EGFR-mediated epidermal stem cell motility drives skin regeneration through COL17A1 proteolysis

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Re: JCB manuscript #202012073

Dr. Daisuke Nanba  
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Dear Dr. Nanba,

Thank you for submitting your manuscript entitled "EGFR-mediated epidermal stem cell motility drives skin regeneration through COL17A1 proteolysis". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers made suggestions to improve the flow of the paper and enhance the discussion and interpretation of the data. In our opinion, the reviewers' comments are constructive and their questions are relatively straightforward to address. We feel that the manuscript would be stronger if their remarks were addressed in full. We'd be happy to further discuss the reviews if you anticipate any issues addressing them or have any questions.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.
As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Cédric Blanpain, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This study entitled "EGFR-mediated epidermal stem cell motility drives skin regeneration through COL17A1 proteolysis" builds on previous publications from the co-corresponding authors (Nanba et al, JCB 2015 and Liu et al, Nature 2019). Previously and using in vivo cultures of human keratinocytes, Nanba et al 2015 described cell movement as a key parameter to predict whether human keratinocytes will give rise to expanding/proliferative colonies (holoclones) that could be used for transplantation purposes. In the other study, using lineage tracing in young and aged mice, Liu et al reported that COL17A1 is differently expressed in young and aged interfollicular epidermis stem cells and show that COL17A1 expression in the stem cells of young mice confers competence to these stem cells to outcompete and eliminate adjacent clones and favours wound healing. In the same study the authors showed that cell competition and contribution to wound healing is limited in skin from aged mice due to low levels of COL17A1 expression in the basal cells. In this submitted study Nanba et al identify the activation of EGFR signalling pathway as a driver of COL17A1 stabilization and show that this stabilization in human keratinocytes promotes holoclone formation and promotes migration in epithelial gap closure assays.

In this study, the authors firstly describe using time-lapse imaging that the keratinocytes from expanding colonies mainly undergo self-renewing divisions, while cells from stacking colonies undergo one or two divisions before terminally differentiating. And also show that cells from expanding colonies show cell locomotion (change in the position of the nuclei) while this locomotion
is reduced in cells from stacking colonies. The authors use an in silico model to study the impact of keratinocyte motility in stacking and expanding colony formation. Secondly, they identify activation of EGFR signalling as a pathway more activated in wounded skin from young (12 weeks) mice than from aged mice (19-25 months). Then the authors study the role of EGFR activation in colony formation using human keratinocytes. They show that addition of recombinant human EGF in human keratinocyte cultures promotes the formation of expanding colonies while diminishing the proportion of stacking colonies in those cultures. Thirdly, using biochemical assays they infer that EGFR activation inhibits COL17A1 proteolysis by inducing the secretion of TIMP1 (a metalloprotease inhibitor). Finally, the authors show that downregulation of COL17A1 expression, using siRNA and shRNA in human keratinocytes in vitro, lead to a decrease in cell motility resulting in a huge decrease in the proportion of expanding colonies in vitro and, reduced migration of the keratinocytes in epithelial gap closure assays.

The topic of this study is of interest and describes a novel axis (EGFR activation) as a regulator of COL17A1 stabilization in keratinocytes. In addition, it provides insights into the role of COL17A1 in keratinocyte motility and sheds light into, a controversial topic, the role of COL17A1 in keratinocyte migration. My main criticism is that it is difficult to follow the flow of the study, the different figures are not well connected and in some cases seem to be addressing different unrelated topics. It is difficult to follow the link between the cell culture experiments, aging and wound healing. In some figures it is also difficult to assess if the data presented is novel or the repetition of previous published experiments (Nanba et al 2015), the authors should state in the text the novelty of the experiments presented.

Remarkably, there are some points that need to be further studied and/or considered:

1. The authors perform time-lapse in expanding and stacking colonies and show that expanding colonies are composed of cells that present higher cell locomotion than cells in stacking colonies. In Figure 2a, it is shown that only some cells from the expanding colony show changes in the position of the nuclei, are these cells proliferative cells of the colony? Can we infer by the absence or presence of cell locomotion or the cell locomotion speed if a cell is proliferative or terminally differentiated? If so, does inhibition of cell motility (motility inhibitor) prevent self-renewal and favour cell differentiation?

2. The authors in Figure 1h show that the cells from expanding colonies mainly perform self-renewing divisions (PP: 98%, PD: 1.3 % and DD: 0,7%) while the cells from stacking colonies give rise to two terminally differentiated (DD) cells in half of their divisions. In Figure 2f &g the authors show that 15 to 20% of cells from expanding colonies undergo stacking mode (one would categorize them as PD or DD) after cell division. How can we reconcile these apparently different results? This should be better explained in the text as it could generate confusion.

3. In Figure 3 the authors provide an in silico model to study the impact of the rotational speed in colony type and characteristics. In Figure 3f and g they show that colonies with low rotation speed are thicker and contain more layers than colonies with higher rotation speed that are more flat and contain less layers. However, in Figure 3j it is the opposite, colonies with higher rotation speed contain more layers and are more stratified. How can the authors explain the differences between Figure 3fg and 3j? Please clarify this confusing point.

4. The authors found that EGFR is activated upon wound healing in young mice but not in aged
mice (Figure 4), however the role of EGFR activation in wound healing and expanding colony formation in keratinocytes from young and aged mice/humans is not explored. Is this difference in EGFR activation in young and aged mice only present during wound healing or also observed in homeostatic conditions? Would activation of EGFR in keratinocytes from aged mice lead to higher proportion of expanding colony formation and higher migration in epithelial gap closure assays? These experiments will be helpful to link wound healing, aging and cell culture experiments and improve the flow of the paper.

5. In Figure 5 the authors show a higher proportion of terminal and stacking colonies using siCOL17A1. One question that arises from these results is: do stacking and expanding colonies show different levels of COL17A1 expression?

6. The authors claim that COL17A1 organizes actin and keratin filament networks. However, the data to illustrate this point is poor, the images are low quality. Confocal images should be used to illustrate colocalization.

Minor points:

1. In Figure 1 e, f and g it is not clear what "missed cells" mean. Please clarify.

2. In page 9 line 7-8 the authors state. "A few cells with longer cell cycle durations were contained in stacking (average 14.1hr) but not in expanding (average 16.4hr) colony-forming keratinocytes (Fig 1i)". In figure 1i cells from stacking colonies have slightly longer cell cycle durations than expanding colonies, please correct the text accordingly.

3. The staining for TIMP1 in figure 5h is blurry and the signal is not clear. Please improve the staining or use in situ hybridization to illustrate this point.

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Nanba and colleagues identifies a signaling network regulating epidermal stem cells behaviors that involves the regulation of COL17A1 proteolysis downstream of EGFR signaling. The authors start by describing two different types of clones they obtain by in vitro culturing human epidermal cells: the expanding clones and the stacking clones. The first type survives long-term and seems to be originated by stem and/or progenitors cells, while the second produces more differentiated cells. Additionally, expanding clones are constituted of cells that are more motile and have higher rotational motion. Computational simulations suggested that higher rotational speed is a necessary characteristic to form an expanding clone. The authors hypothesize that in expanding clones after cell division, daughter cells are more likely remaining attached to the basal membrane because surrounding cells would be more prone to move, leaving them free space in the basal layer, while in stacking clones cells would remain in the suprabasal layer because they would not find space elsewhere. At the molecular level, EGFR is the upstream element that confers motility to the expanding clones by stabilizing COL17A1. EGFR induces transcriptionally TIMP1, a protease able to inhibit the MMPs responsible of the degradation of COL17A1.

The data provided by the authors are rigorous and well explained. However, there are some parts that are only correlative and that would require additional clarifications.
I would suggest the authors to strengthen some analysis before the work can be published in JCB:

1) It is unclear how the rotational speed could influence the motility/migration/locomotion of the cells. In my opinion, these could be two separate entities or at least the authors should better define what they are describing and which is the exact link between the two. I think the confusion is partially arising because the two different phenotypes/properties have two distinct levels of complexity: on one side they are describing the importance of the rotational speed on cell fates decisions, and on the other side they analyse the molecular players involved in cell migration.

2) The effects of EGF on keratinocytes proliferation and migration are very well studied. However, here EGF treatment seems to do not influence the duration of the cell cycle, but it is unclear whether EGF treatment could influence the number of cells that enter the cell cycle or whether there is a switch in the cellular fate. The authors could address these unclear point by performing BrdU or EdU analysis on expanding versus stacking clones treated with EGF and by performing the lineage tracing. The data at the moment shows a decrease in the percentage of the stacking clone upon EGF treatment, but it is unclear whether the cells in these clones are forced to increase their self-renewal properties (i.e. give rise to more PP divisions).

3) It is unclear which is the detailed phenotype of the stacking clones. In Supplementary Figure 1b the cells in the centre (where there are more layers) seems to be Involucrin negative. Which markers are these cells expressing? Could they be more differentiated cells? A simple staining for keratin 1 or keratin 10 would be sufficient to clarify this point.

4) In general it would be important to understand whether cell divisions are evenly distributed in the colonies or whether there are preferential locations e.g. at the centre or at the border of the colonies and whether EFG treatment have any effects on the localisation of proliferation.

5) Following the previous point, in the Figures 6g,i and Supplementary figure 7g,h, only cells at the border of the colony are analysed. Is there a biological reason? Which is the phenotypic effect of COL17A1 down-regulation on PLEC, actin and keratins in the cells at the centre of the colonies?

6) The connections between COL17A1, PLEC, actin and keratins are only correlative. I suggest the authors to tone down their conclusion stating "These results indicate that COL17A1 generates a functional boundary between actin microfilaments and keratin intermediate filaments by forming a complex with PLEC and coordinates cell motility for enhancing cell migration".

7) Is the down-regulation of COL17A1 affecting the renewal activity of the cells in the expanding clones (i.e. give rise to more PP divisions)?

8) It is unclear which are the variable parameters in Figure 3f. Are the three plots referring to three different rotation speeds? Same in Figure 3g.

Overall, this study is interesting and provides novel molecular insights on the connection between a growth factor signalling, EGF, and a structural component such as COL17A1 that is known to be a fundamental regulator of epidermal stem cells. How this network really impact on the behaviour of epidermal stem cells remain unclear at this stage.
Point-by-point Responses to Reviewers

Reviewer #1:

This study entitled "EGFR-mediated epidermal stem cell motility drives skin regeneration through COL17A1 proteolysis" builds on previous publications from the co-corresponding authors (Nanba et al, JCB 2015 and Liu et al, Nature 2019). Previously and using in vivo cultures of human keratinocytes, Nanba et al 2015 described cell movement as a key parameter to predict whether human keratinocytes will give rise to expanding/proliferative colonies (holoclones) that could be used for transplantation purposes. In the other study, using lineage tracing in young and aged mice, Liu et al reported that COL17A1 is differently expressed in young and aged interfollicular epidermis stem cells and show that COL17A1 expression in the stem cells of young mice confers competence to these stem cells to outcompete and eliminate adjacent clones and favours wound healing. In the same study the authors showed that cell competition and contribution to wound healing is limited in skin from aged mice due to low levels of COL17A1 expression in the basal cells. In this submitted study Nanba et al identify the activation of EGFR signalling pathway as a driver of COL17A1 stabilization and show that this stabilization in human keratinocytes promotes holoclone formation and promotes migration in epithelial gap closure assays.

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and shRNA in human keratinocytes in vitro, lead to a decrease in cell motility resulting in a huge decrease in the proportion of expanding colonies in vitro and, reduced migration of the keratinocytes in epithelial gap closure assays.

The topic of this study is of interest and describes a novel axis (EGFR activation) as a regulator of COL17A1 stabilization in keratinocytes. In addition, it provides insights into the role of COL17A1 in keratinocyte motility and sheds light into, a controversial topic, the role of COL17A1 in keratinocyte migration. My main criticism is that it is difficult to follow the flow of the study, the different figures are not well connected and in some cases seem to be addressing different unrelated topics. It is difficult to follow the link between the cell culture experiments, aging and wound healing. In some figures it is also difficult to assess if the data presented is novel or the repetition of previous published experiments (Nanba et al 2015), the authors should state in the text the novelty of the experiments presented.

Remarkably, there are some points that need to be further studied and/or considered:

1. The authors perform time-lapse in expanding and stacking colonies and show that expanding colonies are composed of cells that present higher cell locomotion than cells in stacking colonies. In Figure 2a, it is shown that only some cells from the expanding colony show changes in the position of the nuclei, are these cells proliferative cells of the colony? Can we infer by the absence or presence of cell locomotion or the cell locomotion speed if a cell is proliferative or terminally differentiated? If so, does inhibition of cell motility (motility inhibitor) prevent self-renewal and favour cell differentiation?

Response: Thank you for your constructive comments and suggestions. According to this comment, we measured the cell locomotion speed and cell division cycles during expanding colony formation. As you predicted, locomotive cells were more proliferative than stationary cells even in the expanding colonies. We added this result in new Fig. 2, C and D.

As you suggested, the inhibition of cell motility will prevent the clonal expansion of keratinocyte stem cells. However, we cannot suppress only the cell locomotion of keratinocyte stem cells without affecting their proliferative activity. Molecules related to keratinocyte migration, including Rac1, are also involved in keratinocyte stem cell maintenance (Benitah et al, Science 2005, Nanba et al., EMBO
Motility inhibitors for keratinocytes such as cytochalasin D (Kinoshita et al., Sci Rep 2019) and colcemid (Park et al, Nat Cell Biol 2017) also impact cell proliferation. To understand keratinocyte colony behavior when only cell locomotion is affected, we have performed simulation experiments. To emphasize this, we added the following sentence to the “Results” section (page 9):

“since we cannot modulate cell motility without affecting cell proliferative activity in cell culture experiments.”

*Figure 1 and 2 seem to be similar to those from Nanba et al 2015, I suggest merging the two figures in one and highlighting in the text the novelty of the experiments presented in this study.*

**Response:** Thank you for your suggestion. Our previous study (Nanba et al., J Cell Biol 2015) did not analyze stacking colonies and cell lineages during the colony formation. We also added new data in new Figs. 1 and 2 in this revised manuscript according to the Reviewers’ suggestions. Although we maintained our Figs. 1 and 2 in the manuscript, we now describe the novelty of this study by adding the following sentence to the “Results” section (page 5), according to your suggestion:

“As the expanding and stacking phenotypes of the colonies have phenotypic similarities to the progressive and impaired collective migration of keratinocytes, we analyzed the spatiotemporal dynamics of human keratinocytes during the formation of expanding and stacking colonies.”

2. The authors in Figure 1h show that the cells from expanding colonies mainly perform self-renewing divisions (PP: 98%, PD: 1.3 % and DD: 0.7%) while the cells from stacking colonies give rise to two terminally differentiated (DD) cells in half of their divisions. In Figure 2f &g the authors show that 15 to 20% of cells from expanding colonies undergo stacking mode (one would categorize them as PD or DD) after cell division. How can we reconcile these apparently different results? This should be better explained in the text as it could generate confusion.

**Response:** Thank you for your valuable comment. As you pointed out, our manuscript contains two cell division parameters concerning cell fate patterns (PP, PD and DD divisions) and cell position (expanding and stacking modes), which might generate confusion for readers. PP divisions can also include the stacking mode of cell behavior after division since keratinocytes that move up vertically after cell division sometimes
migrate down to the basal layer and proliferate again there within 48 h. This case was categorized into PP division and stacking mode. Thus, two modes of cell behavior after cell division is associated but not consistent with three types of cell fate determination. To avoid this confusion, we added the details of cell fate and behavior analysis after cell division to the “Materials and methods” section (page 25-26) as follows:

“Three patterns of cell fate determination after cell division were defined according to the classification by Roshan et al. (Roshan et al., 2016). We categorized cultured keratinocytes into two types: proliferative (P) or differentiating (D) cells, which divided or did not divide within 48 h, respectively. Keratinocytes that could not be tracked for 48 h were excluded from further analysis. PP divisions were defined in cases where one proliferating (P) cell symmetrically divides and produces two proliferative cells. DD divisions were defined in cases where one proliferating (P) cell symmetrically divides and produces two differentiating (D) cells. Asymmetric PD divisions were defined in cases where one proliferating (P) cell divides and produces one proliferative (P) and one differentiating (D) cell.

Two modes of keratinocyte behavior after cell division were defined as follows: The expanding mode was defined in cases where two daughter cells produced by planar cell division remain in the basal layer. The expanding mode was defined in cases where one or two daughter cells produced by planar cell division in the basal layer move toward the suprabasal layer and remain there at least several hours. It was sometimes observed that one or two daughter cells in the suprabasal layer generated by the stacking mode of behavior migrate down to the basal layer and proliferate there within 48 h. Therefore, two modes of cell behavior after cell division can occur but are associated with three types of cell fate determination. The frequency of the two modes of cell behavior was calculated from 24 h observations of the formation of each type of colony on culture days 2 and 3, respectively.”

3. In Figure 3 the authors provide an in silico model to study the impact of the rotational speed in colony type and characteristics. In Figure 3f and g they show that colonies with low rotation speed are thicker and contain more layers than colonies with higher rotation speed that are more flat and contain less layers. However, in Figure 3j it is the opposite, colonies with higher rotation speed contain more layers and are more stratified. How can the authors explain the differences between Figure 3fg and 3j? Please clarify this confusing point.
Response: Thank you for your valuable comments. We performed the simulation experiments with two different settings. In Fig. 3, F and G, colonies could grow up in an unlimited space, which simulates cultures where cells are seeded at clonal density. In contrast, in Fig. 3, J, colonies could grow up within a limited area with a 100 μm radius, which simulates high density cell cultures. To avoid any confusion, we added the following sentence to the “Figure legends” section (page 45) for new Fig. 3, H-J.

“(H-J) The cells grew within a limited area with a 100 μm radius to simulate high density cell cultures.”

4. The authors found that EGFR is activated upon wound healing in young mice but not in aged mice (Figure 4), however the role of EGFR activation in wound healing and expanding colony formation in keratinocytes from young and aged mice/humans is not explored. Is this difference in EGFR activation in young and aged mice only present during wound healing or also observed in homeostatic conditions? Would activation of EGFR in keratinocytes from aged mice lead to higher proportion of expanding colony formation and higher migration in epithelial gap closure assays? These experiments will be helpful to link wound healing, aging and cell culture experiments and improve the flow of the paper.

Response: Thank you for your constructive comments. According to your suggestion, we investigated the expression and phosphorylation of EGFR in normal dorsal and tail skin of young and of aged mice using immunofluorescence microscopy. EGFR was mainly localized at the basal layer of the epidermis and was less phosphorylated in the skin of young and aged mice. The localization of EGFR and its low phosphorylation level in the skin of neonatal mice has also been confirmed by Wang et al. (J Cell Biol, 2006). We added this result in new Fig. S4, E.

We also investigated the effect of EGFR activation on human keratinocytes isolated from infants (5-19 months-old) and from aged (70-78 years-old) patients. As you predicted, treatment with EGF increased the ratio of expanding colonies in cultures by enhancing cell migration in aged keratinocytes as well as in infant keratinocytes. We added this result in new Fig. 4, C-F. Thanks to your constructive comments, we could improve the flow of the paper. We really appreciate your valuable suggestions.

5. In Figure 5 the authors show a higher proportion of terminal and stacking colonies using siCOL17A1. One question that arises from these results is: do stacking and
expanding colonies show different levels of COL17A1 expression?

**Response:** Thank you for your constructive question. According to your question, we examined the expression of COL17A1 in expanding and in stacking colonies. We found that COL17A1 was highly expressed by small proliferative cells in expanding colonies. This result was consistent with our previous study by flow cytometry (Liu et al., Nature 2019). We added this result in new Fig. 7, E.

6. The authors claim that COL17A1 organizes actin and keratin filament networks. However, the data to illustrate this point is poor, the images are low quality. Confocal images should be used to illustrate colocalization.

**Response:** Thank you for your comment and suggestion. As you pointed out, the image quality of cytoskeletons was poor although we had used a confocal microscope. According to your suggestion, we have improved the image quality of COL17A1, actin and keratin filaments, and PLECTIN in HaCaT cells using confocal microscopy with a higher magnification and resolution. The data are now presented in new Fig. 8, C, D, F, and G.

**Minor points:**

1. In Figure 1 e, f and g it is not clear what "missed cells" mean. Please clarify.

**Response:** Thank you for pointing this out. We replaced the term “missed cells” with “untraceable cells” in new Fig. 1, F.

2. In page 9 line 7-8 the authors state. "A few cells with longer cell cycle durations were contained in stacking (average 14.1hr) but not in expanding (average 16.4hr) colony-forming keratinocytes (Fig 1i)". In figure 1i cells from stacking colonies have slightly longer cell cycle durations than expanding colonies, please correct the text accordingly.

**Response:** Thank you for your comment. As you pointed out, that sentence was incorrect. We removed that sentence and simply stated that there is no significant difference in cell cycle duration between expanding and stacking colony-forming
keratinocytes as follows in the “Results” section (page 6):

“There was not much difference in the median cell cycle length between expanding (13.8 h) and stacking (14.4 h) colony-forming keratinocytes.”

3. The staining for TIMP1 in figure 5h is blurry and the signal is not clear. Please improve the staining or use in situ hybridization to illustrate this point.

Response: Thank you for your comment. As you pointed out, the image quality of TIMP1 was poor although we had used a confocal microscope. According to your suggestion, we improved the image quality of TIMP1 using confocal microscopy with a higher magnification and resolution. The data are presented in new Fig. 6, G.

We highly appreciate your positive and constructive comments to improve the clarity and impact of our manuscript.
**Reviewer #2**

The manuscript by Nanba and colleagues identifies a signaling network regulating epidermal stem cells behaviors that involves the regulation of COL17A1 proteolysis downstream of EGFR signaling.

The authors start by describing two different types of clones they obtain by in vitro culturing human epidermal cells: the expanding clones and the stacking clones. The first type survives long-term and seems to be originated by stem and/or progenitors cells, while the second produces more differentiated cells. Additionally, expanding clones are constituted of cells that are more motile and have higher rotational motion. Computational simulations suggested that higher rotational speed is a necessary characteristic to form an expanding clone. The authors hypothesize that in expanding clones after cell division, daughter cells are more likely remaining attached to the basal membrane because surrounding cells would be more prone to move, leaving them free space in the basal layer, while in stacking clones cells would remain in the suprabasal layer because they would not find space elsewhere. At the molecular level, EGFR is the upstream element that confers motility to the expanding clones by stabilizing COL17A1. EGFR induces transcriptionally TIMP1, a protease able to inhibit the MMPs responsible of the degradation of COL17A1.

The data provided by the authors are rigorous and well explained. However, there are some parts that are only correlative and that would require additional clarifications. I would suggest the authors to strengthen some analysis before the work can be published in JCB:

1) **It is unclear how the rotational speed could influence the motility/migration/locomotion of the cells. In my opinion, these could be two separate entities or at least the authors should better define what they are describing and which is the exact link between the two.**

I think the confusion is partially arising because the two different phenotypes/properties have two distinct levels of complexity: on one side they are describing the importance of the rotational speed on cell fates decisions, and on the other side they analyse the molecular players involved in cell migration.
Response: Thank you for your valuable comments. In this paper, we propose that alteration of the EGFR-COL17A1 axis, which underlies both rotational stem cell motion and stem cell self-renewal, explains the age-associated impaired wound healing. According to your suggestion, to strengthen the link between rotational speed and cell motility and to increase the readability of the manuscript, we added the following sentence to the beginning of the simulation experiments in the “Results” section (page 9):

“We previously reported a unique rotational motion of two-cell colonies of human keratinocytes, and our simulation experiments revealed that the rotational motion of individual keratinocytes generates cell locomotion in the colony (Nanba et al., 2015).”

We also added the following sentence to the end of the subheading “Decline of EGFR signaling in skin wound healing with aging” part in the “Results” section (page 12) to connect the cell migration and molecular players:

“Collectively, these results strongly suggest that a cell-extrinsic defect of EGFR activation causes the delayed skin wound healing associated with aging.”

2) The effects of EGF on keratinocytes proliferation and migration are very well studied. However, here EGF treatment seems to do not influence the duration of the cell cycle, but it is unclear whether EGF treatment could influence the number of cells that enter the cell cycle or whether there is a switch in the cellular fate. The authors could address these uncertain point by performing BrdU or EdU analysis on expanding versus stacking clones treated with EGF and by performing the lineage tracing. The data at the moment shows a decrease in the percentage of the stacking clone upon EGF treatment, but it is unclear whether the cells in these clones are forced to increase their self-renewal properties (i.e. give rise to more PP divisions).

Response: Thank you for these constructive comments. According to your suggestions, we performed EdU experiments and lineage analysis with and without EGF. As you predicted, treatment with EGF increased the cell cycle entry on culture day 6, and maintained PP division and the self-renewal property of keratinocyte stem cells. We added these results in new Fig. 5, F-I. We thank you again for this comment.

3) It is unclear which is the detailed phenotype of the stacking clones. In Supplementary Figure 1b the cells in the centre (where there are more layers) seems
to be Involucrin negative. Which markers are these cells expressing? Could they be more differentiated cells? A simple staining for keratin 1 or keratin 10 would be sufficient to clarify this point.

Response: Thank you for your questions and suggestion. According to your suggestion, we performed immunostaining of stacking colonies and found that cells aggregating in the center of the stacking colonies expressed keratin 10 and loricrin. Thus, as you predicted, the stacking colonies contained more differentiated cells. We added this result in new Fig. S1, B.

4) In general it would be important to understand whether cell divisions are evenly distributed in the colonies or whether there are preferential locations e.g. at the centre or at the border of the colonies and whether EFG treatment have any effects on the localisation of proliferation.

Response: According to your suggestion, we performed EdU experiments and confirmed that proliferating cells are localized at the periphery of the colonies after expanding colonies grow up. When colonies were small (less than 40 cells), EdU-positive cells were observed in the entire colony. As you predicted, treatment with EGF enhanced the proliferation of cells located at the center of the colony. We added these results in new Fig. 5, H.

5) Following the previous point, in the Figures 6g,i and Supplementary figure 7g,h, only cells at the border of the colony are analysed. Is there a biological reason? Which is the phenotypic effect of COL17A1 down-regulation on PLEC, actin and keratins in the cells at the centre of the colonies?

Response: Thank you for your questions. In this study, we investigated the roles of COL17A1 on cell migration and only analyzed cells located at the periphery of colonies since migratory phenotypes can be easily observed in peripheral cells of the colonies. In cells located at the center of the colonies, actin and keratin filaments are mainly distributed throughout cell-cell contacts, and no structural changes in these cytoskeletons were observed.
following the knockdown of COL17A1. However, knockdown of COL17A1 also delocalized PLEC in cells at the center of the colonies. This result also suggests that COL17A1 regulates PLEC distribution. The accompanying image shows this effect.

6) The connections between COL17A1, PLEC, actin and keratins are only correlative. I suggest the authors to tone down their conclusion stating "These results indicate that COL17A1 generates a functional boundary between actin microfilaments and keratin intermediate filaments by forming a complex with PLEC and coordinates cell motility for enhancing cell migration".

Response: Thank you for your comment and we agree that sentence was overstated. We removed that sentence from the “Results” section in the revised manuscript.

7) Is the down-regulation of COL17A1 affecting the renewal activity of the cells in the expanding clones (i.e. give rise to more PP divisions)?

Response: Thank you for your valuable question. The down-regulation of COL17A1 by siRNA targeting COL17A1 (siCOL17A1) resulted in a decreased size of the colonies and increased stacking colony formation, as shown in new Fig. 7, C and D. We further examined the effect of COL17A1 down-regulation on the self-renewal property of keratinocytes and confirmed that treatment with siCOL17A1 decreased the colony-forming efficiency of human keratinocytes. We added this result in new Fig. 7, F.

8) It is unclear which are the variable parameters in Figure 3f. Are the three plots referring to three different rotation speeds? Same in Figure 3g.

Response: Thank you for your question. As you pointed out, these plots indicate the simulation results with three different rotation speeds. We added the speed information in new Fig. 3, F.

Overall, this study is interesting and provides novel molecular insights on the connection between a growth factor signalling, EGF, and a structural component such as COL17A1 that is known to be a fundamental regulator of epidermal stem cells. How this network really impact on the behaviour of epidermal stem cells remain unclear at this stage.
We highly appreciate your positive and constructive comments to improve the clarity and impact of our manuscript.
Dear Dr. Nanba:

Thank you for submitting your revised manuscript entitled "EGFR-mediated epidermal stem cell motility drives skin regeneration through COL17A1 proteolysis". The paper has now been seen again by the original reviewers and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

**As you will see, reviewer #2 has one final issue that will need to be addressed (or explained) in the final revision. Please be sure to provide a detailed rebuttal indicating how you addressed this final concern.**

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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Articles/Tools may have up to 5 supplemental figures. At the moment, you meet this limit but please
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Please also note that tables, like figures, should be provided as individual, editable files. A summary
of all supplemental material should appear at the end of the Materials and methods section.

9) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements
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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Cédric Blanpain, MD, PhD
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Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

In this revised version of the manuscript, the authors have responded to all of the issues raised in the first submission. I think this is an interesting work that will be of great interest to the readership of JCB. As such I strongly recommend its publication.

Reviewer #2 (Comments to the Authors (Required)):

The current version of the manuscript is significantly improved. The addition of the new experiments added clarity in the description of the two types of colonies, their dynamics and the potential molecular mechanisms involved in stem cells motility with relevant impact on ageing and regeneration. The authors addressed all the criticism and provided the requested experiments. I have only a final point regarding Figure 5I: the percentage on the 2nd division without EGF are not fully in line with the colonies "controls" reported in Figure 1H and the sum of the provided percentage for each type of division is not 100% (PP 26.1% + PD 31.5% + DD 15.6 %). Could the authors clarify it?
For Reviewer #2:

The current version of the manuscript is significantly improved. The addition of the new experiments added clarity in the description of the two types of colonies, their dynamics and the potential molecular mechanisms involved in stem cells motility with relevant impact on ageing and regeneration. The authors addressed all the criticism and provided the requested experiments. I have only a final point regarding Figure 5I: the percentage on the 2nd division without EGF are not fully in line with the colonies "controls" reported in Figure 1H and the sum of the provided percentage for each type of division is not 100% (PP 26.1% + PD 31.5% + DD 15.6%). Could the authors clarify it?

Response: Thank you for pointing this out. It was a simple miscalculation. We replaced “PP 26.1%, PD 31.5%, DD 15.6 %” in the second column of -EGF condition with “PP 26.1%, PD 31.5%, DD 42.4 %” in Fig 5, I. Thanks to you, we could find the error in the manuscript before publication.

We highly appreciate your comment to improve our manuscript.