO_2 for 5−10 min. Check when cells start to rise from the bottom of the well and inactivate the dissociation solution by adding 500 µL of DMEM/20% FBS to each well.

2.11.5. Collect fibroblasts from all the wells into a conical 15 mL conical. Add extra medium to the wells to collect the remaining cells. Centrifuge the tube at 350 x *g* for 5 min.

2.11.6. In the meantime, add 500 µL DMEM/20% FBS to each well of previously gelatin coated plates.

2.11.7. Aspirate medium and resuspend fibroblasts in 6 mL of DMEM/20% FBS.

2.11.8. Add 500 µL of fibroblast suspension to each well (two 6-well plates per sample/donor in total). Incubate cells overnight at 37 °C, 5% CO_2.

2.12. On the next day, add 1 mL of DMEM/20% FBS to each well. Replace medium with 2 mL of fresh DMEM/20% FBS every 2−3 days until wells are 80% confluent.

2.13. Repeat section 2.11 for three 80% confluent wells until third passage is reached.

2.14. Freeze fibroblasts from confluent wells (passages 1 and 3).

2.14.1. Aspirate medium from the wells and wash once with 1 mL of PBS.

2.14.2. Dissociate and collect fibroblasts as described in steps 2.11.4 and 2.11.5.

2.14.3. Count cells with a hemocytometer and centrifuge the tube at 350 x *g* for 5 min.

2.14.4. After centrifugation, aspirate medium and resuspend fibroblasts in FBS with 10% DMSO at a density of 5 x 10^5 cells/mL.

2.14.5. Add 1 mL of the cell suspension per cryovial and freeze cells overnight at -80 °C using a freezing container. Move vials to -150 °C (liquid nitrogen) for long-term storage.

3. Lentiviral production

3.1. Grow HEK293T cells in a 100 mm tissue-culture treated dish with 10 mL of DMEM, at 37 °C, 5% CO_2, until confluency is reached.

3.2. On the day prior to transfection, aspirate medium and wash the plate carefully with 5 mL of PBS.

3.3. After removing PBS, add 1.5 mL of dissociation solution and incubate at 37 °C, 5% CO_2 for 5−10 min to dissociate cells from the plate.

NOTE: It is recommended to warm both PBS and dissociation solution before using, so that cells do not suffer a thermal shock.

3.4. Inactivate dissociation solution with 3 mL of DMEM and transfer the cell suspension to a 15 mL conical tube. Wash plate with 5 mL of DMEM to remove remaining attached cells and transfer

this volume to the 15 mL conical tube.

3.5. Centrifuge cell suspension at 350 x *g* for 5 min.

3.6. Aspirate supernatant and split the cell pellet evenly between six 100 mm tissue-culture treated dishes in a final volume of 10 mL DMEM per plate. Cells should be at a confluency around 60% by the time of transfection.

3.7. On the next day, transfect cells with different plasmid mixes as follows:

NOTE: This part of the protocol describes the production of lentiviruses in one 100 mm tissue-culture treated plate per plasmid mix. To obtain higher volumes of lentiviral supernatant for concentration, use at least four 100 mm HEK293T cell plates per mix.

3.7.1. In a 15 mL conical tube, add 10 µg of the three transfer plasmids together: 3.33 µg of pFUW-tetO-GATA2 (Addgene plasmid #125028)[14], 3.33 µg of pFUW-tetO-GFI1B (Addgene #125597)[14] and 3.33 µg of pFUW-tetO-FOS (Addgene #125598)[14], plus 10 µg of the 2nd generation psPAX2 packaging vector encoding the *Gag*, *Pol*, *Tat* and *Rev* genes (Addgene #12260) and 5 µg of pMD2.G envelope vector encoding the *VSV-G* gene (Addgene #12259). Add water up to 500 µL.

3.7.2. In two new 15 mL conical tubes add 10 µg of FUW-M2rtTA plasmid (Addgene #20342)[17], 10 µg of psPAX2 packaging vector and 5 µg of pMD2.G envelope vector to each tube. Add water up to 500 µL. One tube is going to be used as a control.

3.7.3. To each tube add 62.5 µL of 2 M $CaCl_2$. Next, release bubbles into each mixture using a Pasteur pipet inserted into a pipet controller. While bubbles are forming, pipette 500 µL of *N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffered saline (pH 7.1, 25 °C), with a P1000 pipette, drop-wise against the Pasteur pipet and onto the mixture.

3.7.4. Incubate tubes at room temperature for at least 15 min. The mixtures will appear slightly cloudy after some time.

3.8. Meanwhile, aspirate medium from HEK293T cell plates (passaged the day before) and add 10 mL of DMEM + 10% FBS + 1% L-glutamine (without pen/strep). Be careful and add media slowly to the plates as HEK293T cells are semi-adherent.

3.9. Distribute each individual mixture (approximately 1 mL) evenly and drop-wise into separate plates and incubate overnight at 37 °C, 5% CO_2.

3.10. Replace medium with 4 mL of DMEM, 24 h after incubation. Incubate overnight at 37 °C, 5% CO_2. If available, incubate at 32 °C, 5% CO_2, as the reduced temperature will increase the half-life of the lentiviral particles.

3.11. Collect supernatant with lentiviral particles three times to a 50 mL conical tube. Do not mix different lentiviral particles at this point. Each plate will result in 12 mL of lentiviral supernatant. Four plates of the same viral preparation fit into one 50 mL conical tube.

CAUTION: Perform lentiviral collection in a biosafety level-2 laboratory in a laminar flow hood dedicated for the effect and place viral contaminated waste (tubes, tips, plates) in an appropriate residue container for biohazardous materials.

3.11.1. Do the first collection 16 h after the last incubation and add 4 mL of DMEM. Incubate at 37 °C, 5% CO_2.

3.11.2. Do the second collection 8 h after the first to the same tube, add 4 mL of DMEM and incubate at 37 °C, 5% CO_2.

3.11.3. Do the third collection 16 h after the second to the same tube and discard the plates.

3.12. Filter each lentiviral supernatant using a 0.45 µm low-protein binding filter with a cellulose acetate membrane (**Table of Materials**) to a clean tube.

3.13. Add a maximum of 15 mL of filtered supernatant to a centrifugal filter unit with a regenerated cellulose membrane (**Table of Materials**) and spin at 4000 x *g* for 25 min. Discard flow-through. A viscous liquid containing lentiviruses will remain in the filter unit.

3.14. Repeat step 3.13 by adding 15 mL of supernatant on the top of the filter unit, until there is no more lentiviral supernatant left.

NOTE: When there are only a few milliliters of supernatant to concentrate, decrease the spinning time to 10 min. If there is still extra liquid (non-viscous) on the filter, centrifuge for an additional 10 min.

3.15. Make aliquots (50−200 µL depending on the initial supernatant volume) of each type of concentrated lentiviruses and store at -80 °C for long-term storage (1−2 years) or at 4 °C for short-term storage (1−2 weeks).

NOTE: Concentrated or non-concentrated lentiviruses can also be used fresh. Do not re-freeze and thaw as this results in reduced titer.

4. Hemogenic reprogramming

NOTE: Use HDFs with a passage number of three (P3) or higher (until P10) to perform experiments.

4.1. Coat a 100 mm plate with 5 mL of 0.1% gelatin and incubate at 37 °C for 20 min. Aspirate the remaining gelatin solution.

4.2. Thaw a fibroblast vial and plate cells in the 0.1% gelatin coted dish. Incubate overnight at 37 °C, 5% CO_2. If necessary, expand fibroblasts for a longer period of time until the desired passage and confluency are reached.

4.3. Coat 6-well tissue-culture treated plates with 500 µL of 0.1% gelatin solution and incubate at 37 °C for 20 min. Remove extra gelatin.

4.4. Plate HDFs at a density of 150,000 cells per plate (25,000 cells per well) in a final volume of 2 mL DMEM per well. Incubate overnight at 37 °C, 5% CO_2, to let cells attach.

4.5. Replace medium with 2 mL of DMEM plus 8 µg/mL polybrene. Prepare a 1:1 ratio mix of pool-produced TFs lentiviruses and M2rtTA in a new microcentrifuge tube.

NOTE: In this protocol, pool-production of lentiviruses for the three TFs is performed, which, in authors' hands, results in higher reprogramming efficiency. Alternatively, it is suggested to perform a titration of the individual lentiviral particles by qPCR[18], on a standard cell line. This will be used to define the volume of individual viruses necessary to meet a multiplicity of infection (MOI) optimal for co-transduction and hemogenic reprogramming.

4.6. Distribute 10 to 100 µL of lentiviral mixture per well, to transduce HDFs. This is day -2 of reprogramming.

NOTE: Defining the optimal volume of lentiviral mix for efficient reprogramming, without compromising cell viability, requires optimization (see **Supplementary Figure 1** for more details). HDFs with more than 7 passages may require higher volumes of viruses than cells with lower passages.

4.7. After 16 hours of incubation, remove viruses and add DMEM. Allow cells to recover for 6–8 h.

4.8. After recovery, aspirate medium and add 2 mL of DMEM with 8 µg/mL polybrene.

4.9. Do a second transduction as described in step 4.6 and incubate at 37 °C, 5% CO_2 for 16 h. This is day -1 of reprogramming. The lentiviral mix can be performed on day -2 for both transductions and kept at 4 °C.

4.10. On the next day, remove the viruses and add DMEM supplemented with 1 µg/mL DOX. This is day 0 of reprogramming. Incubate at 37 °C, 5% CO_2 for 48 h.

4.11. At day 2 of reprogramming, split each well in a 1:2 ratio.

4.11.1. Aspirate medium and wash cells with 1 mL of PBS.

4.11.2. Aspirate PBS and dissociate cells with 500 µL of dissociation solution. Incubate 5−10 min at 37 °C, 5% CO_2.

4.11.3. Inactivate the dissociation solution with 1 mL of DMEM and collect cells into a conical tube. Centrifuge at 350 x *g* for 5 min.

4.11.4. Resuspend the pellet in hematopoietic medium (see step 1.3), supplemented with 1 µg/mL DOX, and plate cells into new tissue-culture treated 6-well plates coated with 0.1% gelatin at a final volume of 2 mL per well.

4.12. Change medium (hematopoietic medium plus DOX) twice a week for the duration of the reprogramming cultures (25 days).

4.13. Analyze resulting reprogrammed cells at different time points by brightfield or fluorescence microscopy (see **Supplementary Figure 2**), flow cytometry, bulk and single-cell mRNA sequencing, and transplantation assays for the acquisition of hematopoietic morphology, presence of endothelial and hematopoietic markers, acquisition of endothelial/hematopoietic gene expression profile and regeneration capacity[14].

5. Protocol optimization for assessing transcription factor binding sites at the onset of hemogenic reprogramming

5.1. Plate 300,000 HDFs (<P8) in 0.1% gelatin coated tissue-culture treated 6-well plates with DMEM to a final volume of 2 mL per well. Incubate overnight at 37 °C, 5% CO_2.

5.2. On the following day, replace medium with DMEM supplemented with 8 µg/mL polybrene.

5.3. Transduce cells with individual factors: pFUW-tetO-FOS[14], pLV-tetO-HA-GFI1B (Addgene #125599)[14] and pFUW-tetO-3xFLAG-GATA2 (Addgene #125600)[14] or with a pool of the three factors, plus FUW-M2rtTA in a 1:1 ratio. Use 10−20 µL total virus (individual TF + M2rtTA or three TFs + M2rtTA). Incubate cells overnight at 37 °C, 5% CO_2.

NOTE: It is recommended to use twelve 6-well plates per condition (for each individual TF and the three TFs combined).

5.4. Remove lentiviruses and add DMEM 16 h after the first transduction. Let cells recover for 6−8 h.

5.5. Transduce cells a second time with the same amount of virus per condition and incubate at 37 °C, 5% CO_2.

5.6. On the next day remove viruses and add fresh DMEM. Incubate at 37 °C, 5% CO_2 for 24 h.

5.7. Re-plate each well into a 0.1% gelatin coated tissue-culture treated 100 mm dish with DMEM

to a final volume of 10 mL per dish. This represents approximately a 1:6 passage.

5.8. Allow cells to grow for 6 days at 37 °C, 5% CO_2.

5.9. On day 6 after re-plating, aspirate medium and add DMEM with 1 µg/mL DOX. Incubate cells at 37 °C, 5% CO_2 for 2 days.

5.10. Collect fibroblasts and analyze genomic binding sites of the three TFs transduced individually or in combination, by ChIP-seq 2 days after DOX supplementation[14].

NOTE: The final seventy-two 100 mm dishes will contain between 20−50 x 10^6 cells, sufficient to perform ChIP-seq experiments and replicates.

REPRESENTATIVE RESULTS:

A schematic representation of the reprogramming approach using HDFs is illustrated in **Figure 1A**. Fibroblasts are acquired from commercial sources or collected from human donors and expanded in vitro previous to reprogramming. After plating, cells are transduced twice with GATA2, GFI1B and FOS (and M2rtTA) lentiviruses, and doxycycline is added at day 0 of reprogramming. On day 2, cells are split and plated in hematopoietic medium until day 25 of culture. Reprogrammed cells may be generated at different time points for multiple applications including transplantation in immunocompromised mice, single-cell mRNA-sequencing (scRNA-seq) of FACS purified cell populations (day 2 unsorted, day 15 CD49+ and day 25 CD49f+CD34+ cells), as well as microscopy and flow cytometry analysis for the cell surface markers CD49f, CD34, CD9 and CD143. Representative cytometry plots show ~17% of reprogrammed cells expressing both CD49f and CD9 (**Figure 1B, left panel**), after 25 days of reprogramming. The majority of double positive cells express CD143 (~86%), and a small population express CD34 (0.9%), suggesting a dynamic hemogenic fate induction. These markers are not activated in M2rtTA transduced HDFs cultured for 25 days (**Figure 1B, right panel**). Immunofluorescence images confirm expression of CD9 and CD143 in adherent and round cells, morphologically distinct from fibroblasts that are negative for these markers (**Figure 1C**). Human hemogenic colonies also express CD49f and CD34[14]. Single cell analysis of HDFs, day 2 (unsorted), and purified reprogrammed cells day 15 (CD49f+CD34-) and day 25 (CD49f+CD34+) show a stepwise increase in CD49f, CD9 and CD143 expression from day 2 to day 25. CD49f and CD9 positive cells appear first during the reprogramming process, between day 2 and 15, indicating that these molecules may represent markers of early human hemogenesis. CD143 expression starts to be detected at day 15 and CD34 expressing cells are detected only at later time points (day 25). CD34+ umbilical cord blood (UCB) cells were used as reference (**Figure 1D**).

Figure 2A describes a modified protocol to generate sufficient number of cells for ChIP-seq analysis at the initial stages of hemogenic reprogramming (day 2). First, HDFs are plated at a density two times higher than in the standard protocol (300,000 cells versus 150,000 cells per plate). After transduction, each well is re-plated into a 100 mm dish allowing cells to expand for 6 days before supplementing media with DOX. Cells are analyzed 2 days after adding DOX and consequent TF expression. **Figure 2B** shows genome browser profiles of GATA2 binding to

genomic regulatory regions of *ITGA6* and *ACE* when cells are co-transduced with the three factors (3TFs) or GATA2 individually. GATA2 also binds to open chromatin regions of *CD9* and *CD34* genes[14].

FIGURE LEGENDS:

Figure 1: Induction of hemogenic fate in human dermal fibroblasts. (**A**) Experimental strategy for hemogenic reprogramming of human dermal fibroblasts (HDFs). Fibroblast from skin punch biopsies are collected from donors, expanded and transduced with GATA2, GFI1B, FOS and M2rtTA lentiviruses. Doxycycline (DOX) is added to the culture at day 0 of reprogramming and cells are analyzed at several time points until day 25. scRNA-seq, single cell mRNA-sequencing. FACS, Fluorescence-Activated Cell Sorting. (**B**) Gating strategy used to evaluate the expression of hemogenic/hematopoietic markers by flow cytometry at day 25 after transduction with the three transcription factors (3TFs). Cytometry plots show percentage of double positive cells for CD49f and CD9, gated in the live-cell population (DAPI-negative). Within the double positive population, the expression of CD143 and CD34 is shown. HDFs transduced only with M2rtTA virus under the same culture conditions are used as control. (**C**) Immunofluorescence images of day 25 reprogrammed colonies confirming the expression of CD9 (upper panel) and CD143 (lower panel) Independent wells were stained with individual antibodies (**Table of Materials**) diluted 1:100 in PBS with 2% FBS with mouse serum (1:100), incubated 20 min at 37 °C, 5% CO_2, washed three times and imaged in PBS with 2% FBS. Phase, phase-gradient contrast. Scale bars, 50 µm. (**D**) scRNA-Seq analysis of 253 cells at different time points. Expression of *ITGA6*, *CD9*, *ACE* and *CD34* is activated during reprogramming. Cells are collected at day 2 (unsorted), day 15 (CD49f+CD34-) and day 25 (CD49f+CD34+). HDFs and CD34+ umbilical cord blood (34+UCB) cells are used as references.

Figure 2: Expansion of human dermal fibroblasts for ChIP-seq analysis. (**A**) Experimental strategy depicting a modified protocol to generate high numbers of transduced human dermal fibroblasts (HDFs) for ChIP-seq at day 2 of reprogramming. 300,000 cells are plated in 6-well plates and transduced twice with individual factors (pFUW-tetO-FOS, pLV-tetO-HA-GFI1B pFUW-tetO-3xFLAG-GATA2) or a combination of the three factors (plus M2rtTA). After removing viruses, fibroblasts are expanded for six days in 100 mm dishes. Doxycycline (DOX) is added at day 0 and cells are collected two days after DOX addition. (**B**) Genome browser profiles highlighting GATA2-binding sites (grey boxes) at *ITGA6* and *ACE loci* two days after transduction with the three transcription factors (3TFs) or with GATA2 alone. The total number of mapped reads is represented on the y-axis.

Supplementary Figure 1: Defining an optimized lentiviral volume for efficient hemogenic reprogramming. Increasing volumes of concentrated (10 to 100 µL) pool-produced lentiviral particles (3TFs: GATA2, GFI1B and FOS) are used to transduced HDFs, together with M2rtTA in a ratio of 1:1, following steps 4.5−4.12 of the protocol. Reprogrammed cells are analyzed at day 25 to define an optimal volume of transduction for hemogenic reprogramming, given by the percentage of CD49f+CD9+ cells gated in live-cells (DAPI-negative). Cell viability can be assessed

by quantifying the absolute number of live cells at day 25. HDFs transduced with M2rtTA (100 µL) are used as control.

Supplementary Figure 2: Morphological characteristics during hemogenic reprogramming of human dermal fibroblasts. Human Dermal Fibroblast (HDF) cultures are imaged at the day of the first transduction (day -2), when DOX is added to the cultures (day 0), two days after DOX supplementation (day 2) and at the end-point of the experiment (day 25). Hemogenic colonies at day 25 are highlighted. Scale bars, 100 µm.

DISCUSSION:

In this article, a method is described to generate hematopoietic progenitor cells directly from human fibroblasts, which go through and HP cell intermediate, similarly to definitive HSCs[14].

Pool-production of lentiviral particles encoding GATA2, GFI1B and FOS was preferred over individual production, since in our hands it results in higher reprogramming efficiencies (unpublished data). Lentiviruses, as members of the *Retroviridae* family, normally contain two copies of positive single-stranded RNA[19]. The increased reprogramming efficiency may be due to packaging of two different transgenes in the same lentiviral particle, resulting in increased number of cells co-transduced with the three transcription factors. To ensure the success of this protocol, it is necessary to transduce HDFs with adequate amount of virus depending on the cell passage to obtain an optimal balance between reprogramming efficiency and cell viability, as recommended in step 4.6. Moreover, fresh non-concentrated viruses can be used. It is recommended to transduce cells with 0.5-3 mL of 3TFs pool and M2rtTA. Also, cell density should be adjusted according to the application. 150 000 HDFs per 6-well plate (step 4.4) provided the optimal density to perform FACS, transplantation and flow cytometry analysis of reprogrammed cells. For ChIP-seq experiments, more cells were required from the beginning (step 5.1). It is important to check cells regularly for morphological changes and replace hematopoietic medium twice a week to support the emergence of induced hematopoietic cells. Addition of hematopoietic cytokines or co-culture in feeder layers may increase reprogramming efficiency.

With this method, it was possible to demonstrate the expression of new hematopoietic markers that are dynamically expressed during hemogenic reprogramming. CD9, which was shown to be up regulated in reprogrammed cells at the transcriptional level[14], is rapidly activated at the initial phases of reprogramming and is expressed at the cell surface of CD49f+CD143+ cells, serving as a novel marker of human HSC precursors. We also show that *ITGA6* and *ACE* are direct targets of GATA2 during the initial stages of hemogenic reprogramming, in addition to *CD9* and *CD34*[14], providing a direct mechanistic link between human hemogenic precursor phenotype and GATA2. One advantage of this system resides in the use of relatively homogeneous fibroblast cultures. While PSCs are easily expanded and maintained *in vitro,* differentiation protocols generate heterogeneous populations that include hematopoietic progenitors, which engraft poorly[5–7]. Moreover, there is a risk of tumorigenesis when transplanting PSC-derived HSPCs, since undifferentiated PSCs may still remain in culture after the differentiation protocol. Alternatively to fibroblasts, direct reprogramming to HSCs has been applied to blood-committed progenitors[20] and endothelial cells[21]. However, starting with blood-restricted progenitor cells hinders

therapeutic application of the resulting HSCs if the patient carries mutations that affect the stem/progenitor hematopoietic population[22]. In the case of endothelial cells, these are more difficult to obtain compared to fibroblasts, and constitute a very heterogeneous cell population in terms of phenotype, function and structure, which are organ-dependent[23]. Other studies have succeeded in reprogramming mouse fibroblasts into engraftable hematopoietic progenitors[24,25] yet, so far, no other protocol describes the generation of HSPC-like cells from human fibroblasts.

This approach, coupled with pharmacological inhibition, gene knock-out, or knock-down permits to define individual or combination of factors that are required to directly induce human HSCs. Employing high efficiency screening methodologies based on recent CRISPR-Cas9 technologies in HDFs prior to reprogramming, represents an exciting endeavor for defining novel regulators of human definitive hematopoiesis. In the future, reprogramming non-blood related human cell types such as fibroblasts will serve as a platform to generate healthy patient-tailored hematopoietic progenitor cells for clinical applications.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1
Figure 1

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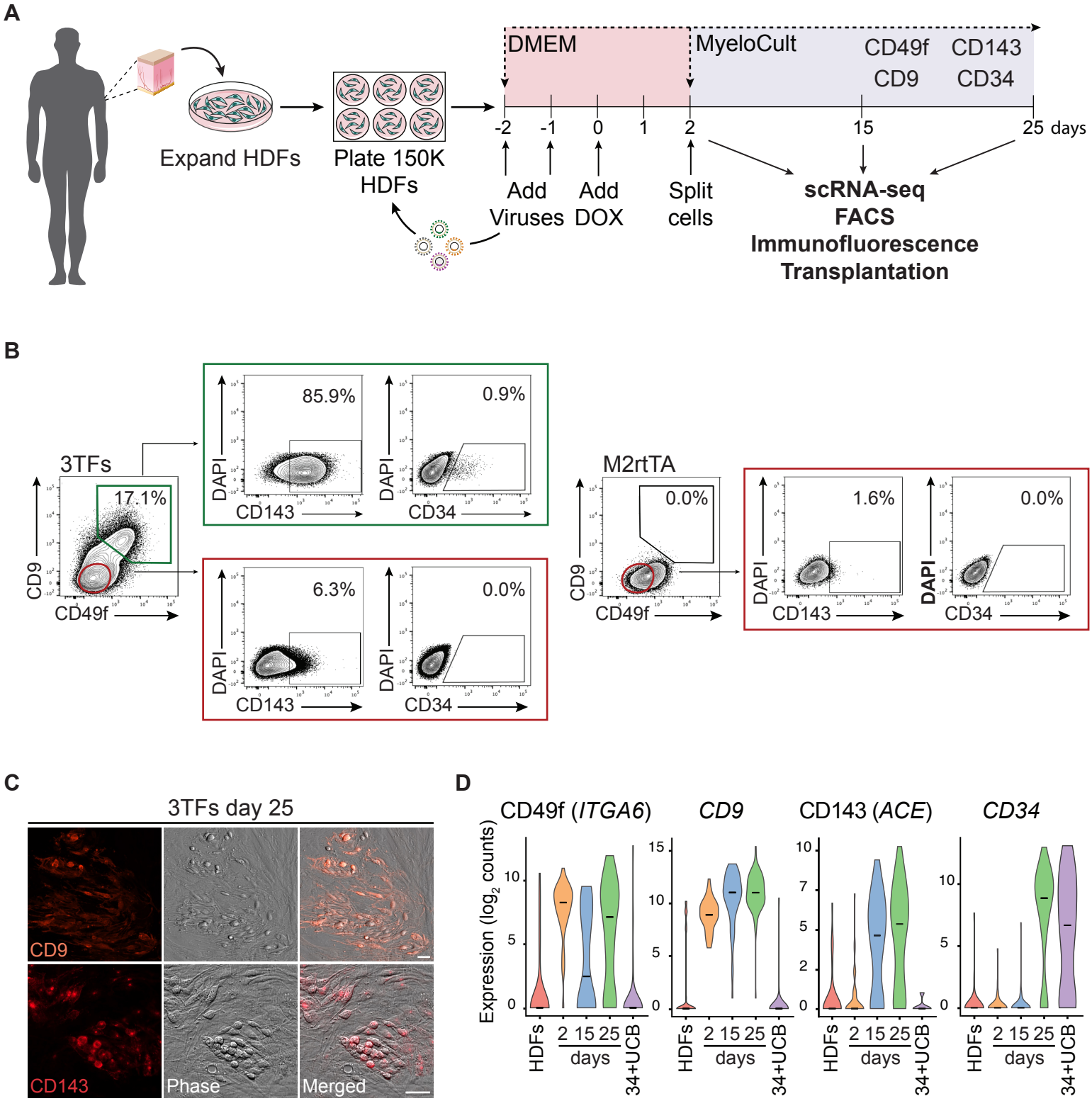

Figure 2
Figure 2

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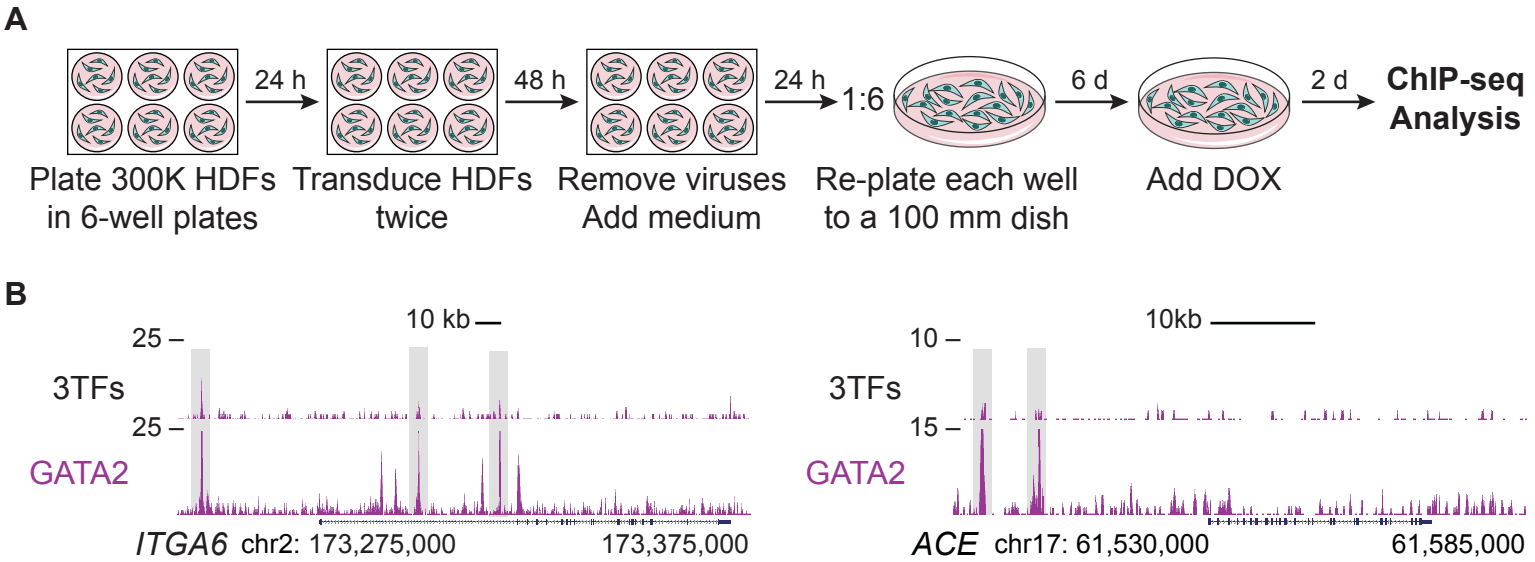
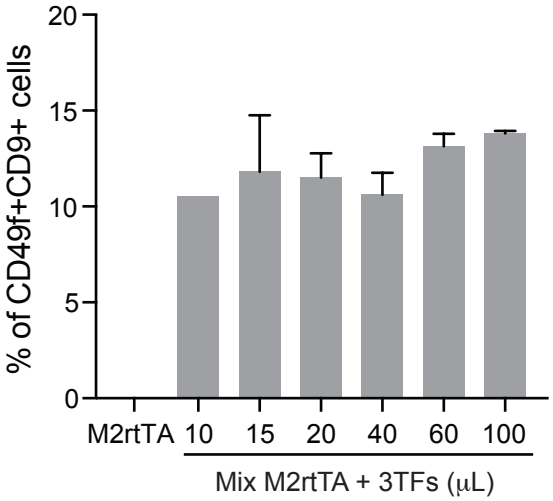
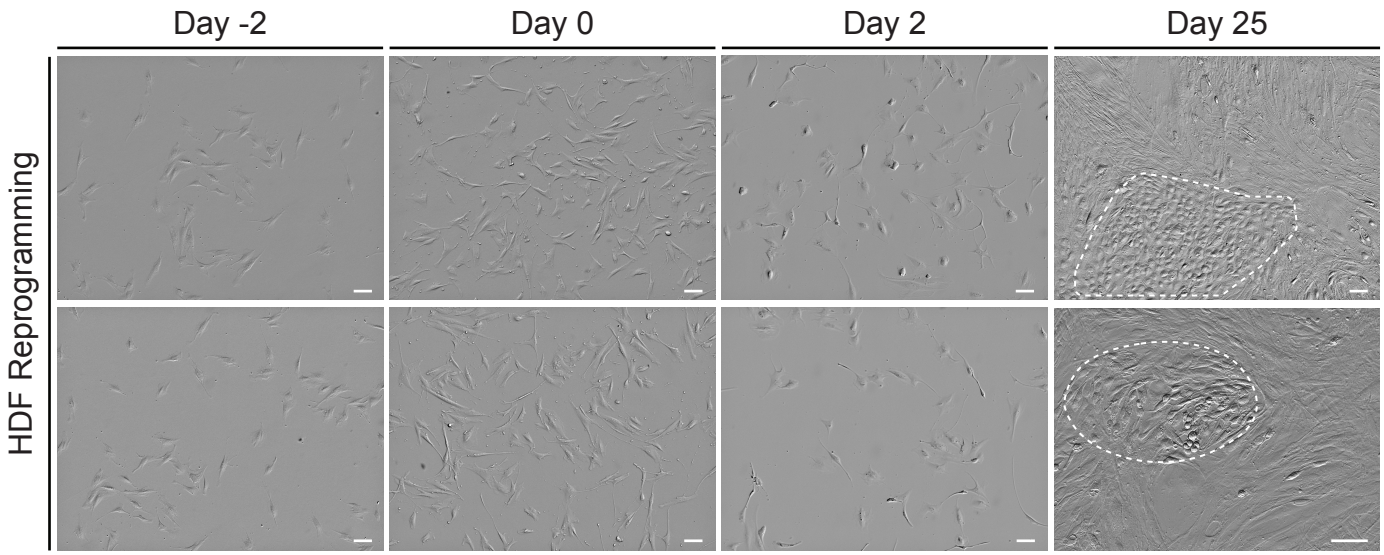


Supplementary Figure 1



Supplementary Figure 2



Reagent and Materials	Company	Catalog Number
0.45 µm low-protein binding filter, 150 mL Bottle Top Vacuum Filter	Corning	#430625
2-Mercaptoethanol	Sigma-Aldrich	#M6250
Alexa Fluor 488 anti-human CD34 clone 581	BioLegend	#343518
BD Pharmingen APC Mouse Anti-Human Angiotensin Converting Enzyme (CD143) clone B	BD Biosciences	#557929
BES buffered saline	Sigma-Aldrich	#14280
Calcium chloride (CaCl2)	Sigma-Aldrich	#449709
Centrifugal filter unit, Amicon Ultra-15 Centrifugal Filter Unit	Sigma-Aldrich	#UFC903096
Dissociation solution, TrypLE Express Enzyme (1X) no phenol red	Gibco	#12604-021
Doxycycline hyclate (DOX)	Sigma-Aldrich	#D9891
eBioscience CD49f (Integrin alpha 6) Monoclonal Antibody (eBioGoH3 (GoH3)), PE-Cyanine	Invitrogen	#25-0495-82
FUW-M2rtTA	Addgene	#20342
Gelatin from Porcine Skin Type A	Sigma-Aldrich	#G1890
Gibco L-Glutamine (200 mM)	ThermoFisher Scientific	#25030-024
Gibco MEM Non-Essential Amino Acids Solution (100X)	ThermoFisher Scientific	#11140-035
Hematopoietic medium, MyeloCult H5100	STEMCELL Technologies	#05150
Hexadimethrine bromide (polybrene)	Sigma-Aldrich	#H9268
Human Dermal Fibroblasts (HDFs)	ScienCell	#2320
HyClone Dulbecco's Modified Eagles Medium (DMEM)	GE Healthcare	#SH30243.01
HyClone Fetal Bovine Serum (FBS)	GE Healthcare	#SV30160.03
HyClone Penicillin Streptomycin 100X Solution (Pen/Strep)	GE Healthcare	#SV30010
HyClone Phosphate Buffered Saline solution (PBS)	GE Healthcare	#SH30256.01
Hydrocortisone	STEMCELL Technologies	#7904
Mouse serum	Sigma-Aldrich	#M5905
PE anti-human CD9 Antibody clone HI9a	BioLegend	#312105
pFUW-tetO-3xFLAG-GATA2	Addgene	#125600
pFUW-tetO-FOS	Addgene	#125598
pFUW-tetO-GATA2	Addgene	#125028
pFUW-tetO-GFI1B	Addgene	#125597
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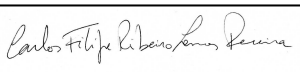
Signature:	
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Hemogenic Reprogramming of Human Fibroblasts by Enforced Expression of Transcription Factors

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All relevant details including steps and sub-steps were highlighted.

12. References: Please do not abbreviate journal titles.
The journal titles were corrected and written in full.

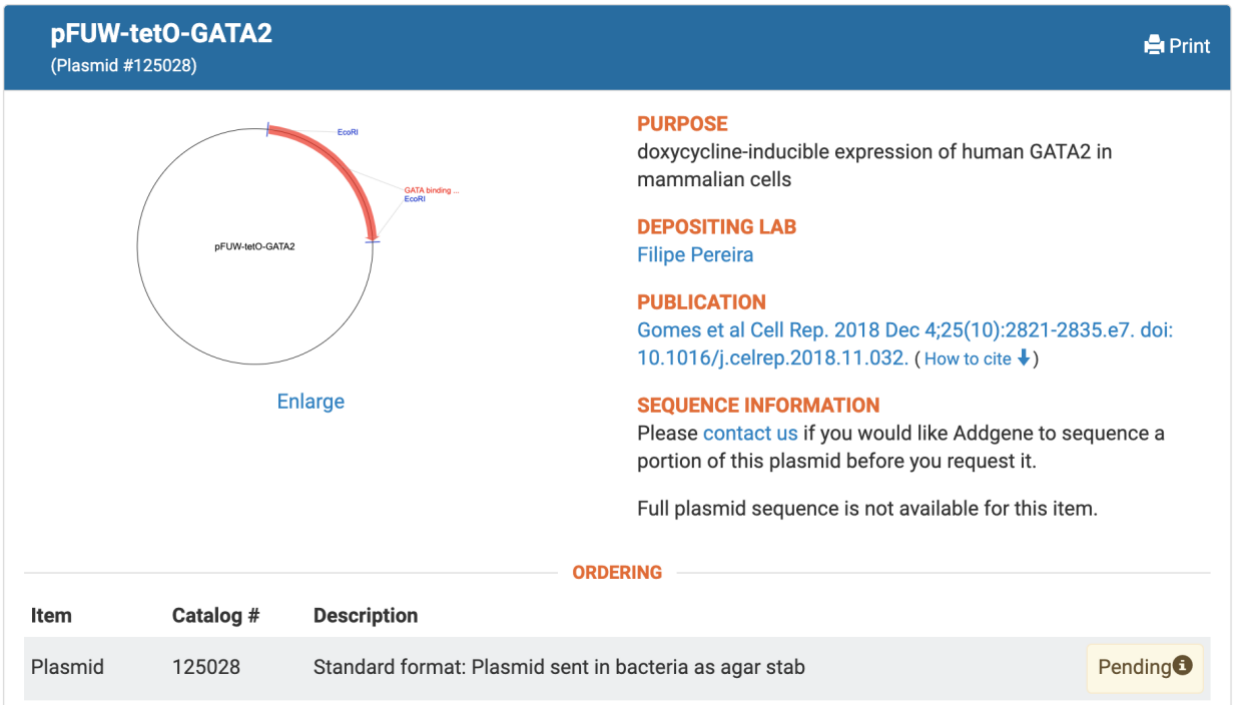
Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this work, the authors describe a protocol for programming human skin fibroblasts to hemogenic progenitors by lentiviral vector-based, doxycyline-inducible expression of the transcription factors GATA2, GFI1B and FOS.
The title and abstract are appropriate for this article, the individual steps are clearly explained in the text and will likely lead to the described hemogenic conversion of fibroblasts in the hands of an experienced researcher, provided that the following points are carefully addressed:

We would like to thank this reviewer for the positive outlook and for finding the title and abstract appropriate, as well as, for all the suggestions provided. The comments and concerns addressed helped us improving our paper greatly.

Major Concerns:

1) The materials necessary for lentiviral vector production are incomplete: the Addgene numbers for pFUW-tetO-3xFLAG-GATA2 (#125600), pFUW-tetO-FOS (#125598), pFUW-tetO-GATA2 (#125028), pFUW-tetO-GFI1B (#125597) and pFUW-tetO-HA-GFI1B (#125599) are wrong; at least the given numbers don´t exist in the Addgene-database. Those should be checked and corrected.

All plasmids were submitted to Addgene in parallel to manuscript submission. We have double checked and the IDs are correct. The distribution is still pending (see print screen below with Gata2 plasmid example). We have contacted Addgene and they are now waiting for the sequencing results to make these plasmids available to the community in the near future. For now, it is only possible to see the information regarding each plasmid through the corresponding author's account.



2) A critical part of this protocol is the efficient transduction of primary fibroblasts with the lentiviral vectors, as the authors state themselves (page 9, lines 374/375; "To ensure the viability of this protocol, it is necessary to transduce cells with a proper amount of virus depending on the cell passage to obtain the best results, as recommended in step 4.6.1."). The necessary amount of a virus is usually defined as "multiplicity of infection" (MOI), i.e. the amounts of infectious units per target cell. To be able to define the volume of virus-containing supernatant necessary to achieve a desired MOI, titration needs to be done beforehand. In addition, a titration step will also provide information whether virus production has worked at all, which is especially important to avoid possible wasting the valuable primary fibroblasts in the next step. Unfortunately, neither a titration protocol nor a minimal MOI necessary to achieve successful co-transduction with all four vectors is

provided. Instead, only volumes of collected and concentrated vector-containing medium are given (page 6, 4.6.1., from line 250 on).
It would be very helpful to at least recommend titrating the virus on cells of a standard cell line before proceeding to fibroblast transduction. Calculation can be done either by flow-cytometry (if a fluorescence marker is co-expressed) or by qRT-PCR using vector-specific primers. In case of the pooled virus-production described in step 3.7.1, titration may be somewhat difficult, indeed. However, to increase the likelihood of a successful implementation of this protocol by colleagues, one could recommend to produce and titrate the viral vectors separately, instead.

We understand the reviewer's concern about the efficiency of transduction of fibroblast cultures and thank this reviewer for the suggestions.
In line with the above comment, we have restructured steps 4.5 to 4.12 to include the reviewer's suggestions regarding an alternative approach that includes the titration of individual vectors by qPCR necessary to achieve a desired MOI, and a supplementary figure (Supplementary Figure 1) to illustrate the reprogramming efficiency using different volumes of pool-produced lentiviruses, representing a "functional titration". The rephrased steps can be found here:

4.5 Replace medium with 2 mL DMEM plus 8 µg/mL polybrene and prepare a 1:1 ratio mix of pool-produced TFs lentiviruses and M2rtTA in a new microfuge tube.
NOTE: In this protocol, we perform pool-production of lentiviruses for the three TFs that, in our hands, results in higher reprogramming efficiency. Alternatively, it is suggested to perform a titration of the individual lentiviral particles by qPCR[1], on a standard cell line. This will be used to define the volume of individual viruses necessary to meet a multiplicity of infection (MOI) optimal for co-transduction and hemogenic reprogramming.

4.6 Distribute 10 to 100 µL of lentiviral mixture per well, to transduce HDFs. This is day -2 of reprogramming.
NOTE: Defining the optimal volume of lentiviral mix for efficient reprogramming, without compromising cell viability, requires optimization (see Supplementary Figure 1 for more details). HDFs with more than 7 passages may require higher volumes of viruses than cells with lower passages.

4.7 After 16 hours of incubation, remove viruses and add DMEM. Allow cells to recover for 6-8 h.

4.8 After recovery, aspirate medium and add 2 mL DMEM with 8 µg/mL polybrene.

4.9 Do a second transduction as described in step 4.6 and incubate at 37 °C, 5% CO_2 for 16 h. This is day -1 of reprogramming. The lentiviral mix can be performed on day -2 for both transductions and kept at 4 °C.

4.10 On the next day, remove the viruses and add DMEM supplemented with 1 µg/mL DOX. This is day 0 of reprogramming. Incubate at 37 °C, 5% CO_2 for 48 hours.

4.11 At day 2 of reprogramming, split each well in a 1:2 ratio.

4.11.1 Aspirate medium and wash cells with 1 mL PBS.

4.11.2 Aspirate PBS and dissociate cells with 500 µL dissociation solution. Incubate 5-10 min at 37 °C, 5% CO2.

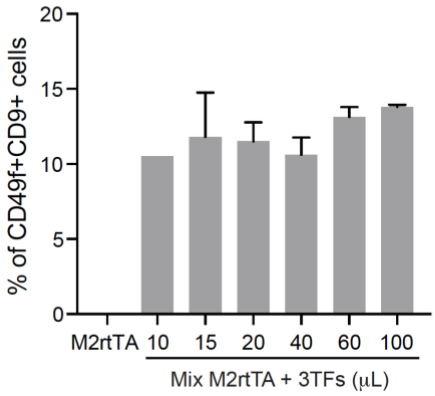
4.11.3 Inactivate the dissociation solution with 1 mL DMEM and collect the cells

into a conical tube. Centrifuge at 350 x *g* for 5 min.

4.11.4 Resuspend the pellet in hematopoietic medium (see step 1.3), supplemented with 1 µg/mL DOX, and plate cells into new tissue-culture treated 6-well plates coated with 0.1% gelatin at a final volume of 2 mL per well.

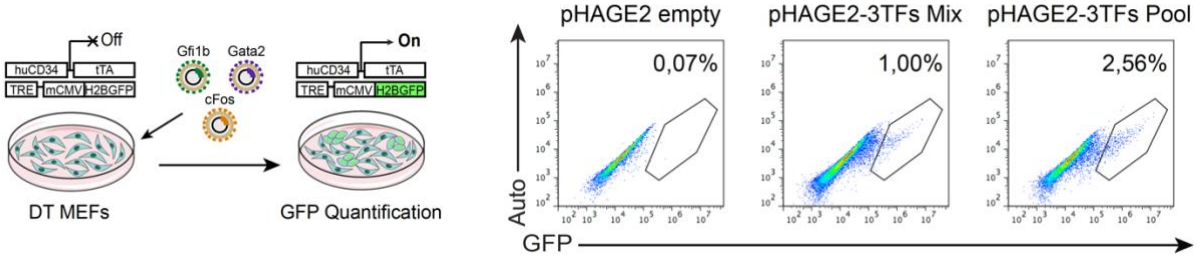
4.12 Change medium (hematopoietic medium plus DOX) twice a week for the duration of the reprogramming cultures (25 days).

Supplementary Figure 1



Supplementary Figure 1. Defining an optimized lentiviral volume for efficient hemogenic reprogramming. Increasing volumes of concentrated (10 to 100 µL) pool-produced lentiviral particles (3TFs: GATA2, GFI1B and FOS) are used to transduced HDFs, together with M2rtTA in a ratio of 1:1, following steps 4.5 to 4.12 of the protocol. Reprogrammed cells are analyzed at day 25 to define an optimal volume of transduction for hemogenic reprogramming, given by the percentage of CD49f+CD9+ cells gated in live-cells (Dapi-negative). Cell viability can be assessed by quantifying the absolute number of live cells at day 25. HDFs transduced with M2rtTA (100 µL) are used as control.

In this protocol, we opted to describe pool-production of lentiviral particles, as we have shown in a previous experiment that this results in higher reprogramming efficiencies. We include below this data: the mouse versions of Gata2, cFos and Gfi1b were expressed using a constitutive vector (pHAGE2) in double transgenic (huCD34-tTA/TRE-H2BGFP) mouse embryonic fibroblasts (MEFs). Reprogramming efficiency, measured in this case by the percentage of GFP positive cells [2], was lower when lentiviruses were produced separately and then combined (pHAGE2-3TFs Mix), compared to when the three factors were co-transfected in HEK293T cells (pHAGE2-3TFs Pool).



Percentage of reprogrammed cells when mouse Gata2, Gfi1b and cFos lentiviruses are produced individually in HEK293T cells and mixed for co-transduction or are pool-produced (co-transfected). GFP expression is driven by the activation of the human (hu)CD34 promoter in mouse fibroblasts. Empty vector was used as control.

Lentiviruses, as members of the *Retroviridae* family, normally contain two copies of positive single-stranded RNA[3].The increased reprogramming efficiency may result from packaging of two different transgenes in the same lentiviral particle, resulting in an increased number of cells co-transduced with the 3 transcription factors.

We have included a sentence in the discussion section regarding the explanation above (lines 388-397): "Pool-production of lentiviral particles encoding GATA2, GFI1B and FOS was preferred over individual production, since in our hands it results in higher reprogramming efficiencies (unpublished data). Lentiviruses, as members of the *Retroviridae* family, normally contain two copies of positive single-stranded RNA[19]. The increased reprogramming efficiency may be due to packaging of two different transgenes in the same lentiviral particle, resulting in increased number of cells co-transduced with the three transcription factors. To ensure the success of this protocol, it is necessary to transduce HDFs with adequate amount of virus depending on the cell passage to obtain an optimal balance between reprogramming efficiency and cell viability, as recommended in step 4.6. Moreover, fresh non-concentrated viruses can be used. It is recommended to transduce cells with 0.5-3 mL of 3TFs pool and M2rtTA."

Minor Concerns:

3) Page 5, 3.7.1, line187: It would be helpful to mention that a second generation gag-pol expression plasmid is used (psPAX2) and that the pMD2.G encodes for VSV-G envelope protein, as this information is relevant for the necessary biosafety level in which all work needs to be done.

This information is indeed important and was added to step 3.7.1, as follows:

"In a 15 mL conical tube, add 10 µg of the three transfer plasmids together: 3.33 µg of pFUW-tetO-GATA2 (Addgene plasmid #125028)[3], 3.33 µg of pFUW-tetO-GFI1B (Addgene #125597)[3] and 3.33 µg of pFUW-tetO-FOS (Addgene #125598)[3], plus 10 µg of the 2nd generation psPAX2 packaging vector encoding the *Gag*, *Pol*, *Tat* and *Rev* genes (Addgene #12260) and 5 µg of pMD2.G envelope vector encoding the *VSV-G* gene (Addgene #12259). Add water up to 500 µL."

4) Page 5, 3.7.3, line 194: even if a 2xBES-solution suitable for Ca3(PO4)2 transfections can be purchased, it would be favorable to define the concentration and pH of the solution, for those who wish to make their own buffer.

The pH of the BES solution is 7.1 at 25 ºC. This information was added to step 3.7.3 (line 192).

4a) Page 5, 3.12., line 291: it would be helpful to provide information on the type of membrane filter used in the low-protein centrifugal filter unit. Is it Polyethersulfone (PES), Cellulose Acetate (CA), PVDF, ... ?

The low-protein binding filter used to filter each lentiviral supernatant is made of cellulose acetate (added to line 218). Moreover, the centrifugal filter unit used to concentrate lentiviral particles is made of regenerated cellulose (added to line 220). Both materials were added to the Table of Materials where the respective catalogue numbers are included.

4b) There is a mistake in the abbreviation of the pore size of the filter. It should probably

read 0.45 µm instead of µM (micromolar).
The typo has been corrected (line 217).

5) Results section, page 8, line 325: the information which type of single cell analysis is performed, namely scRNAseq, is only given in the legend of Fig 1D. Please also add it to the main text.
The type of single cell analysis was scRNAseq and is included in the main text sentence "Reprogrammed cells may be generated at different time points for multiple applications including transplantation in immunocompromised mice, **single-cell mRNA-sequencing (scRNA-seq)** of FACS purified cell populations (day 2 unsorted, day 15 CD49+ and day 25 CD49f+CD34+ cells), as well as microscopy and flow cytometry analysis for the cell surface markers CD49f, CD34, CD9 and CD143.", in the "Representative Results" section, lines 315-319 of the revised manuscript.

In the figure legend, please add information on the total number of single cells analyzed.
A total of 253 cells were analyzed. This information was added to the legend of Figure 1D and can now be found in line 358.

Please correct the y-axis: if this is log to the base of 10, please add the base. If so, $\log_{10}$ 0 is 1, which also would have to be corrected in the graph.
The expression values are represented in log to the base of 2. This information was added to eh y-axis of the graph in Figure 1D.

5) Figure legend 1C, page 9, line 353ff: information on the anti CD9 antibody used here is missing. And a brief staining protocol should be included. Was the antibody simply added to the medium? In which concentration?
We thank the reviewer for this suggestion that will improve our paper. A short staining protocol was added to the legend of Figure 1C. In lines 355 and 357 the following sentence addresses this concern: "Independent wells were stained with individual antibodies (Table of Materials) diluted 1:100 in PBS 2%FBS with mouse serum (1:100), incubated 20 min at 37 ºC, 5% CO2, washed three times and imaged immediately in PBS 2%FBS."

6) In figure legend 2, details on the y-axis of the ChIPseq data are missing. What do the numbers represent? Are these counts from the sequencing data? Please complete.
The y axis represents the total number of reads that were mapped by ChIP-sequencing. This information was now added to lines 370 and 371 in the revised text as follows: "The total number of mapped reads is represented on the y-axis.".

7) Discussion section, page 10, line 394. When cultivating primary cells from skin biopsies, the outgrowing cells only appear homogeneous because of their overall morphology. Although the composition of the culture is certainly by far not as heterogeneous as that of differentiating PSC-cultures, they can contain different cell types, such as mesenchymal stroma cells, endothelial cells, melanocytes, keratinocytes and, of course, fibroblasts. All of those may show different programming efficiencies after transduction. As such cultures are commonly called "fibroblasts" and to avoid (unnecessary) confusion, one could perhaps simply write "One advantage of this system resides in the use of relatively homogeneous skin fibroblast cultures..." (or similar).

We appreciate the reviewer's insight in this comment. We agree with the reviewer that a skin biopsy has indeed several skin-related cell types other than fibroblasts, in particular keratinocytes. Nevertheless, high glucose DMEM 20% FBS supports fibroblast grow compared to keratinocytes[4]. We modified lines 412 to 413 according to reviewer's suggestion: "One advantage of this system resides in the use of relatively homogeneous fibroblast cultures."

8) line 397-398. It is stated that transplantation of PSC-derived HSPCs is associated with an increased risk of tumorigenesis due to PSC-plasticity. To my knowledge, PSC-plasticity as such is not the real problem, but, instead, undifferentiated PSCs which may have remained in the cultures during the differentiation process.
As the reviewer stated, our concern truly lies in the PSCs that keep their undifferentiated state and might reside in the cultures during the differentiation process. Lines 415-416 of the revised manuscript was altered to make this concept clearer, as follows: "Moreover, there is a risk of tumorigenesis when transplanting PSC-derived HSPCs, since undifferentiated PSCs may still remain in culture after the differentiation protocol."

Reviewer #2:
Manuscript Summary:
Recent work has shown, in a number of models, that direct reprogramming methods exist allowing cells to transit from one cell type to another upon expression of reprogramming factors. The current manuscript describes such a protocol to generate hematopoietic stem and progenitor-like cells from human fibroblasts through the transgenic expression GATA2, GFI1B and FOS transcription factors.

This work is important as it constitutes a model to modelize the very first steps of embryonic hemogenic endothelium differentiation in a human setting. The authors have previously shown this in a mouse model (1) and a human model (2), and in this manuscript describe in detail the necessary steps.

The authors cite an example of further work which can be performed based on this protocol consisting in detecting GATA2 targets during the early phases of reprogramming through chromatin immunoprecipitation-sequencing assays. The authors describe 4 essential steps of the protocol, two of which (Lentiviral Production and Hemogenic Reprogramming) are proposed for a video protocol.

*Human Dermal Fibroblast Isolation
*Lentiviral Production
*Hemogenic Reprogramming
*Protocol Optimization for Assessing Transcription Factor Binding Sites at the Onset of Hemogenic Reprogramming

We are grateful to this reviewer for considering our work important and for trusting on the future impact of our system as a model to study human hematopoietic development. We thank the reviewer for the comments which were used to improve the quality of our manuscript.

Major Concerns:
None

Minor Concerns:
A few typographical mistakes should be corrected:

*Abstract
L44 : reprograming > reprogramming
This typo has been corrected in line 42 (revised manuscript).

*Introduction
L74 :The sentence is difficult to understand :
"Recently, we have demonstrated that human versions of the three TFs, GATA2, FOS and GFI1B reprogram human dermal fibroblasts (HDFs) into HPs that originated hematopoietic progenitors with short-term engraftment capacity."
It may be better to replace with :
"Recently, we have demonstrated that human versions of the three TFs, GATA2, FOS and GFI1B reprogram human dermal fibroblasts (HDFs) into HPs with short-term engraftment capacity."
We thank the reviewer for the suggestion and we have modified the text accordingly.

L79 : contain > contained
Typo was corrected in line 75.

L87 : promotor > promoter
Typo was corrected in line 84.

* Protocol
L109 : Human dermal fibroblast can be purchased from Sciencel or ATCC: could a reference/catalog number be cited in order to ascertain the replication of the experiments?
As requested in the editorial comments, commercial language was removed from main text and it is now referred as “certified suppliers (see Table of Materials)” in line 108. Nevertheless, in the Table of Materials we have included the supplier of HDFs as well as catalogue number (ScienCell, #2320).

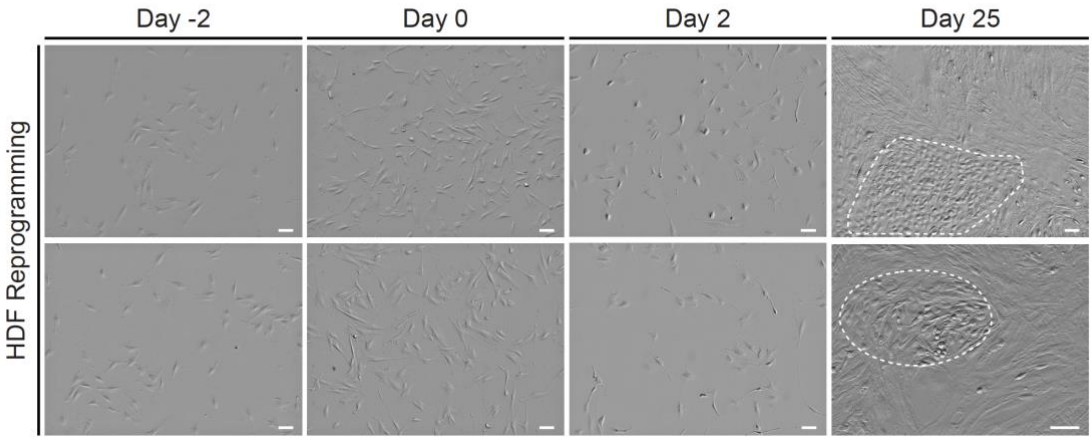
L126 and 134: cover slip > coverslip
Typo is now corrected in both lines 125 and 133.

L219 : please precise a reference for the 0.45um filter, as some materials may less compatible with viral particles.
That information was now added to “Table of Materials”. A reference to table of materials was added to line 218.

* Discussion
L371: Prefer "In this article, a method is described to generate…"
Thank you for the suggestion. We have modified the text accordingly (line 386-387).

*Additional Comments to Authors:
One important aspect of this protocol to the researchers reproducing the experiment is the morphological changes demonstrated by the cells during the reprogramming. It would be important and relevant in the results of the"Hemogenic Reprogramming " (Fig1) to show the morphological characteristics and confluency of the cells at days -2, 0, 2, 15, 25. A table with a representative photographs at these different stages would be useful, and the morphology of the cells should also be visible in the video.
We appreciate the reviewer's comment and agree that it is important to show the morphological changes that occur during the hemogenic reprogramming process.
We have generated a supplementary figure including the morphological characteristics and confluency of the cells at days -2, 0, 2 and 25. This new figure was added as Supplementary Figure 2 in the manuscript and referenced in step 4.13 of the protocol.

Supplementary Figure 2



Supplementary Figure 2. Morphological characteristics during hemogenic reprogramming of human dermal fibroblasts. Human Dermal Fibroblast (HDF) cultures are imaged at the day of the first transduction (day -2), when DOX is added to the cultures (day 0), two days after DOX supplementation (day 2) and at the end-point of the experiment (day 25). Hemogenic colonies at day 25 are highlighted. Scale bars, 100µm.

Due to short deadline for paper re-submission we were unable to include day 15 time-point. This time-point will be added to the video protocol.

References

1. Kutner, R. H., Zhang, X. & Reiser, J. Production , concentration and titration of pseudotyped HIV-1-based lentiviral vectors. *Nature Protocols* **4**, 495–505 (2009).
2. Pereira, C. F. *et al.* Induction of a Hemogenic Program in Mouse Fibroblasts. *Cell Stem Cell* **13**, 205–218 (2013).
3. Suzuki, Y. S. & Suzuki, Y. Gene Regulatable Lentiviral Vector System. in *Viral Gene Therapy* (ed. Ke, X.) 286–308 (IntechOpen, 2011).
4. Vangipuram, M., Ting, D., Kim, S., Diaz, R. & Schüle, B. Skin Punch Biopsy Explant Culture for Derivation of Primary Human Fibroblasts. *Journal of Visualized Experiments* e3779 (2013).