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Hair follicle stem cell cultures reveal self-organizing plasticity of stem cells and their progeny

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

05 July 2016

Thank you for the submission of your manuscript (EMBOJ-2016-94902) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #1 points out that some conclusions are not sufficiently well supported by the current data (ref #1, pts. 2, 6, 7, 18). In particular, this referee states the need for you to provide further molecular proof of the occurrence of HFSCs in the culture system (see ref #1, pts. 10, 11, 17, 18). Referees #2 and #3 agree that more molecular insights would be needed to substantiate the characterisation of the cell populations reported and to exemplify the applicability of your system (see ref #2, pt. 4; ref #3). In addition, referees #1 and #2 list a number of technical issues and controls regarding the *in vitro* reconstitution and other points, that would need to be addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I agree that it would be essential to corroborate the molecular identity of the stem cells in your culture and their interconversion from their progeny. Also, I agree that the manuscript would strongly benefit from more refined characterization of the signaling involved in these cellular transitions.

REFEREE REPORTS

Referee #1:

Comments

The manuscript by Chacon and colleagues describe a new culture condition to maintain and expand hair follicle stem cells and an accompanying cell population. Authors characterize this population using *in vitro* colony forming, *in vivo* grafting and RNA sequencing experiments. They notice that two subpopulations present in culture can interconvert and maintain equilibrium, which they model using their culture data. Finally, some insights into the mechanism of the inter-conversion process, driven by Shh and Bmp, are presented. While the study paves a nice starting point, in my judgement the manuscript is too premature to be published in EMBO Journal. My major criticisms are the lack of experimental proof for the identity of cultured cells, lack of a novel biological finding based on the model that is vigorously corroborated and the absence of *in vivo* relevance of the described findings. Overall, there is no significant biological discovery concerning the niche and stem cell dynamics made using the described culture system. Claiming that the niche is reconstructed *ex vivo* and that the authors reveal self-organising plasticity of stem cells (as mentioned in the title) is an overstatement of the data included in the manuscript. I would be glad to see the manuscript again shall the authors come back with an improved version (major revisions). However, I am afraid it will take quite a few months before the work can be finalized and the authors might benefit from submitting the work to another journal. Below are my comments, which I hope the authors find useful in improving their work.

Specific comments:

1.) How do the cultures change over time with respect to population doubling/proliferation? Overall, what is the fold increase in absolute cell numbers over time? A better description of how spheroid size change should be provided. There seems to be a transient increase in absolute cell numbers that by 10 days reach a plateau, raising questions about how much the culture can be expanded (what is the replating efficiency?).
2.) In page 5, lines 2-6; how many days after culture was a 7-fold increase? For 2D vs 3D comparison figure 1B is cited; however, media composition of how these cells are cultured is different (KGM vs 3C). Thus the conclusion that 3D is essential indicated cannot be drawn from this experiment.
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4.) Is fgf-2 the only Fgf family members capable of supporting growth? And what is the rationale behind choosing the Fgf?
5.) In Figure 1D, what do the authors want to show with BF and PH panels? It is very hard to judge the morphology of these spheroids from these pictures, which look rather irregular
6.) In S1C; authors claim of 'encapsulation' is not substantiated. What are the chances of laminin from the matrigel being left on the surface of the spheroids, which would look like encapsulation? This could be excluded with a staining on collagen grown spheroids
7.) In page 5; authors conclude prematurely that their culture includes HFSCs. While Sox9 and keratin 15 are enriched in HFs, it is not entirely restricted to it. While CD34 is a bulge marker (not necessarily a stem cell marker), its expression might be altered in culture. The claim is repeated in page 6 after reconstitution experiments, where virtually any SC/progenitor population from epidermis has been shown to repopulate the skin. While cells with SC characteristics seem to grow in culture, their identity HFSCs
8.) In general, quantification of the flow cytometry analysis should be provided
9.) The PCA plot in supplementary figure 3 is unlike any I have seen before; is this indeed the correct panel? Why can't we see the 9 populations described in the text on this figure? How could

the authors conclude a resemblance from this analysis?

10.) The design and analysis of the RNA sequencing experiments are poor. In the molecular analysis part, the claim 'resembled more closely than freshly isolated...' does not prove similarity of 3C culture to HFSCs. One way is to include other epidermal populations for comparison. For the purpose of this study, basal cells from the interfollicular epidermis and from the isthmus should be included. Pearson correlation between samples should be included to reveal the degree of similarity, as well as reproducibility (among samples of the same group).

11.) CD34+a6+ cells are presented as undisputed HFSCs in vivo. The increase in CD34 does not necessarily mark these cells as bulge cells. According to Figure 3B, only 270/792 genes enriched in 3C is shared with CD34+a6+ cells, which share 270/1250 with the former. This shows clear differences between the molecular signatures of both populations, arguing against the main message of the authors.

12.) In page 7, an overlap between expression patterns is reported. Which test is used to obtain the p values described?

13.) The qPCR analysis in 3C also suggest a very mild enrichment of Sox9 (2x) and CD34 (3x), which are used to claim 3C cells as HFSCs.

14.) In the text describing the data displayed in figure 3D and 3E (page 7), authors mention that most upregulated genes in 3C versus CD34+a6+ HFSCs resembled the TAC signature, but not the quiescent HFSC signature. The text is followed by the claim that 3C cultures resemble 'bona fide HFSCs'. There is very clear contrast between these two sentences and raises doubts about how critical the molecular data is analyzed. Similarly, any overlap displayed in panels D and E is a small portion of the total population. While authors provide statistical analysis (I could not find the test used is for the analysis), the authors should not overlook large differences between these populations.

15.) Do cultures start from a single sorted cell? This issue is detrimental to understand the source of the culture

16.) Authors hint to the presence of HFSC progeny in culture, yet there is no proof provided. Are they describing the hair producing progeny? They should provide strong molecular proof to claim reprogramming of the progeny back into the stem cell state

17.) Both populations can interconvert, which could simply represent different position within a spheroid or contact to the neighboring cells or extracellular matrix. Given the importance of the interconversion of CD34+ and CD34- populations in 3C, the authors should identify the molecular differences/similarities between these populations.

18.) Authors use CD34+ as a stem cell marker; yet they quickly predict that both populations could interconvert and include this in their model. The end-point analysis of panel 4E suggests 80% and 33% CD34 positivity upon 2 weeks in culture. Both numbers are different from the data and the simulation results provided in 4D where around 50% of the cells are CD34+a6+, which is also predicted by the model. This is in clear contrast to authors claim that this experiment provides experimental proof of the system. Authors need to provide strong proof their model indeed predicts the reality. Live imaging experiments on reporter lines would be a good choice

19.) While the conversion of CD34+ and CD34- populations might be of interest, the relevance of these populations to hair follicle genesis remains obscure. The identity of CD34- cells remains obscure. Are these indeed hair generating progeny of stem cells?

20.) Does conversion between these two populations (or states) happen in vivo in equilibrium?

Referee #2:

In this work Chacón-Martínez et al describe the specific culture conditions that allow the long-term

expansion and maintenance of functional murine hair follicle stem cells (HFSCs). This is a very important study, that will pave the way to study in vitro the HSCs biology, as well as, provide a platform for drug screening.

In this study, the authors firstly identify the culture conditions and combination of morphogens (referred as 3C) that allow the enrichment and maintenance of cells expressing CD34+a6+, two markers expressed in bulge stem cells. Then, they assess the proliferation potential of 3C cultured cells using colony-forming assays and their multipotency and functionality showing that they are able to generate hairs using full-thickness reconstitution assays. Interestingly, the authors show that the proportion of CD34+a6+ cells in their culture is conserved after several passages and after being frozen and thawed. Secondly, the authors study the transcriptome of the 3C cultured cells and compare it to the one of FACS-purified CD34+a6+ HFSCs cells and published signature of HFSC. They show that some upregulated genes in the 3C culture cells are also found upregulated in FACS-purified CD34+a6+ and in published HFSC signature. Thirdly, the authors study in detail the cellular dynamics of their culture providing a mathematical model that describes the equilibrium and bidirectional conversion that exist between CD34+a6+ and CD34-a6+ cells when cultured using the 3C strategy. They elegantly validate one of the hypothesis of their model, the interconversion of CD34-a6+ to CD34+a6+, by showing that FACS-isolated CD34-a6+ cells are able to give rise to CD34+a6+ as well as CD34-a6+. Finally, the authors show that alterations in the Shh and BMP signalling pathways, pathways that have been reported to mediate HFSC maintenance and quiescence respectively, alter the cellular dynamics through modifying proliferation or differentiation of CD34+a6+ cells.

This is a nice study and the topic is of interest. However, there are some important points that need to be further characterised before the paper can be considered for publication in EMBO J.

1. In Fig1, the authors need to quantify the proportion of Sox9 in 3C and control condition (KGM 2D).
2. The authors perform full-thickness skin reconstitution assays using 3C cultured cells (42% CD34+a6+) and freshly isolated epidermal cells (5.6% CD34+a6+). They state that their 3C cultures are more efficient regarding their HF regeneration capacity as compared to freshly isolated epidermal cells (Fig 2b). However, since the cells were not FACS isolated, this could simply reflect the fact that there is higher proportion of CD34+ cells in the 3C. The authors should determine whether the FACS isolated CD34+ cells are more efficient at HF regeneration in 3C condition versus native state.
3. The authors claimed that there is no exhaustion over passage. However, this statement has not been quantified. This should be done.
4. The molecular profiling and the comparison with native bulge cells is too superficial. They should better describe the genes in common as well as the genes specifically expressed by either the 3C CD34+ and the native bulge cells. Also, they should use the original paper describing bulge cells including Tumber et al, Morris et al, and Blanpain et al. 2004. Is there any particular category of genes maintained or lost in 3C? They proposed that cells are activated, how is the expression of cell cycle regulators?
5. The model proposed in figure 4 is confusing. The authors should not analyse the bulk of population but rather plate FACS isolated a6+CD34- and a6+CD34+ in 3C and assess the proportion of CD34+ cells overtime. If they want to determine the role of mixing two populations on the CD34 proportion, the individual populations should be marked with different fluorescent proteins. They should then analyse for each of them the apoptosis, proliferation rate and rate of interconversion.
6. It would be interesting if the authors could show that upon freezing and thawing 3C cultured cells retain their functionality using full-thickness skin reconstitution assays. This would reinforce the robustness of the described culture system.

Overall, this is an interesting and relevant study that could be valuable to the research community. I recommend publication of this paper in EMBO journal once they address the key points highlighted above.

Referee #3:

In this work, Chacón-Martínez and colleagues have developed a 3D culture system to maintain hair

follicle stem cells (integrin alpha6 bright/CD34+) and their progeny (integrin alpha6 bright/CD34-) without the need to co-culture them with any other cell type. Importantly, they show that HFSCs can be maintained long-term under these conditions, with high self-renewing capacity and in vivo stem potential. In addition to this, they provide intriguing data supporting the idea that stem cells and their progeny can interconvert, and that this is regulated by Bmp and Shh signals that control quiescence and activation. This indicate, as it had been previously suggested by in vivo work by other groups, that the stem cell state is much more flexible than previously thought.

I think this is a very solid and interesting work. The quality of the data is impeccable, and the results are novel and timely. As such I do not have any criticism regarding the data provided. I do think that the authors should provide more mechanistic data on the Bmp and Shh part though, since this is precisely the strength of their system: it allows to perform more mechanistic studies that would otherwise be much more difficult to do with more conventional in vivo approaches. The authors should analyse the global gene expression changes by RNA-seq that occur upon modulation of Bmp and Shh signalling of the 3C cultures to drive the cell state equilibrium towards the SC state to the TAC state. This will provide very interesting and useful mechanistic information to the field on how these cell transitions occur and how these signalling pathways function.

1st Revision - authors' response

06 October 2016

While the study paves a nice starting point, in my judgement the manuscript is too premature to be published in EMBO Journal. My major criticisms are the lack of experimental proof for the identity of cultured cells, lack of a novel biological finding based on the model that is vigorously corroborated and the absence of in vivo relevance of the described findings. Overall, there is no significant biological discovery concerning the niche and stem cell dynamics made using the described culture system. Claiming that the niche is reconstructed ex vivo and that the authors reveal self-organising plasticity of stem cells (as mentioned in the title) is an overstatement of the data included in the manuscript. I would be glad to see the manuscript again shall the authors come back with an improved version (major revisions). However, I am afraid it will take quite a few months before the work can be finalized and the authors might benefit from submitting the work to another journal. Below are my comments, which I hope the authors find useful in improving their work.

We thank the reviewer for the critical comments that greatly helped us to improve our manuscript. In response to the comment on novelty, we would like to emphasize that this is the first report in the literature of successful expansion and long-term maintenance of CD34⁺α6⁺ HFSCs *ex vivo* and therefore will provide an important research tool for the field. The lack of terminal differentiation in this system together with the tunable, plastic balance between HFSCs and non-HFSCs by known stem cell-regulatory factors (Shh and BMP) further distinguishes this system from classical stem cell organoid cultures that represent a very successful research paradigm for stem cell differentiation. This distinction together with the immediate research need for an in vitro tool for HFSC biology highlights the relevance of this discovery. In addition to this unique research tool, we report two important biological discoveries. First, we demonstrate that a three-dimensional ECM in combination with a defined set of growth factors, in the absence of heterologous cell types, is sufficient to drive reprogramming of CD34⁻α6⁺ cells to the CD34⁺α6⁺ HFSC state. This is important, as it has been demonstrated that progenitors can return to the bulge niche and reassume a stem cell fate (Hsu et al., 2011, Rompolas et al., 2013), but due to the complexity of in vivo niches (multiple heterologous cell types, extracellular matrix), the signals that drive this reprogramming have remained completely unknown until now. Second, we demonstrate that the dynamic population equilibrium is generated and maintained through mathematically tractable interconversion, which to our knowledge has not been reported for adult stem cell niches. We are therefore convinced that the manuscript represents a significant advance in the field of hair follicle stem cell research.

Specific comments:

1.) How do the cultures change over time with respect to population doubling/proliferation? Overall, what is the fold increase in absolute cell numbers over time? A better description of how spheroid size change should be provided. There seems to be a transient increase in absolute cell numbers that by 10 days reach a plateau, raising questions about how much the culture can be

expanded (what is the replating efficiency?).

Data already included in the first submission showed the absolute changes of cell numbers over time (Fig. 1C) and showed a 5x fold increase of after 14 days of culture (Fig. 1C). The time resolved changes were also shown in Fig. 4A. As we agree with the reviewer that this is an important point we have now, as requested, furthermore included the fold increase in absolute cell number in long-term cultures (replating efficiency). These data show that the fold increase of CD34⁺α6⁺ cells does not decline during long-term passaging, indicating that the cultures can be expanded without loss of CD34⁺α6⁺ cell proliferative potential (new Fig. S2B).

As the reviewer notes, there is an initial phase of exponential increase in cell numbers (Fig. 4A), which coincides with high proliferation rates (new Fig. S5A). The proliferation rates later decline, resulting in a plateau of absolute cell numbers around day 10-12. This coincides with a plateau in the size of the clusters, the quantification of which is now included as new Fig. S4A. Nevertheless, upon passage of the 3C cultures, absolute numbers of cells consistently again increase exponentially (Fig. S4B) and, as already mentioned, the fold increase in absolute numbers of CD34⁺α6⁺ cells remains stable (Fig S2B), showing that this plateau is not due to exhaustion of proliferative potential of these cells.

Together these data show that the 3C cultured cells maintain long-term proliferative potential, whereas the cluster size is likely to be a rate limiting factor for growth, probably due to limited diffusion of nutrients or contact inhibition, a phenomenon that is also observed in standard 2D epithelial cell cultures.

2.) In page 5, lines 2-6; how many days after culture was a 7-fold increase? For 2D vs 3D comparison figure 1B is cited; however, media composition of how these cells are cultured is different (KGM vs 3C). Thus the conclusion that 3D is essential indicated cannot be drawn from this experiment.

The 7-fold increase was observed after 14 days, we have now clarified this in the text on p. 5. The reviewer might have overlooked that in addition to KGM we also analyzed the effect of the 3C medium on 2D cell cultures and showed that enrichment of CD34⁺α6⁺ cells is only seen in 3D conditions (Fig. 1B, 3rd condition from the left is 3C medium in 2D), allowing us to conclude that the 3D configuration is essential.

3.) The VEGF used (a,b,c?) should be mentioned in the text

We have used VEGF-A and indicate this in the revised manuscript.

4.) Is fgf-2 the only Fgf family members capable of supporting growth? And what is the rationale behind choosing the Fgf?

We chose FGF-2 as it has been shown to support growth of various types of stem cells in culture, including pluripotent stem cells (e.g. Yu et al., Cell Stem Cell 2011, Brons et al., Nature 2007). However, we have now also added data to show that other FGF family members (FGFs 7, -10, and -18) can also support growth of the CD34⁺α6⁺ population (new Fig. S1B).

5.) In Figure 1D, what do the authors want to show with BF and PH panels? It is very hard to judge the morphology of these spheroids from these pictures, which look rather irregular

To improve clarity, we have replaced the BF/PH panels in Fig. 1D with a single bright field image to better illustrate morphology of the clusters.

6.) In SIC; authors claim of 'encapsulation' is not substantiated. What are the chances of laminin from the matrigel being left on the surface of the spheroids, which would look like encapsulation? This could be excluded with a staining on collagen grown spheroids

As requested, we have performed this experiment and show Laminin 332 deposition also in 3C cultures grown in collagen I (new Fig. S1E). In addition, we have reformulated the conclusion to remove encapsulation and to state “expression and deposition of a laminin-332 meshwork”.

7.) In page 5; authors conclude prematurely that their culture includes HFSCs. While Sox9 and keratin 15 are enriched in HF, it is not entirely restricted to it. While CD34 is a bulge marker (not necessarily a stem cell marker), its expression might be altered in culture. The claim is repeated in page 6 after reconstitution experiments, where virtually any SC/progenitor population from epidermis has been shown to repopulate the skin. While cells with SC characteristics seem to grow in culture, their identity HFSCs

We agree with the reviewer that other cells than HFSCs can express SOX9, K15, alpha6 integrin and CD34. However, to our knowledge only HFCSs express all 4 markers, and the combination of these markers is widely used to identify HFSCs *in vivo*. In response to the criticism, we have toned down the conclusion on p.5 and p6 and refer to these cells as “cells with HFSC characteristics”.

8.) In general, quantification of the flow cytometry analysis should be provided

Our apologies that not all quantifications were clearly indicated as to our knowledge all cytometry plots were accompanied by quantifications. We have now made sure that for all flow cytometry experiments presented in the revised manuscript the quantification is clearly presented.

9.) The PCA plot in supplementary figure 3 is unlike any I have seen before; is this indeed the correct panel? Why cant we see the 9 populations described in the text on this figure? How could the authors conclude a resemblance from this analysis?

The PCA plot in the Supplementary Fig. S3 was a standard PCA plot that we derived using the cummeRbund R package, but it did indeed not depict the individual biological replicates. We have now replaced this previous plot with a new PCA plot that displays individual replicates (new Fig. S3A). This new plot shows that biological replicates of the same population cluster together showing good reproducibility and technical quality. Importantly, the results presented in the plot show that the transcriptomes of the freshly purified CD34⁺α6⁺ samples cluster much more closely with the 3C transcriptomes, than the epidermal cell mixture, further supporting our conclusion that these two populations are closely related.

10. The design and analysis of the RNA sequencing experiments are poor. In the molecular analysis part, the claim 'resembled more closely than freshly isolated...' does not prove similarity of 3C culture to HFSCs. One way is to include other epidermal populations for comparison. For the purpose of this study, basal cells from the interfollicular epidermis and from the isthmus should be included. Pearson correlation between samples should be included to reveal the degree of similarity, as well as reproducibility (among samples of the same group).

With all due respect to the reviewer, we strongly feel that it is essential to directly compare the freshly isolated epidermal cell mixture with the 3C cells and with freshly isolated *in vivo* CD34⁺α6⁺ as this very same mixture was used to inoculate the 3C cultures, which were subsequently sequenced after 14 days of culture (RNA isolation, library preparation and sequencing of all samples was done together to avoid batch effects). This direct comparison allows us to carefully and in an unbiased way monitor the global transcriptional changes that occur in the entire epidermal cell population upon 14 days of 3C cell culture. Most importantly, it allowed us to directly compare the transcriptional identity of the 3C culture with their cells of origin as well as with freshly isolated bulge stem cells, the only stem cells *in vivo* that express both CD34⁺ and α6⁺.

The fact that the transcriptomes of 3C cells that are a 50:50 mixture of CD34⁺α6⁺ and CD34⁻α6⁺ cells, shift from resembling the cells of origin (which in the adult epidermis consists mainly of basal progenitors, 80-90% express α6 integrin as shown in Fig. 1A and by Cotsarelis, Fuchs, and Watt labs among others) toward that of HFSCs that are only 5-10% of the total epidermal population, as shown by 1) PCA, Pearsons correlations, and Euclidian distance, and 2) with specific upregulation of master transcriptional regulators and identity genes of HFSCs, provides important proof for the conclusion that the 3C cultures enrich for HFSCs. This is also in clear contrast to recent studies showing that multiple purified epidermal stem cell populations completely lose their molecular identity after 14 days of 2D cell culture (Gunnarson et al., Stem Cell Res 2016) indicating that standard cell culture conditions do not promote expression of epidermal stem cell genes, further

highlighting the specificity of our system.

It should be noted that in our initial bioinformatics analyses we compared the 3C signature also to published signature of the upper isthmus (Lrig+, Page et al., Cell Stem Cell 2013), but as we did not observe significant overlap with the Lrig+1 cells (that do not express CD34+), we decided not to include this comparison in the manuscript.

We have further strengthened this point by directly comparing gene expression of the 3C cells to FACS-purified epidermal progenitors (CD34⁺α6⁺ cells) and *in vivo* HFSCs (CD34⁺α6⁺) (new Fig. 3G, H). This analysis, that we describe this analysis in detail below in our answer to the next point #11, further confirms the transcriptional similarity of 3C- CD34⁺α6⁺ cells to *in vivo* CD34⁺α6⁺ HFSCs.

The heatmap algorithm that we used to graphically represent the transcriptomes in Fig. 3A was based on calculating Pearson's correlation distance (indicated by the Z-score) that gives a numerical value for the similarity of the transcriptomes. Based on the correlation distance that clustered freshly isolated HFSCs and 3C cultures, together with the PCA that gave a similar result, we could conclude that the transcriptomes of the two populations are more similar to each other than to the epidermal cell mixture.

We have now further quantified similarity using Euclidian distance. Both methods yielded very similar results and confirmed the similarity of freshly isolated CD34⁺α6⁺ cells and 3C cultures (as well as the excellent reproducibility of the biological replicates), and the separation of epidermal cells. To maintain manuscript clarity we have included the requested Pearson's correlation distance as a heatmap matrix including replicates (new Fig. S3B), and to avoid redundancy, replaced the previous Fig. 3A heatmap with one where the dendrogram represents Euclidian distance and the color code FPKM values (new Fig. 3B).

11.) CD34+α6+ cells are presented as undisputed HFSCs in vivo. The increase in CD34 does not necessarily mark these cells as bulge cells. According to Figure 3B, only 270/792 genes enriched in 3C is shared with CD34+α6+ cells, which share 270/1250 with the former. This shows clear differences between the molecular signatures of both populations, arguing against the main message of the authors.

It is essential to point out that the 3C conditions are a ~50:50 mixture of CD34⁺α6⁺ and CD34⁻α6⁺ cells, both of which actively cycle, so a perfect transcriptional overlap with freshly sorted telogen-stage quiescent HFSCs would be highly unlikely.

Importantly, the gene expression signature shown in the former Figure 3B (Fig. 3C in the revised manuscript) do not represent transcripts that would be specific for one of the two cell populations. They are genes that are found in both cell populations but with >log2 increase in expression compared to the total epidermis. Given the sensitivity of RNAseq (with >6000 genes differentially expressed in 3C vs Epi d0), we actually find it remarkable that 40% of the most significantly upregulated genes in 3C (a ~50:50 mixture of CD34⁺α6⁺ and CD34⁻α6⁺ cells that has been in culture for 14 days) represent genes highly enriched in HFSCs ("signature genes"). This is also reflected in the very low p-values obtained for these overlaps, obtained by hypergeometric analyses. To improve clarity, we have now substituted the term "gene expression signature" with "gene expression enrichment profiles".

In addition, we have taken several experimental approaches to strengthen this point:

A) We have performed new RNAseq experiments where we compared the transcriptomes of FACS-purified CD34⁺α6⁺ and CD34⁻α6⁺ populations from the 3C cultures. As expected and subsequently validated by RT-qPCR (new Figs. 3E-H and S3G), the 3C-CD34⁺α6⁺ population is enriched for a number of HFSC markers and transcription factors that have been shown to be directly involved in establishing HFSC lineage identify or regulating their fate (*Sox9*, *Lhx2*, *Tcf3*, *Nfatc1*, *Id1*, 2 and 3), with 47 % of the upregulated genes representing genes enriched in bona fide HFSCs. In contrast, the 3C-CD34⁻α6⁺ population shows upregulation of genes shown to be upregulated in HFSC progeny (TACs, hair germ, hair matrix; Greco et al., 2009, Lien et al., 2011).

B) We have performed additional RT-qPCR experiments where we directly compare freshly isolated

FACS-purified *in vivo* CD34⁺α6⁺ and CD34⁻α6⁺ cells to 3C CD34⁺α6⁺ and CD34⁻α6⁺ cells. As expected, HFSC identity genes are upregulated in both freshly isolated CD34⁺α6⁺ and 3C-CD34⁺α6⁺ cells compared to the freshly isolated CD34⁻α6⁺ and 3C-CD34⁻α6⁺ cells (new Fig. 3G), and many of these key factors are expressed at comparable level in 3C and *in vivo* CD34⁺α6⁺ HFSCs, further validating the 3C-CD34⁺α6⁺ as true HFSCs. Interestingly, the 3C-CD34⁻α6⁺ cells show intermediate expression of this panel of HFSC identity genes when compared to the *in vivo* HFSCs and progenitors, and quite mild upregulation of lineage progression genes (new Fig. 3G, Fig. S3G), clearly indicating that these cells are progenitors and not differentiated cells. On the other hand, some cell cycle regulators as well as some markers of HFSC immediate progeny (*Ccnd1*, *Ccne1*, *Msx2*, *Wnt7a*, *Wnt10a*; new Fig. 3H) are mildly upregulated in both 3C populations compared to both freshly isolated cell populations, most likely reflecting the actively cycling nature of these cells.

C) We have performed more detailed analyses of the genes differentially expressed in 3C cultures compared to *in vivo* CD34⁺α6⁺ cells. These analyses show that cell cycle regulators, extracellular matrix proteins and components of the Wnt signaling pathway are most significantly differentially regulated in 3C versus *in vivo* CD34⁺α6⁺ HFSCs (new Figs. S3C-F), which is in line with our findings that the 3C cultures contain actively cycling HFSCs in contrast to the telogen CD34⁺α6⁺ HFSCs. In addition, providing abundant ECM proteins in Matrigel is likely to influence the expression of ECM components.

Collectively these data provide very strong evidence for the HFSC identity of the 3C- CD34⁺α6⁺ cells and support the conclusion from other data (lineage tracing, derivation of the 50:50 population equilibrium from pure CD34⁺α6⁺ cells) that the 3C-CD34⁻α6⁺ cells represent immediate progeny of CD34⁺α6⁺ cells that still display some molecular characteristics of HFSCs (for example intermediate levels of *Sox9*, *Tcf3* etc). We are grateful for the reviewer for this criticism and suggestions.

12.) In page 7, an overlap between expression patterns is reported. Which test is used to obtain the *p* values described?

We used hypergeometric distribution probability. Similar to all statistical tests used in our experiments, this is indicated in the corresponding figure legend.

13.) The qPCR analysis in 3C also suggest a very mild enrichment of *Sox9* (2x) and *CD34* (3x), which are used to claim 3C cells as HFSCs.

As pointed out in our response to an earlier point, the 3C cultures are a ~50:50 mixture of CD34⁺α6⁺ and CD34⁻α6⁺ cells, and thus the upregulation is expected to be relatively mild. Importantly, however, when we FACS-purify and analyze these populations separately, several key HFSCs identity genes show further upregulation in the 3C-CD34⁺α6⁺ population compared to 3C-CD34⁻α6⁺ cells and many of these key identity regulators such as *Sox9* display comparable mRNA levels in freshly purified *in vivo* HFSCs and 3C CD34⁺α6⁺ cells (new Fig. 3G).

14.) In the text describing the data displayed in figure 3D and 3E (page 7), authors mention that most upregulated genes in 3C versus CD34⁺α6⁺ HFSCs resembled the TAC signature, but not the quiescent HFSC signature. The text is followed by the claim that 3C cultures resemble 'bona fide HFSCs'. There is very clear contrast between these two sentences and raises doubts about how critical the molecular data is analyzed. Similarly, any overlap displayed in panels D and E is a small portion of the total population. While authors provide statistical analysis (I could not find the test used is for the analysis), the authors should not overlook large differences between these populations.

As stated in a previous response, the statistical significance arises from the fact that these genes arise from an extremely large pool of quantified transcripts, and although the overlaps might seem visually unimpressive, the probability that such overlaps would occur by chance is extremely small. Nevertheless, we appreciate this criticism. As the new RNA sequencing of CD34⁺α6⁺ and CD34⁻α6⁺ populations from the 3C cultures together with the additional qPCR analyses provides more solid and detailed characterization of the cell populations and their molecular identity, we have removed the Venn diagram comparisons to TACs and activated HFSCs (Fig 3D, E in the previous version) from the revised manuscript. Instead, we present substantial amounts of new data as described in the

answers to the previous points.

15.) Do cultures start from a single sorted cell? This issue is detrimental to understand the source of the culture

The cultures are inoculated from isolated total epidermal cell suspension that were not sorted, where a suspension of 80000 cells in 40 μ l of Matrigel is dispensed in a cell culture well as described in Materials and Methods. However, the individual cell clusters also form from single cells as shown in the new Supplementary Fig S5H and Movie S1.

16.) Authors hint to the presence of HFSC progeny in culture, yet there is no proof provided. Are they describing the hair producing progeny? They should provide strong molecular proof to claim reprogramming of the progeny back into the stem cell state

We termed these CD34⁻ α 6⁺ cells HFSC progeny as we observed that a) they can arise from a pure population of CD34⁺ α 6⁺ cells, and b) the CD34⁺ α 6⁺ cells proliferate slightly faster than the CD34⁻ α 6⁺ cells, whereas the apoptotic rates are similar. Thus, the CD34⁺ α 6⁺ cells should outcompete the CD34⁻ α 6⁺ cells in the cultures in a few days unless they would constantly give rise to CD34⁻ α 6⁺ cells. Our new RNA sequencing data further confirms that whereas the CD34⁺ α 6⁺ cells are enriched for HFSC identity genes, CD34⁻ α 6⁺ cells show lower expression levels of these genes and mild upregulation of lineage progression genes (new Figs. 3E- G, Fig. S3G and Table S3).

To further strengthen this point we have performed additional lineage tracing experiments where we FACS-purify GFP⁺ and GFP⁻ CD34⁺ α 6⁺ and CD34⁻ α 6⁺ cells and culture them as pure populations and mixtures to genetically track the fate of these cells. The results of these experiments confirm the interconversion of the two cell populations as indicated by the presence of GFP⁺ CD34⁺ α 6⁺ cells in cultures inoculated with GFP⁻ CD34⁺ α 6⁺ cells and GFP⁺ CD34⁻ α 6⁺ cells and vice versa (new Fig. 5F, G and Fig. S5F) These experiments further reveal that the interconversion rates are independent of the relative ratios of CD34⁺ α 6⁺ and CD34⁻ α 6⁺ cells within the initial cell mixtures (new Fig. 5I, J). This finding excludes that secondary factors such as cell positioning close or distant from the matrix contact alone would determine the expression of CD34 as this phenomenon would be sensitive to the relative numbers of CD34⁺ α 6⁺ and CD34⁻ α 6⁺ cells in the mixture.

17.) Both populations can interconvert, which could simply represent different position within a spheroid or contact to the neighboring cells or extracellular matrix. Given the importance of the interconversion of CD34⁺ and CD34⁻ populations in 3C, the authors should identify the molecular differences/similarities between these populations.

As discussed in our response to the previous point, we have now performed RNA sequencing of CD34⁺ α 6⁺ and CD34⁻ α 6⁺ populations and indeed show that the transcriptomes of these two populations are different with several key lineage identity genes upregulated in the CD34⁺ α 6⁺ population (new Figs 3E-G, S3G). This clearly excludes that the two populations would simply reflect differential expression of CD34.

18.) Authors use CD34⁺ as a stem cell marker; yet they quickly predict that both populations could interconvert and include this in their model. The end-point analysis of panel 4E suggests 80% and 33% CD34 positivity upon 2 weeks in culture. Both numbers are different from the data and the simulation results provided in 4D where around 50% of the cells are CD34⁺ α 6⁺, which is also predicted by the model. This is in clear contrast to authors claim that this experiment provides experimental proof of the system. Authors need to provide strong proof their model indeed predicts the reality. Live imaging experiments on reporter lines would be a good choice

To more stringently test the validity of the model, we have performed additional time course experiments on 3C cultures established from FACS-purified CD34⁺ α 6⁺ and CD34⁻ α 6⁺ cells. Here, the populations were FACS-purified from 3C cultures to avoid the double stress of FACS purification and tissue dissociation. These new experiments confirm that a ~50:50 population equilibrium is also generated from the purified populations, as predicted by the model. The new data further fit the theoretical assumptions of the initial steady state model in that the observed proliferation and apoptosis rates together with constant interconversion rates are necessary to explain the observed stable population equilibrium of CD34⁺ α 6⁺ and CD34⁻ α 6⁺ cells. We have

further used this time course data to refine our model parameters and present new simulations for purified cell populations in the new Fig. 5D, E, S5C-E.

The differences to the initial model (Fig. 4D) arises from the fact that this modeling was done on steady state cultures based on cell numbers, proliferation and apoptosis data collected from day 6 to day 14 (see x-axis of Fig. 4D). The reason for modeling steady state behavior can be seen in Fig 4A, where the absolute number of cells over time is shown. The cell numbers dramatically decrease during the first 2 days, which is due to the fact that many cells do not survive the stress of tissue dissociation, a phenomenon familiar to all who work with primary cell cultures. It is also important to acknowledge that FACS purification is a significant stress for cells and therefore cells that have been FACS-purified will invariably display lower survival rates during the first 1-2 days than cells that have not been FACS. As the cell survival during the first days is unpredictable and depends on technical variation and not a biologically relevant process, it is impossible to provide numerical data for this initial phase that would serve as a meaningful, robust basis for modeling.

19.) While the conversion of CD34⁺ and CD34⁻ populations might be of interest, the relevance of these populations to hair follicle genesis remains obscure. The identity of CD34⁻ cells remains obscure. Are these indeed hair generating progeny of stem cells?

As described in our responses to previous points, the RNA sequencing experiments of CD34⁺α6⁺ and CD34⁻α6⁺ populations show that the transcriptomes of these two populations are different in exactly the ways one would predict from them representing HFSCs and non-HFSCs, respectively. As expected, a large panel of HFSC markers and identity regulators are upregulated in the CD34⁺α6⁺ population, whereas the CD34⁻α6⁺ population represents an early lineage committed state that still maintains some characteristics of HFSCs but also upregulates, for example, hair keratins and other markers of hair germ/matrix cells (new Fig. 3E-G, S3G).

20.) Does conversion between these two populations (or states) happen in vivo in equilibrium?

This is a very interesting question that can now be addressed based on the observations in the current manuscript. It is clear that interconversion occurs in vivo as reported by laser ablation and lineage tracing experiments (see for example Mesa et al., Nature 2013). Quantification of equilibrium state behaviors in vivo require very sophisticated, technically demanding and time consuming experiments, such as long-term quantitative live imaging of reporter mice, and is therefore beyond the scope of the current manuscript.

Referee #2:

This is a nice study and the topic is of interest. However, there are some important points that need to be further characterised before the paper can be considered for publication in EMBO J.

We thank the reviewer for finding our work interesting and for the critical comments that greatly helped us to improve our manuscript.

1. In Fig1, the authors need to quantify the proportion of Sox9 in 3C and control condition (KGM 2D).

We have now performed these quantifications (new Fig.S1G) that show that the proportion of SOX9-high cells is the same as the proportion of CD34⁺α6⁺ cells in 3C culture, whereas no SOX9-high cells are detected in the control condition (we used 3C medium in 2D conditions based on comments from reviewer #1).

2. The authors perform full-thickness skin reconstitution assays using 3C cultured cells (42% CD34⁺α6⁺) and freshly isolated epidermal cells (5.6% CD34⁺α6⁺). They state that their 3C cultures are more efficient regarding their HF regeneration capacity as compared to freshly isolated epidermal cells (Fig 2b). However, since the cells were not FACS isolated, this could simply reflect the fact that there is higher proportion of CD34⁺ cells in the 3C. The authors should determine whether the FACS isolated CD34⁺ cells are more efficient at HF regeneration in 3C condition versus native state.

We agree with the reviewer that the increased efficiency of 3C cells to produce hair in comparison to total epidermis is very likely due to the increased numbers of CD34⁺α6⁺ in this mixture. We do not want to claim that the 3C CD34⁺α6⁺ cells are superior in HF reconstitution assays when compared to the *in vivo* CD34⁺α6⁺ cells. We have clarified this in the text.

As suggested by the reviewer we also performed transplantation experiments to directly compare the ability of CD34⁺α6⁺ cells freshly FACS-purified from epidermis and 3C CD34⁺α6⁺ cells to generate hair. The results support the notion that the 3C-cultivated CD34⁺α6⁺ cells perform equally well as the directly purified *in vivo* CD34⁺α6⁺ cells (see Figure below). Unfortunately, several nude mice died in the course of this experiment, likely as it is extremely difficult to maintain complete sterility during the FACS purification procedure of the cells, and we therefore lack a sufficient number of biological replicates to show statistical relevance. Unfortunately, German law is very strict with animal numbers used in these experiments and it would require at least another 6 months to get permission. In addition, as replication of this experiment would require sacrificing 27 experimental animals (6 nude recipient mice, 6 HFSC adult donor mice and 15 newborn fibroblast donor mice), and we also find this ethically somewhat problematic given the confirmatory nature of the experiment. We therefore respectfully request to omit this requested experiment.

Sorted 3C-CD34⁺α6⁺ Sorted *in vivo*-CD34⁺α6⁺**Figure for reviewer:**

Transplantation assays demonstrate that freshly purified *in vivo* CD34⁺α6⁺ cells and 3C CD34⁺α6⁺ cells are equally competent in generating hair

3. The authors claimed that there is no exhaustion over passage. However, this statement has not been quantified. This should be done.

We have now calculated the fold increases of absolute HFSC numbers over time to demonstrate that these cells maintain their proliferative potential (new Fig S2B).

4. The molecular profiling and the comparison with native bulge cells is too superficial. They should better describe the genes in common as well as the genes specifically expressed by either the 3C CD34⁺ and the native bulge cells. Also, they should use the original paper describing bulge cells including Tumber et al, Morris et al, and Blanpain et al. 2004. Is there any particular category of genes maintained or lost in 3C? They proposed that cells are activated, how is the expression of cell cycle regulators?

Based on the reviewers comment we have included a much more in depth overview of the molecular profile of the 3C cells and how they relate to bulge HFSCs. Importantly, we have now also included new RNA sequencing data to further substantiate the similarities between the 3C CD34⁺ cells and the HF bulge CD34⁺ stem cells. Specifically, using multiple different approaches we have done the following:

A) We have first analyzed the global patterns of differentially expressed genes among the three conditions (native HFSCs, 3C cultures and total freshly isolated epidermal mixtures used to start the 3C cultures). Using gene-by-gene quantification of Euclidian distance we have identified clusters of genes that exhibit similar expression levels in native HFSCs, 3C cultures but are different in the total epidermal mixtures (new Fig. S3C). GO term analysis of these clusters show that genes involved in protein/vesicle transport and protein localization and translation display similar expression in *in vivo* HFSCs and 3C cultures, whereas they are differentially expressed in the epidermal mixtures. In contrast, genes involved in transcription, DNA replication and repair as well as cell cycle are differentially expressed in 3C cultures compared to *in vivo* HFSCs, in agreement with the observation that the 3C CD34⁺ cells are actively cycling cells compared to the telogen-quiescent stage of the *in vivo* HFSCs

B) We have performed more detailed analysis of the differentially expressed genes in 3C cultures compared to *in vivo* HFSCs. These analyses show that cell cycle regulators and components of the Wnt signaling pathway seem to be differentially regulated in 3C versus HFSCs (new Fig. S3D, F), which provides further support that 3C cultures enrich for more activated HFSCs. Furthermore, genes of the extracellular matrix are downregulated in 3C cultures compared to bulge HFSC (new Fig. S3E, F), indicating that key ECM components are already provided in excess by the Matrigel in the 3C conditions.

We originally chose the Lien et al signature as this was also generated through RNA seq, whereas all other available datasets are from microarray data and therefore cannot directly be compared. However, as requested, we have now merged the Lien signature with those from Tumber et al. 2004 and Morris et al. 2004 into a combined HFSC signature (Supplementary Table S2). These data again demonstrate that the 3C cultures enrich for cells with a HFSC signature. We were unable to include the data from the Blanpain et al. 2004 paper, as the complete microarray dataset is not publically available.

5. The model proposed in figure 4 is confusing. The authors should not analyse the bulk of population but rather plate FACS isolated α6⁺CD34⁻ and α6⁺CD34⁺ in 3C and assess the proportion of CD34⁺ cells overtime. If they want to determine the role of mixing two populations on the CD34 proportion, the individual populations should be marked with different fluorescent proteins. They should then analyse for each of them the apoptosis, proliferation rate and rate of interconversion.

We have now performed additional experiments to further substantiate the proposed model. As suggested by the reviewer, we have performed time course analyses of 3C cultures established from FACS-purified populations and show that these populations evolve into a 50:50 population equilibrium in a similar manner as the mixed cultures (new Fig. 5B, C). We have further measured their apoptosis and proliferation rates (new Fig. S5A, B). Using this new dataset, we have performed additional simulations that demonstrate, as with the mixed cultures, that bidirectional interconversion is necessary to explain the observed evolution of the population equilibrium (new Fig. 5D, E, S5D, E).

Furthermore, to additionally demonstrate the interconversion, we have performed the suggested lineage tracing experiments, where we mix purified EGFP+ CD34⁺α6⁺ with purified unlabeled CD34⁻α6⁺ cells in various ratios, and vice versa, mix purified EGFP+ CD34⁻α6⁺ with unlabeled CD34⁺α6⁺ cells (new Fig. 5F-J, S5F, G). Importantly, the results demonstrate that not only EGFP+ CD34⁺α6⁺ can generate EGFP+ progeny as expected, but more importantly, show that EGFP-labeled CD34⁻α6⁺ give rise to EGFP+ CD34⁺α6⁺ cells (new Fig. 5F, G and S5F), indicative of interconversion of the two cell populations.

Interestingly, these experiments further reveal that the 50:50 population equilibrium is reached at the same rate regardless of the relative amounts of CD34⁺α6⁺ and CD34⁻α6⁺ cells in the initial mixture and that interconversion rates of each cell population are independent of the presence of the other cell population (new Fig 5I, J). This is in agreement with live imaging data showing that the individual 3C clusters within the matrigel initiate from single cells, thus explaining their cell autonomous behavior in reaching the 50-50 population equilibrium.

6. It would be interesting if the authors could show that upon freezing and thawing 3C cultured cells retain their functionality using full-thickness skin reconstitution assays. This would reinforce the robustness of the described culture system.

We have performed these experiments and observe that cells that have undergone a freeze-thaw cycle are still capable of generating hair (new Fig S2D).

Overall, this is an interesting and relevant study that could be valuable to the research community. I recommend publication of this paper in EMBO journal once they address the key points highlighted above.

We thank the reviewer for this assessment.

Referee #3:

I think this is a very solid and interesting work. The quality of the data is impeccable, and the results are novel and timely. As such I do not have any criticism regarding the data provided.

We thank the reviewer for this positive assessment of our work.

I do think that the authors should provide more mechanistic data on the Bmp and Shh part though, since this is precisely the strength of their system: it allows to perform more mechanistic studies that would otherwise be much more difficult to do with more conventional in vivo approaches. The authors should analyse the global gene expression changes by RNA-seq that occur upon modulation of Bmp and Shh signalling of the 3C cultures to drive the cell state equilibrium towards the SC state to the TAC state. This will provide very interesting and useful mechanistic information to the field on how these cell transitions occur and how these signalling pathways function.

As suggested, we have performed additional RNA sequencing on 3C cultures upon inhibition of BMP and Shh pathways. To capture early signaling events induced by modulation of Shh and BMP pathways, we performed these analyses 48h after applying the inhibitor (new Fig. 6D, E, S6A). Interestingly, the Id factors 1,2, and 3 were the most prominently altered group of genes (new Fig. 6D, E). This is intriguing as these genes represent direct BMP and Smad 1/5 targets, are highly

transcribed in HFSCs, downregulated during telogen to anagen transition, as well as functionally implicated in HFSC activation and TAC specification (Genander et al., 2014). This data strengthens our conclusion that the 3C cultures faithfully recapitulate *in vivo* signaling responses of HFSCs, and highlights the role of the BMP-Id signaling axis in HFSC activation.

2nd Editorial Decision

24 October 2016

Thank you for submitting the revised version of your manuscript. It has now been seen by the three original referees, whose comments are enclosed below.

As you will see the referees find that their concerns have been overall sufficiently addressed and are now broadly in favour of publication. However, while referee #2 agrees in that the manuscript has strongly improved, this referee also points to remaining concerns related to the mathematical modeling on interconversion between the CD34pos/neg cell populations (ref #2, second paragraph). Please note, that while this point is well taken, taking into account the positive comments of referee #1 and referee #3, we have decided that pending a satisfactory revision, we would go ahead with acceptance of this manuscript as soon as possible. Thus, I ask you to revise your manuscript regarding the points raised by referee #2 and evaluate, whether you would be able to add supportive complementary data, or, alternatively, relativise your statements and introduce caveats where appropriate.

Thus, I invite you to submit a revised version of the manuscript together with a pt-by-pt response addressing the referees' concerns using the link provided below.

REFEREE REPORTS

Referee #1:

The authors have addressed all points I have raised during the first round of revisions. I believe the manuscript should be published in its current form.

Referee #2:

The manuscript has greatly improved compared to the initial version. The authors addressed in the revised manuscript most of the initial comments providing new experiments and a more precise transcriptional analysis of their cultures, which greatly clarifies the study. Chacón-Martínez and colleagues should be complimented for their effort in the revision of the manuscript.

However, my concern is that the revised version does not validate properly the mathematical model of the cultures, which to my opinion is not at all essential for the conclusion that CD34+ and CD34- population interconvert *in vitro*. To be precise the author should use lineage tracing/fate mapping strategies. I strongly recommend to challenge/ validate the model by labelling the two populations or tone down this part of the manuscript and remove the model from the manuscript. I suggest addressing this point before the paper can be considered for publication in EMBO journal.

Referee #3:

I already thought this manuscript was very interesting and solid in the first round of submission. The authors have now performed the transcriptome analysis that I suggested in the first revision of their manuscript. This new data highlights and confirms that the *in vitro* system they have developed faithfully recapitulates the molecular identity and regulation observed for hair follicle stem cells *in vivo*. Hence, this is a very novel and interesting model that is very likely to allow researchers in the field to study in much better detail the molecular mechanisms underlying epidermal stem cell function.

Referee #1: The authors have addressed all points I have raised during the first round of revisions. I believe the manuscript should be published in its current form.

We thank the reviewer for this assessment.

Referee #2: The manuscript has greatly improved compared to the initial version. The authors addressed in the revised manuscript most of the initial comments providing new experiments and a more precise transcriptional analysis of their cultures, which greatly clarifies the study. Chacón-Martínez and colleagues should be complimented for their effort in the revision of the manuscript. We appreciate that the reviewer is overall satisfied with the revised manuscript and for the critical comments that helped us to improve it.

However, my concern is that the revised version does not validate properly the mathematical model of the cultures, which to my opinion is not at all essential for the conclusion that CD34+ and CD34- population interconvert in vitro. To be precise the author should use lineage tracing/fate mapping strategies. I strongly recommend to challenge/ validate the model by labelling the two populations or tone down this part of the manuscript and remove the model from the manuscript. I suggest addressing this point before the paper can be considered for publication in EMBO journal.

It should be emphasized that we present a very simple mathematical model in order to estimate the degree to which interconversion occurs. The model entails three fundamental processes, namely (i) cell proliferation, (ii) apoptosis and (iii) interconversion. Appropriate assays (EdU incorporation and Annexin staining) allowed quantification of the proliferation and apoptosis rates of CD34+ and CD34- cells. Additional time course experiments where we quantified these parameters together with absolute cell numbers further allowed us to estimate the rate of interconversion by fitting the mathematical model to the measured data. When we simulate the behavior of the two populations using the resulting parameters, the simulations mimic the experimental findings and result in formation of a ~50:50 population equilibrium.

To further support our line of argument we provide additional model simulations where we apply the same fitting procedures as in the submitted manuscript, with the important difference that we do not allow conversion from CD34- to CD34+ cells. As indicated in the supplied figure, it is not possible to re-establish a mixed population of CD34+ and CD34- from purified CD34- cells on the time scale that this was observed to occur in our experiments. In this respect, the mathematical model serves as an essential tool to estimate the extent to which interconversion contributes to the establishment of a ~50:50 population equilibrium.

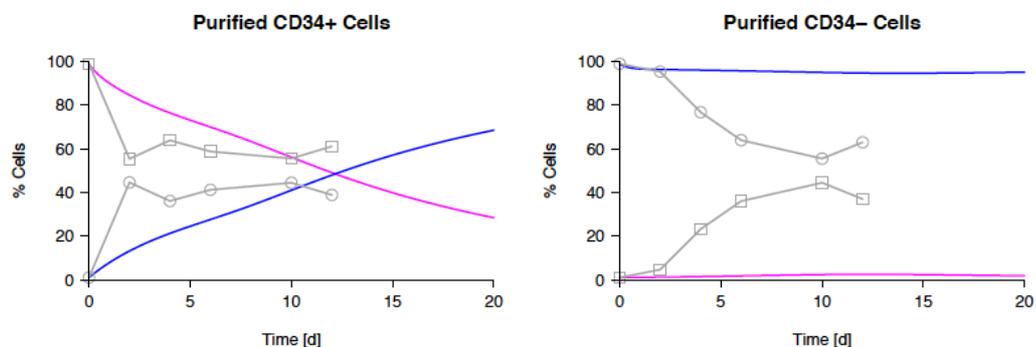


Figure for the reviewer. Simulations with the same parameters as in manuscript with the exception that interconversion rate of CD34- cells (in cyan) to CD34+ cells (in magenta) is set to 0. Grey lines show experimental data.

We agree with the reviewer that further validation of the model is important, and labeling the two

populations is an ideal experiment to do so. Therefore we had, already in the previous manuscript version, FACS-purified CD34⁺ cells that were genetically labeled with EGFP, mixed them with non-labeled FACS-purified CD34⁻ cells in various ratios, and traced the fate of the EGFP-labeled cells over time (Fig 5H-J, and Appendix figure S5F). As additional control, we had performed the reverse experiment where purified CD34⁺ cells were unlabeled and purified CD34⁻ cells were genetically labeled with EGFP. We then traced the fate of the labeled cells over time and quantified the number of CD34⁺ cells that had remained CD34⁺ as well as the number of these cells that had converted into CD34⁻ cells (Fig. 5I). Likewise, we quantified the number of CD34⁻ cells that had remained CD34⁻ and the number that had converted into CD34⁺ cells (Fig. 5J). The results of these experiments demonstrate that: 1) A ~50:50 equilibrium is established, regardless of the amount of CD34⁺ and CD34⁻ cells at the onset of the culture (Fig. 5H), as predicted by the model 2) The conversion rate of CD34⁺ cells into CD34⁻ and vice versa is independent of the ratio of CD34⁺ and CD34⁻ cells in the system (Fig. 5I-J), as predicted by the model 3) The conversion rate of CD34⁺ to CD34⁻ cells is higher (around 50% convert) than the conversion rate of CD34⁻ to CD34⁺ cells (around 30% convert) (Fig. 5I-J), as predicted by the model. This data therefore provides strong validation for the model. We have now edited the manuscript in order to clarify this point and to avoid over interpretation.

Referee #3: I already thought this manuscript was very interesting and solid in the first round of submission. The authors have now performed the transcriptome analysis that I suggested in the first revision of their manuscript. This new data highlights and confirms that the in vitro system they have developed faithfully recapitulates the molecular identity and regulation observed for hair follicle stem cells in vivo. Hence, this is a very novel and interesting model that is very likely to allow researchers in the field to study in much better detail the molecular mechanisms underlying epidermal stem cell function.

We thank the reviewer for this assessment.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sara Wickström

Journal Submitted to: EMBO JOURNAL

Manuscript Number: EMBOJ-2016-94902R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/ varied/ perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to determine sample size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical method was used to determine sample size
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were not randomized
For animal studies, include a statement about randomization even if no randomization was used.	Samples were not randomized
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was applied
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was applied
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	normal distribution and equal variance were assessed by Kolmogorov-Smirnov test and F-test, respectively
Is there an estimate of variation within each group of data?	yes
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

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<http://ijb.biochem.sun.ac.za>

http://oba.od.nih.gov/biosecurity/biosecurity_documents.html

<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Immunofluorescence: rabbit anti-SOX9 (H-90,sc20095, Santa Cruz Biotechnology), mouse anti-Keratin15 (LHK15, CBl272 NeoMarkers-Millipore), mouse eFluor660-CD34 (eBioscience;50-0341-82 (clone RAM34)), Laminin 332 (gift from R.E. Burgeson, Rousselle et al., J Cell Biol 1991)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Flow cytometry: mouse eFluor660- or FITC-CD34 (clone RAM34, eBioscience50-0341-82), mouse BV421-CD34 (clone RAM34, BD Biosciences 562608),rat eFluor450-, PE-Cy7- or FITC-α6 Integrin (clone GoH3, eBioscienc 12-0451), PE-Cy7-EpCAM (clone G8.8, eBioscience 25-5791-80).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Epidermal cells were isolated from 21d-old or 48d- to 60d-old C57BL/6j mice or LifeAct-EGFP mice (Riedl et al, 2010). Primary dermal fibroblasts were isolated from 2d-old C57BL/6j mice. Here genders were randomly distributed among the groups. 7-9 week-old female BALB/c nude mice (CAnN.Cg-Foxn1nu/Crl; Charles River, Germany) were used as recipients in transplantation experiments. Animals were housed and maintained according to FELASA guidelines in the animal facility of the Max Planck Institute for Biology of Ageing, Cologne, Germany. All experiments were approved by the Animal Welfare authorities of the state of North-Rhine-Westphalia (permit # 2014.A491)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The transplantation experiments fully comply with ethical regulations and were approved by the authorities of the state of North-Rhine-Westphalia (permit # 2014.A491)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All animal experiments were adequately reported and documented

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human subjects were involved
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNA sequencing data has been deposited to NCBI-GEO (accession # GSE76779).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	RNA sequencing data has been deposited to NCBI-GEO (accession # GSE76779).
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: Primary Data Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 Referenced Data Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4Q26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We have not included this section
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as BioModels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Model for simulations presented in Figures 4 and 5 can be downloaded in SBML format from the BioModels website (accession number pending)

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No
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