Hemogenic Reprogramming of Human Fibroblasts by Enforced Expression of Transcription Factors

Response to Reviewers and Editors:
We would like to thank the reviewers and editors for their comments and recommendations. In this letter we will address each individual concern and provide detailed answers. Figures representing new data are included.

Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Agreed. We have proofread the manuscript and spelling and grammar issues are corrected and tracked in the manuscript.

2. Please define all abbreviations (DMEM, PBS, etc.) before use.
All abbreviations in the manuscript are now defined before they are used for the first time. The following abbreviations were defined in step 1 (1. Reagent Preparation, lines 97-106):

- Dulbecco's Modified Eagle Medium (DMEM)
- Fetal Bovine Serum (FBS)
- Penicillin-Streptomycin (Pen/Strep)
- Phosphate Buffered Saline (PBS)

3. Please use the period symbol (.) for the decimal separator.
The decimal separator (,) was replaced with (.) throughout the text.

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.
The following ethics statement was added before the numbered protocol: "Ethics statement: This protocol was preformed according to Lund University's human research ethics committee guidelines and should be done in accordance with each institutional guideline."

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: MyeloCult, ScienCell, ATCC, TrypLE, Amicon, etc.
To fit journal guidelines MyeloCult was replaced with "hematopoietic medium". TrypLE Express is now "dissociation solution". ScienCell and ATCC are now "certified suppliers". Amicon was removed from the text and now it is only referred as "centrifugal filter unit". Eppendorf tube was removed and is now referred as "microcentrifuge tube".
"Table of Materials" was modified to contain the commercial name next to the generic term of the reagents.

6. 3.11.1 and 3.11.2: Please specify incubation conditions.
The incubation conditions (37 °C, 5% CO2) were added to steps 3.11.1 (line 212) and 3.11.2 (line 214).

7. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Eight shorter steps were merged with related steps throughout the manuscript.

8. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.
Manuscript is now formatted with single-line spacing.

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
The relevant steps in the protocol that should be included in the video are highlighted in yellow and make up less than 2.75 pages for the Protocol.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
Highlighted sections comprise completed sentences with at least one action in the imperative tense. No notes were highlighted.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
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.

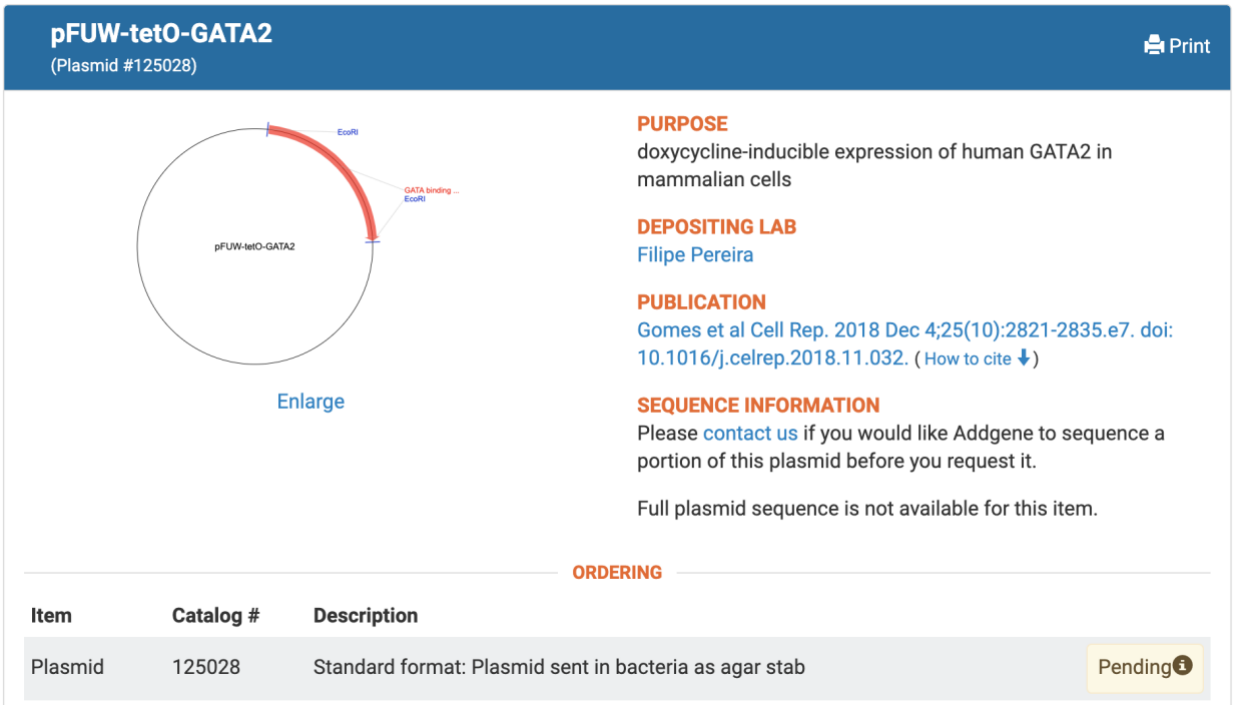
Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this work, the authors describe a protocol for programming human skin fibroblasts to hemogenic progenitors by lentiviral vector-based, doxycyline-inducible expression of the transcription factors GATA2, GFI1B and FOS.
The title and abstract are 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.

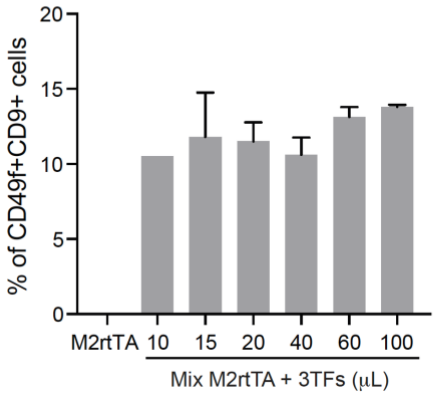
4.11.3 Inactivate the dissociation solution with 1 mL DMEM and collect the cells

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.

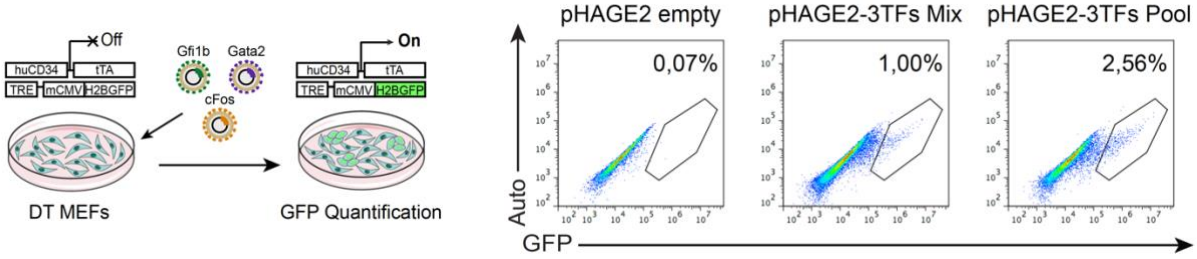
4.12 Change medium (hematopoietic medium plus DOX) twice a week for the duration of the reprogramming cultures (25 days).

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).

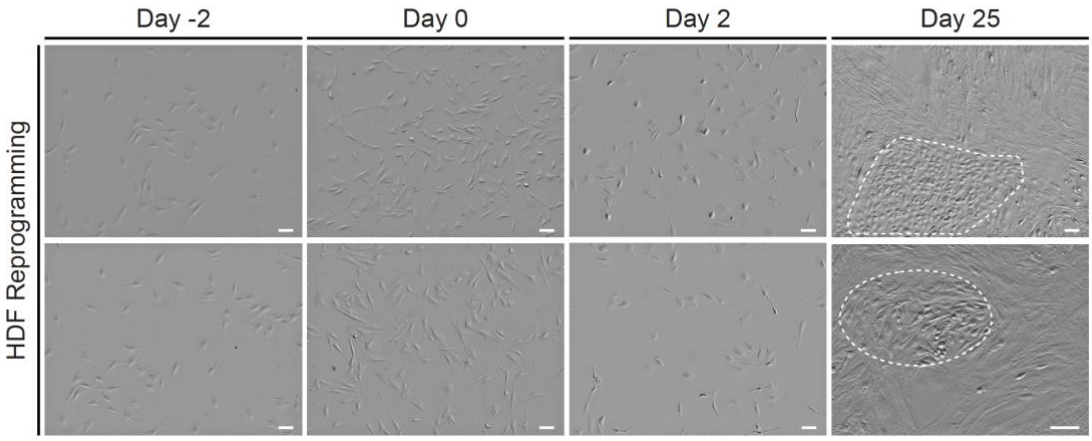
L126 and 134: cover slip > coverslip
Typo is now corrected in both lines 125 and 133.

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).