EMBO reports

# The extracellular matrix fibulin 7 maintains epidermal stem cell heterogeneity during skin aging

Erna Raja, Gopakumar Changarathil, Lalhaba Oinam, Jun Tsunezumi, Yen Ngo, Ryutaro Ishii, Takako Sasaki, Kyoko Imanaka-Yoshida, Hiromi Yanagisawa, and Aiko Sada
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Thank you for transferring your manuscript from Review Commons to EMBO reports. I went through your manuscript, the referee reports (attached again below), and your revision plan, and decided to ask an expert advisor for an independent opinion. Taking into consideration his/her feedback (you will also find below), I would like to invite you to revise your manuscript with the understanding that all referee concerns (as indicated in your revision plan) and the points of the advisor must be addressed in the revised manuscript or in the final point-by-point response.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the author contributions section.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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Advisor:

The authors present an analysis of two populations of basal epidermal cells that they have previously discovered in the tail skin of mice. The existence of these two populations has not been well documented elsewhere in the skin, which makes the manuscript less interesting for a broader audience (the authors should also more clearly state that they are studying mouse tail skin as this is a special region of the skin that behaves deferentially due to the scales etc). Some of the central experiments have key technical caveats, some of which are nicely acknowledged by the authors, which is great but nevertheless make interpretations of the data difficult.

Loss of the fast cycling population most likely reflects the fact that these cells represent a more committed cell population - as has been shown for the back skin using Involucrin-Cre (Blanpain lab, more recently Greco&Kasper), so it is not surprising that these clones are "lost" over time as they are on average more differentiated at the moment of labeling. Thus, a loss of these clones does not necessarily mean that stem cell heterogeneity is impacted by aging as the authors propose, it just means that most of the labeled cells in this population were not stem cells to begin with.

As the authors themselves disclose in the limitations of the study, comparing "label retaining cells" as proxies for two distinct stem cell populations is hugely problematic: aging changes the overall rates of tissue turnover, the cells that retain label in the aged mice can be very different from the young mice, substantially limiting the interpretability of the RNAseq data.

An additional caveat of the RNAseq is that its done from back skin, which as said is very different from tail. Given the two technical caveats of the RNAseq, the main solid findings in this dataset have already been reported: increased DNA damage, metabolism and ecm. Delay in wound healing upon aging has been demonstrated in multiple studies, as are the changes in inflammation and extracellular matrix, as mentioned above.

The two reviewers focus on improving the analysis on the role of Fibulin. Addressing this will indeed strengthen the manuscript. If the experimental plan really works out as the authors propose (including the in vitro expriments and the new antibody, which I think are critical), I would think that the manuscript might be suitable.

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Referee #1:

The authors previously showed that the skin epidermis contains 2 distinct populations of stem cells: slow cycling stem cells (Dlx1+) that persist over time, and faster cycling stem cell populations (marked by Slc1a3) that disappear in aged skin. What determines whether a stem cell proliferates faster or slower remains unclear and is an important question in skin biology. To address this point and to characterize these populations, the Sada & Yangisawa groups lineage traced both populations and subjected them to RNA seq. As expected, principal component analysis grouped the different stem cell populations into distinct clusters. Gene ontology analysis revealed that genes involved in DNA repair & replication, telomere function and chromatin regulation were significantly reduced in aged slow cycling stem cells, whilst genes related to immune response were upregulated.

Generally, aged slow and fast cycling cells were characterized by changes in cell metabolism, cell adhesion, ECM, inhibition of proliferation, activation of differentiation and hair follicle development.

The authors then found that the secreted glycoprotein and ECM component fibulin 7 (Fbln7) was upregulated in fast cycling stem cells of 2 year old mice. To determine the significance of this finding, lineage tracing of different stem cell populations was conducted in Fbln7a knockout mice. 1 year old Fbln7a ko mice exhibited a decreased number (and size) of fast cycling stem cell clones (as compared to WT or young Fbln7a deficient mice). In contrast, Fbln7a depletion does not appear to affect slow cycling stem cells. In addition, Fbln7a depletion exacerbates wound healing deficiencies in aged (but not young) mice and triggered increased expression of inflammatory response genes. The MAPK pathway and cytokine production was upregulated while chemotaxis genes were downregulated. To test whether there was lineage misspecification, they examined K14 expression. There appeared to be an increase in basal to suprabasal expansion of K14+ cells in Fbln7a ko mice. This seems to be progressive, confined to scale region and was only observed in aged mice.

To identify potential factors that interact with fibulin 7, conditioned media from fib7 overexpressing cells was co-eluted with fibulin 7 from an affinity column and identified via mass spec. Fib7 interacts with collagen IV, tenascin, periostin and Ccdc80. Depletion

of fib7 leads to transcriptional upregulation of CoIIV and a slight thickening of CoIV in the basement membrane in scale skin. Similarly, CoIIV increases in aged basement membrane of skin. Lastly, overexpression of Fib7 in human primary keratinocytes decreased differentiation markers and slowed proliferation. This phenotype was dependent on its CC domain. In conclusion the authors identified Fib7a as an important ECM component involved in "regulating" slow stem cell proliferation in mouse skin.

This reviewer has a few points that may improve the paper and provide further mechanistic insight:

The overall gist of this paper is that fibulin 7 plays an important role in regulating proliferation of stem cells.

A major concern is that the authors do not show where fibulin is expressed in mouse skin. Most of the data is based on transcriptional analysis and should be further verified /strengthend by immunofluorescence staining or western blotting.

Another key question is: How does fibulin depletion diminish the number of fast cycling stem cells in aged mice. Does fibulin depletion take off the (proliferation) breaks in fast cycling stem cells in younger mice? The authors conduct BrdU incorporation in young and aged mice (Figure S3). The data is not conclusive - possibly because they looked at overall BrdU incorporation, rather than distinguishing the fast and slow cycling populations (by IF) and analyze BrdU incorp in the different populations. Speeding up proliferation - thereby ending up with more replication cycles (and telomere shortening) would be in agreement with fibulin depletion upregulating the MAPK pathway (Fig 4B).

Many of the figures rely heavily on the analysis of transcriptome data (RNAseq) and lack further mechanistic follow up studies. For instance, on page 9, the authors mention that GO analysis show that genes involved in DNA repair, telomere maintenance etc are downregulated in 2 years old LRC. Is there any evidence that these cells accumulate DNA damage? This could be analyzed by DNA damage response markers y-H2AX and 53BP1.

How does fibulin 7 depletion affect deposition of other key components of the ECM, for instance collagen XVII?

**Minor points:**

Figure 6: Overexpression of fibulin in vitro slows down proliferation. I think it is important to provide western blots to compare the levels of ectopically expressed fibulin 7 and CC mutant in comparison to endogenous levels. Similarly, have the authors looked at the consequences of fibulin depletion in vitro? Will the cells proliferate faster?

It would be interesting to investigate the consequences of fibulin 7 overexpression in vivo. Would it enhance wound repair in aged mouse skin or affect skin or hair development? These experiments would increase the impact of this paper, but given the time consuming nature of such experiments (2 years), they are well beyond the scope of the current manuscript.

Results section: first subtitle: can the authors rephrase this title? "Fast cycling stem cells are gradually lost and compartments of distinct stem cell populations impaired during aging" maybe it can be changed to "Fast cycling stem cells are gradually lost during aging".

In the discussion, please elaborate on the increased expression of Hair follicle development pathways in aged LRC. Does this bear any physiological relevance?

The color legends of Figures S3B,C,E,F do not match the colors of the bars. As mentioned earlier, this analysis looks at overall proliferation. Could the authors focus on the fast-cycling stem cell population?

Page 8: Please provide full name of LRC: it probably stands for label retaining cells (LRC) vs non-label retaining cells (nLRC).

**Significance**

Overall, this is a very well written paper. The results are presented in a clear and concise manner. The topic is of importance for skin regeneration and skin aging.

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Referee #2:

In this manuscript, the authors demonstrated that fast-cycling epidermal stem cells are gradually depleted with aging, and Fibulin-7, an ECM component, is involved in this age-dependent stem cell depletion.

**Major points**

1. In Fig 2, the authors identified several ECM genes and selected Fbln7 gene for further analysis. For the readers, the authors also should reveal the other ECM genes that were upregulated in old nLRCs. In addition, the expression of Fibulin-7 protein in the skin should be also analyzed in young and old mice.

2. In Fig 3F and G, the authors clearly demonstrated delayed wound healing in Fbln7 hetero and KO mice. However, Fibulin7 might have a function in the dermis, which is involved in delayed wound healing. Histological analysis should be performed to indicate that delayed wound healing results from impaired reepithelialization.
3. In Fig 6, the authors demonstrated that Fibulin-7 maintains epidermal stem cells at the undifferentiated state. The authors also suggested that Fibulin-7 modulates the COLIV and maintains epidermal stem cells in Fig. 5. Mouse keratinocytes can be cultured on a COLIV-coated dish. Can overexpression of Fibulin-7 modulate keratinocyte differentiation on the COLIV-coated dish? This experiment clearly demonstrates the Fibulin-7/COLIV axis for epidermal stem cell maintenance.
4. The fast-cycling epidermal stem cells are depleted in old mice and Fibulin-7 KO mice. These results suggest that Fibulin-7 is required for the maintenance of the fast-cycling epidermal stem cells in old mice. However. fibulin-7 is increased in old nLRCs (fast-cycling epidermal stem cells).

**Minor points**

1. On page 12, the sentence "Intriguingly, ...." is duplicated.
2. On page 24, the authors should describe which proteins are coated on the dishes for the culture of mouse keratinocytes. This information is crucial since this study focuses on the role of ECM proteins in keratinocyte stem cell regulation.
3. In Fig 5D and E, the authors displayed uneven distribution of COLIV in Fbln7 KO and old mice. But, it was not clear. Does the uneven distribution mean excess deposition of COLIV or discontinuous staining of COLIV?
2. Significance:

**Significance**

This study has originally extended the previous report (Sada et al, Nat Cell Biol 2016) and contains novel findings. In particular, the age-associated regulation of epidermal stem cells by Fibulin-7 is worth to share the research community. I recommend that this manuscript should be published if the authors address the above points.

**Manuscript number:** EMBOR-2022-55478V1 (RC-2022-01328)
**Corresponding author(s):** Aiko Sada, Hiromi Yanagisawa

## 1. General Statements

We are grateful for the constructive insights and comments that the reviewers provided. We have addressed all these comments by performing the proposed experiments in the previous revision plan.

## 2. Point-by-point description of the revisions

**Advisor:**
The authors present an analysis of two populations of basal epidermal cells that they have previously discovered in the tail skin of mice. The existence of these two populations has not been well documented elsewhere in the skin, which makes the manuscript less interesting for a broader audience (the authors should also more clearly state that they are studying mouse tail skin as this is a special region of the skin that behaves deferentially due to the scales etc). Some of the central experiments have key technical caveats, some of which are nicely acknowledged by the authors, which is great but nevertheless make interpretations of the data difficult.

We thank the advisor for the critical comments. Although differentiation lineages of the scale and interscale structures are unique to the tail skin, the fast- and slow-cycling stem cell populations are not only found in the tail skin but also observed in the human skin (Ghuwalewala et al., *EMBO J*. 2022). In addition, the eye corneal/conjunctival epithelium is also regenerated from the compartmentalized stem cell populations like the tail skin epidermis (Altshuler et al., *Cell Stem Cell*. 2021; Ishii et al., *Development*. 2020).

So, although we use the tail skin as a primary study model, we believe that the principal findings from these two stem cell populations during aging may also be relevant in the human skin and other epithelial tissues.

We added explanations in the introduction of the manuscript to clarify this point better (page 5, line 18-20).

Loss of the fast cycling population most likely reflects the fact that these cells represent a more committed cell population - as has been shown for the back skin using Involucrin-Cre (Blanpain lab, more recently Greco&Kasper), so it is not surprising that these clones are "lost" over time as they are on average more differentiated at the

moment of labeling. Thus, a loss of these clones does not necessarily mean that stem cell heterogeneity is impacted by aging as the authors propose, it just means that most of the labeled cells in this population were not stem cells to begin with.

We thank the advisor for raising this point. The non-LRC or fast-cycling population was characterized previously as undifferentiated as the LRC or the slow-cycling stem cells by microarray gene expression data (Sada et al. NCB, 2016) and more recently by single-cell RNA sequencing (Ghuwalewala et al., EMBO J, 2022). This is in contrast to the Inv-positive progenitor cells, as reported previously.

We added explanations in the introduction of the manuscript about this point (page 5, line 22-24).

As the authors themselves disclose in the limitations of the study, comparing "label-retaining cells" as proxies for two distinct stem cell populations is hugely problematic: aging changes the overall rates of tissue turnover, and the cells that retain the label in the aged mice can be very different from the young mice, substantially limiting the interpretability of the RNAseq data.

Although the stem cells in the aged population may be different from the young ones, which we have acknowledged as a limitation of our study, aged slow- or fast- cycling stem cells still hold some differences in their proliferation rates (Figure EV2A). Changes in the gene expression of the surviving aged stem cells (fast or slow) are as described, and we believe it is still informative and may be indicative of some aging mechanism that has occurred in the aged stem cells with lower or higher proliferation rates.

An additional caveat of the RNAseq is that its done from back skin, which as said is very different from tail. Given the two technical caveats of the RNAseq, the main solid findings in this dataset have already been reported: increased DNA damage, metabolism and ecm. Delay in wound healing upon aging has been demonstrated in multiple studies, as are the changes in inflammation and extracellular matrix, as mentioned above.

The comparison between young and aged LRC or nLRC was made using RNAseq from the tail skin. The RNAseq from Fbln7 WT vs. KO was indeed from the back skin, although it was also previously characterized that the back skin similarly contains the fast- and slow-cycling stem cell populations (Sada et al., NCB 2016).

The slow-cycling nature of SC is thought to reduce replication stress and inhibit aging, but conversely, mutations acquired in slow-cycling SC may accumulate over time due to an error-prone DNA repair pathway (Tumpel & Rudolph, 2019). It remains unclear whether this slow cycle rate maintains the long-term potential for SC and serves as a mechanism to delay stem cell aging due to the lack of a study model. In this study, we demonstrated that the fast-cycling population was indeed depleted while the slow-cycling population was maintained in the same tissue during a two-year chronological aging process. We believe this finding provides insight into the significance of proliferative heterogeneity of tissue stem cells in aging.

We clarified the above points in the manuscript's introduction (page 5, line 6-11).

In addition, it has not been previously reported that fibulin 7 is an ECM that functions in the skin. Our data that fibulin 7 supports stem cell heterogeneity during aging is a novel aspect of this study.

The two reviewers focus on improving the analysis on the role of Fibulin. Addressing this will indeed strengthen the manuscript. If the experimental plan really works out as the authors propose (including the in vitro expriments and the new antibody, which I think are critical), I would think that the manuscript might be suitable.

We have performed additional experiments suggested by the reviewers and revised our manuscript. We are pleased to report that we addressed all the comments we proposed in the revision plan.

**Referee #1:**
The authors previously showed that the skin epidermis contains 2 distinct populations of stem cells: slow cycling stem cells (Dlx1+) that persist over time, and faster cycling stem cell populations (marked by Slc1a3) that disappear in aged skin. What determines whether a stem cell proliferates faster or slower remains unclear and is an important question in skin biology. To address this point and to characterize these populations, the Sada & Yangisawa groups lineage traced both populations and subjected them to RNA seq. As expected, principal component analysis grouped the different stem cell populations into distinct clusters. Gene ontology analysis revealed that genes involved in DNA repair & replication, telomere function and chromatin regulation were significantly reduced in aged slow cycling stem cells, whilst genes related to immune response were upregulated.

Generally, aged slow and fast cycling cells were characterized by changes in cell metabolism, cell adhesion, ECM, inhibition of proliferation, activation of differentiation and hair follicle development.

The authors then found that the secreted glycoprotein and ECM component fibulin 7 (Fbln7) was upregulated in fast cycling stem cells of 2 year old mice. To determine the significance of this finding, lineage tracing of different stem cell populations was conducted in Fbln7a knockout mice. 1 year old Fbln7a ko mice exhibited a decreased number (and size) of fast cycling stem cell clones (as compared to WT or young Fbln7a deficient mice). In contrast, Fbln7a depletion does not appear to affect slow cycling stem cells. In addition, Fbln7a depletion exacerbates wound healing deficiencies in aged (but not young) mice and triggered increased expression of inflammatory response genes. The MAPK pathway and cytokine production was upregulated while chemotaxis genes were downregulated. To test whether there was lineage misspecification, they examined K14 expression. There appeared to be an increase in basal to suprabasal expansion of K14+ cells in Fbln7a ko mice. This seems to be progressive, confined to scale region and was only observed in aged mice.

To identify potential factors that interact with fibulin 7, conditioned media from fib7 overexpressing cells was co-eluted with fibulin 7 from an affinity column and identified via mass spec. Fib7 interacts with collagen IV, tenascin, periostin and Ccdc80. Depletion of fib7 leads to transcriptional upregulation of ColIV and a slight thickening of CoIV in the basement membrane in scale skin. Similarly, ColIV increases in aged basement membrane of skin. Lastly, overexpression of Fib7 in human primary keratinocytes decreased differentiation markers and slowed proliferation. This phenotype was dependent on its CC domain. In conclusion the authors identified Fib7a as an important ECM component involved in "regulating" slow stem cell proliferation in mouse skin.

We appreciate the reviewer’s positive comments and suggestions. We have performed additional experiments and revised the manuscript, as suggested by the reviewer.

This reviewer has a few points that may improve the paper and provide further mechanistic insight:

The overall gist of this paper is that fibulin 7 plays an important role in regulating proliferation of stem cells.

A major concern is that the authors do not show where fibulin is expressed in mouse skin. Most of the data is based on transcriptional analysis and should be further verified /strengthend by immunofluorescence staining or western blotting.

We have validated the new monoclonal antibody using tail skin from *Fbln7* WT vs. KO and observed its localization in the basement membrane (New figures 2H and EV3A, B). When comparing C57BL/6J WT mice from 2-3 months old to 2 years old, we saw downregulation of fibulin 7 protein expression (New figures 2H, I). Despite the upregulation of the mRNA expression in the aged fast-cycling stem cells, which we have not understood the mechanism yet, the decrease in fibulin 7 protein in aged skin would explain its function in maintaining the fast-cycling stem cells, and its loss of expression led to early depletion of these stem cells.

In the manuscript, we clarified the above points in the results and discussion (page 10, line 16-22, page 11 line 1-2).

Another key question is: How does fibulin depletion diminish the number of fast cycling stem cells in aged mice. Does fibulin depletion take off the (proliferation) breaks in fast cycling stem cells in younger mice? The authors conduct BrdU incorporation in young and aged mice (Figure S3). The data is not conclusive - possibly because they looked at overall BrdU incorporation, rather than distinguishing the fast and slow cycling populations (by IF) and analyze BrdU incorp in the different populations. Speeding up proliferation - thereby ending up with more replication cycles (and telomere shortening) would be in agreement with fibulin depletion upregulating the MAPK pathway (Fig 4B).

We thank the reviewer for their suggestion. We examined proliferation in sagittal skin sections and counted Ki67 positive cells within the Slc1a3-CreER labeled fast-cycling basal stem cells at 1-week post-tamoxifen injection. Our new data suggest that indeed Fbln7 KO resulted in more proliferation in the young adult tail skin (2-3m old), which may contribute to the diminishing fast cycling stem cells in the aged Fbln7 KO skin (New figure 3D, E).

In the manuscript, we clarified the above points in the results and discussion (page 11 line 9-11).

Many of the figures rely heavily on the analysis of transcriptome data (RNAseq) and lack further mechanistic follow up studies. For instance, on page 9, the authors mention that GO analysis show that genes involved in DNA repair, telomere maintenance etc are downregulated in 2 years old LRC. Is there any evidence that these cells accumulate

DNA damage? This could be analyzed by DNA damage response markers y-H2AX and 53BP1.

As suggested, we assessed DNA damage markers such as γ-H2AX and 8-oxo-dG in the tail skin of young adult (2-3 months) vs. aged (2 years old) mice. Staining with γ-H2AX yielded no signal even in 2 years old tail skin. 8-oxo-dG staining showed some positive cells in the interscale of 2 years old tail skin, albeit at a rare frequency (New figure EV2 H, I). This may be attributed to the elimination of DNA-damaged stem cells by differentiation, as also observed by suprabasal cell staining.

In the manuscript, we clarified the above points in the results and discussion (page 9 line 5-7).

How does fibulin 7 depletion affect deposition of other key components of the ECM, for instance collagen XVII?

As suggested by the reviewer, we evaluated if collagen XVII level changes upon loss of *Fbln7*. Col XVII staining shows that its level is decreased in both scale and interscale of *Fbln7* KO mice at 1-year-old (New figures EV5 G-I). This is in line with the previous finding that Collagen XVII is downregulated in aging skin. In C57BL/6J WT mice, the decreasing trend in Col XVII was seen in the scale and interscale but interestingly more significant in the interscale region of the 2-year-old mice.

We also showed that laminin, a major component of basement membrane that is also a fibulin 7 interactor (based on our mass spec screen), is downregulated in the absence of *Fbln7* at 1-year-old (New figures EV5 D-F).

These points were addressed in results and discussion page 14, line 12-20

**Minor points:**

Figure 6: Overexpression of fibulin in vitro slows down proliferation. I think it is important to provide western blots to compare the levels of ectopically expressed fibulin 7 and CC mutant in comparison to endogenous levels. Similarly, have the authors looked at the consequences of fibulin depletion in vitro? Will the cells proliferate faster?

The endogenous mRNA expression of fibulin 7 in our primary newborn keratinocytes is very low or zero according to the Ct values we obtained (very close to Ct 40 or

undetected). We think that we will not be able to detect endogenous levels of fibulin 7 protein in these cells.

Due to this reason, we have not performed the reverse experiment to deplete fibulin 7 expression in vitro and look at proliferation. We attempted to isolate primary newborn keratinocytes from WT and *Fbln7* KO mice; however, it failed even in the WT, and we believe due to a different genetic background (129EvSv; C57BL6/J) from C57BL6/J. We have also tried to isolate primary keratinocytes from the tail of young adult or aged mice, but these cells have so far been unable to grow continuously in culture.

It would be interesting to investigate the consequences of fibulin 7 overexpression in vivo. Would it enhance wound repair in aged mouse skin or affect skin or hair development? These experiments would increase the impact of this paper, but given the time consuming nature of such experiments (2 years), they are well beyond the scope of the current manuscript.

As the reviewer kindly indicated, the time required to complete this experiment would be too long, and we think it is beyond the scope of this manuscript.

Results section: first subtitle: can the authors rephrase this title? "Fast cycling stem cells are gradually lost and compartments of distinct stem cell populations impaired during aging" maybe it can be changed to "Fast cycling stem cells are gradually lost during aging".

We agreed and changed it as suggested by the reviewer (page 6, line 10).

In the discussion, please elaborate on the increased expression of Hair follicle development pathways in aged LRC. Does this bear any physiological relevance?

We have not observed any hair follicle-specific proteins in the IFE of aged mice even though their genes were upregulated according to the RNAseq results. We think this could be the mis-regulation of lineage genes at the genomic or epigenetic level as a reflection of increasing genomic instability during aging. However, not necessarily translated into protein expression changes or lineage modifications.

The color legends of Figures S3B,C,E,F do not match the colors of the bars. As mentioned earlier, this analysis looks at overall proliferation. Could the authors focus on the fast-cycling stem cell population?

We corrected the bar colors (new Figure EV3G, H, J, K). We analyzed the proliferation of the fast-cycling stem cell population by measuring Ki67 in the Slc1a3-positive tdTomato clones in the young adult mice (new Figure 3D, E).

In the manuscript text, this was indicated in page 11, line 9-11.

Page 8: Please provide full name of LRC: it probably stands for label retaining cells (LRC) vs non-label retaining cells (nLRC).

We indicated the full name of LRC (label-retaining cells) and nLRC (non label-retaining cells) on page 8, line 2-4.

**Significance**

Overall, this is a very well written paper. The results are presented in a clear and concise manner. The topic is of importance for skin regeneration and skin aging.

We are thankful to the reviewer for the positive comments and constructive feedback.

**Referee #2:**

In this manuscript, the authors demonstrated that fast-cycling epidermal stem cells are gradually depleted with aging, and Fibulin-7, an ECM component, is involved in this age-dependent stem cell depletion.

**Major points**

In Fig 2, the authors identified several ECM genes and selected Fbln7 gene for further analysis. For the readers, the authors also should reveal the other ECM genes that were upregulated in old nLRCs. In addition, the expression of Fibulin-7 protein in the skin should be also analyzed in young and old mice.

There were a few other ECM genes upregulated in old nLRCs with a p-value <0.05. Among these were *Col4a2*, *Col6a1*, *Lgals1*, *Postn*, *Fn1*, *Col8a1*. *Fn1* was expressed at a low RPKM value and was excluded. We examined the Periostin expression pattern in young vs. old skin, and although the dermal expression was reduced, no apparent changes were seen in the basal layer of the aged epidermis. Col8a1 is part of the basement membrane collagens and was already reported to be increased in the aging human skin (McCabe et al., 2020; Li et al., 2021). We decided then to explore fibulin 7,

which has no reported function in the skin. We summarized these results in the new figure EV2K.

The expression of fibulin 7 in the young and old mice was tested using a newly raised monoclonal antibody (New figures 2H, I). Fibulin 7 protein expression was decreased in the epithelium and basement membrane of old tail skin (2-year-old compared to 2-3 months old), in line with the decreasing skin function and fast-cycling stem cells in the aged skin. The mechanism of the increased *Fbln7* mRNA is not understood but could be part of a response to the increased inflammatory environment in aging skin.

This was mentioned in the results and discussion of the manuscript on page 17, line 8-11.

In Fig 3F and G, the authors clearly demonstrated delayed wound healing in Fbln7 hetero and KO mice. However, Fibulin7 might have a function in the dermis, which is involved in delayed wound healing. Histological analysis should be performed to indicate that delayed wound healing results from impaired reepithelialization.

**Response:** We performed H&E staining to see if re-epithelialization is impaired. H&E staining results and quantifications illustrate the thicker and longer epithelial `tongue` at the healing front of *Fbln7* WT skin, suggesting a better re-epithelialization process in the presence of *Fbln7* (New figures 3H-J).

In the manuscript, it was addressed on page 12, line 10-12.

In Fig 6, the authors demonstrated that Fibulin-7 maintains epidermal stem cells at the undifferentiated state. The authors also suggested that Fibulin-7 modulates the COLIV and maintains epidermal stem cells in Fig. 5. Mouse keratinocytes can be cultured on a COLIV-coated dish. Can overexpression of Fibulin-7 modulate keratinocyte differentiation on the COLIV-coated dish? This experiment clearly demonstrates the Fibulin-7/COLIV axis for epidermal stem cell maintenance.

We performed the proposed experiment and evaluated the effect of fibulin7 gain-of-function on differentiation in the presence or absence of ColIV coating on the dish. New experiments to differentiate keratinocytes in the presence or absence of Col IV suggests that Fibulin 7 overexpression could suppress differentiation (as measured by Krt1 and Krt10 markers) even with the addition of the inducer of differentiation such as $CaCl_2$ (New figures 5J, K). Col IV coating enhances $CaCl_2$-induced differentiation of

keratinocytes, and fibulin-7 overexpression inhibited it significantly in the presence of Col IV.

Nevertheless, with or without Col IV coating, fibulin 7 could inhibit $CaCl_2$-induced differentiation. Keratinocytes also express endogenous Col IV. Therefore, it is difficult at this time using this experiment to address if Col IV works together with fibulin 7.

In the manuscript, these experiments were mentioned in results and discussion page 16, line 2-6.

The fast-cycling epidermal stem cells are depleted in old mice and Fibulin-7 KO mice. These results suggest that Fibulin-7 is required for the maintenance of the fast-cycling epidermal stem cells in old mice. However. fibulin-7 is increased in old nLRCs (fast-cycling epidermal stem cells).

Fibulin 7 protein staining suggests that its expression was decreased in the old tail skin epithelium and basement membrane (New Figure 2H, I). This resolves the paradox of its increased mRNA and loss of fast-cycling stem cells in the aged mice. We added a new description on page 10, line 20-22.

The increased mRNA could be due to many factors, such as cells attempting to compensate for changes in the ECM composition during aging, aging-associated inflammatory environment, etc. How fibulin 7 protein expression is decreased would be an interesting follow-up study.

**Minor points**

On page 12, the sentence "Intriguingly, ...." is duplicated.

We thank the reviewer for the correction. It has been deleted.

On page 24, the authors should describe which proteins are coated on the dishes for the culture of mouse keratinocytes. This information is crucial since this study focuses on the role of ECM proteins in keratinocyte stem cell regulation.

The dishes were not coated for the culture of mouse keratinocytes. As the reviewer suggested, we performed experiments with Col IV coating (New figures 5J, K). *Fbln7* overexpression suppressed keratinocytes differentiation potentiated by Col IV.

In the manuscript, these experiments were mentioned in results and discussion page 16, line 2-6.

In Fig 5D and E, the authors displayed uneven distribution of COLIV in Fbln7 KO and old mice. But, it was not clear. Does the uneven distribution mean excess deposition of COLIV or discontinuous staining of COLIV?

**Response:** Overall, there was a trend of increased (excess) deposition of Col IV although not always distributed uniformly throughout the IFE, i.e., there are thicker and thinner areas stained by Col IV. This was especially noticed in the 1-year *Fbln7* KO mice. This paragraph was re-phrased in the results and discussion section to simplify our descriptions (page 14, line 7-13).

**Significance**

This study has originally extended the previous report (Sada et al, Nat Cell Biol 2016) and contains novel findings. In particular, the age-associated regulation of epidermal stem cells by Fibulin-7 is worth to share the research community. I recommend that this manuscript should be published if the authors address the above points.

We are thankful to the reviewer for the positive comments.

Dear Dr. Sada,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that I asked to re-evaluate your study, you will find below. As you will see, both referees now fully supports the publication of your study.

Before proceeding with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors: https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- In the "Data Availability section" (DAS) please add direct links to the datasets and remove any referee tokens and make sure these are public latest upon publication of the study.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main and EV figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics (see Fig. EV3K). It seems presently some graphs still have only partial statistics. Please check.

- Please make sure that all figure panels are called out, that they are called out separately and sequentially. Presently, callouts for Fig. 3B, 3C, 5h seem missing. Moreover, Fig. 4H is called out before 4G. Please check.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. The funding info needs updating in the submission system. Funders should be placed in the first column, and grant reference numbers in the second.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file (using the attached file as basis) with track changes, in order that we can see any modifications done.

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- two to four short (!) bullet points highlighting the key findings of your study (two lines each).
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Best,

Achim Breiling
Senior Editor
EMBO Reports

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Referee #1:

The authors have done an excellent job addressing the points that I raised during my initial review of the manuscript. They conducted additional staining with ColXVII, Fbln7 and a DNA damage marker and quantified the staining intensity. The

manuscript is well-written and the data presented in a concise manner. I have no further comments and recommend publication of the manuscript.

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Referee #2:

The authors have addressed all of my concerns. The revised manuscript is suitable for the publication in EMBO reports.

The authors have addressed all minor editorial requests.

Dr. Aiko Sada
Kumamoto University
International Research Center for Medical Sciences (IRCMS)
2-1-1 Honjo
Chuo-ku
Kumamoto City, Kumamoto 860-0811
Japan

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**Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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:
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  - are tests one-sided or two-sided?
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|---|---|---|
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|---|---|---|
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| Include a statement about **blinding** even if no blinding was done. | Yes | Materials and Methods |
| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Yes | Figure legends |
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| If publicly available data were reused, provide the respective **data citations in the reference list**. | Not Applicable | |