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Senescent human melanocytes drive skin aging via paracrine telomere dysfunction

Stella Victorelli, Anthony Lagnado, Jessica Halim, Will Moore, Duncan Talbot, Karen Barrett, James Chapman, Jodie Birch, Mikolaj Ogrodnik, Alexander Meves, Jeff S. Pawlikowski, Diana Jurk, Peter D. Adams, Diana van Heemst, Marian Beekman, P. Eline Slagboom, David A. Gunn, João F Passos

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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 4th Apr 2019

Thank you for the submission of your manuscript (EMBOJ-2019-101982) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #2 points to major limitations of the cell culture models employed, which in his/her view undermine the impact and physiological relevance of your findings. This referee is also concerned about the mechanistic support for the CXCR3-ip10-ROS axis. Referee #1 agrees in that the detail of ROS activity require further investigation (ref#1, pt. 18) and details a number of complementary experiments to consolidate the link to paracrine senescence (ref#1, pts. 4,7,9). In line, referee #3 asks you to interrogate DNA damage in the different cell types (ref#3, pts. 2,4). In addition, the referees point to issues related to literature and methods annotation as well as to-be-expanded discussion of the findings that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I need to stress though that we do need strong support from the referees on a revised version of the study in order to move on to publication of the work.

REFEREE REPORTS:

Referee #1:

Victorelli et al for EMBO J

Cellular senescence has been demonstrated to cause functional decline of mammalian skin during aging, yet details, such as the contribution of specific cell types, the causes of cellular senescence, and the pathways involved in senescence induction are currently unclear. This is an important and novel study as it begins to address these unresolved questions. The authors reveal an aging-associated increase in melanocytes with features of senescence in human skin, demonstrate that senescent melanocytes can induce paracrine telomere dysfunction in fibroblasts and senescence thorough mechanisms involving IP10-CXCR3-ROS signaling, provide evidence that this mechanism of inducing paracrine senescence in keratinocytes is active also in aged skin and melanoderms, and suggest that senescent melanocytes are a cause of aging associated skin thinning using melanoderms as a model system. Finally, the authors demonstrate that senescent melanocyte-induced skin thinning can be rescued using a senolytic and ROS scavenger. The study therefore not only provides further evidence that cellular senescence is causative of aging associated decline of mammalian tissue and organ systems but it also reveals therapeutic opportunities to alleviate such decline. This is a well-executed study, yet some issues should be resolved in order to strengthen it. Major and minor issues are listed below.

1. Figure 1A: The authors state "all p16 positive cells ...were also positive for melanocyte markers (Figure 1A)". However, the Figure shows no p16 positive cells in the young donor and only one p16 positive cell in the old donor. The authors therefore either should show a micrograph in which approximately 5-10% of melanocytes are p16 positive, which is the average percentage based on the graph in Fig 1B, and/or state that "the p16 positive cell ...was also positive for melanocyte marker..."

2. Figure 1B and D (and in general thorough the study where subjective quantitations were performed, the following information should be provided): A description is needed that explains how p16/Sirt1 positive cells were quantified. This should include how many separate individuals performed the analysis, whether this was conducted in a blinded manner, etc.... Also, since Sirt1 and/or p16 levels are not always detectable (positive) or not (negative), but also likely sometimes contain a faint, intermediate signal, a description is needed that states what criteria were used to determine whether the cells were considered p16/Sirt1 positive and negative?

3. An aging-associated increase in 53BP1 positive melanocytes in skin of human donors has previously been demonstrated and should thus be cited here (Suram et al., 2012 EMBO J. 31 2839)

4. It seems that not all DDR or TAF positive cells are also positive for p16 since the percentages of DDR positive cells (7% young/10% old) are greater compared to p16 positive cells (0% young/7% old). What is the correlation between the induction of the DDR/TAF and the senescence status in melanocytes? Is the induction of DDR/TAF independent of p16 and senescence in dermal melanocytes? One way to address this is to co-immunostain tissue section with MelanA/H2AX (or TAF)/p16 and determine whether DDR positive melanocytes are also p16 positive.

5. Telomeric signals in the micrograph from the old donor (Figure 1E old) appear to cluster into very large foci. Is this a common feature of melanocytes in old donors? If so, what is the significance of this? Does this clustering potentially create such large telomeric foci that the overlap with DDR foci becomes coincidental? How did the authors control for this? Does this clustering affect q-FISH analysis? If not, how can the authors be certain of that?

6. Figure 1K-M: The authors should rephrase the two sentences describing telomere lengths in TAF as their statements suggest that telomeres in TAF are longer than any other telomere in a melanocyte. See: "...by quantifying telomere FISH intensities of telomeres co-localising with γH2AX (TAF) and those that did not co-localise with γH2AX (non-TAF) in melanocytes, we found that telomeres associated with γH2AX were significantly longer than those not co-localising with γH2AX (Figure 1J-L)". This is an incorrect statement since the authors demonstrate in Figure 1M that many TAF have signal intensities less than non-TAF telomeres.

7. Figure 2: The authors suggest that melanocytes and keratinocytes containing TAF are senescent. This has not been directly demonstrated. It is therefore unclear whether cells with H2AX foci (or potentially TAF) in tissue (2A) or in melanoderms (2I) have the ability to cause paracrine senescence. Can the authors show that TAF containing melanocytes express SA-betaGal activity/p16/low laminB1 etc?

8. The authors show that melanocytes with H2AX foci (or TAF) have the ability to cause paracrine TAF/senescence. It is curious why TAF containing senescent keratinocytes don't also cause paracrine TAF and senescence in neighboring keratinocytes. Do the authors observe larger clusters of TAF containing keratinocytes that spread beyond just once cell distance from a TAF containing melanocyte? Why or why not?

9. Figure 2A (and 2I): Although the authors suggest that TAF in melanocytes causes paracrine TAF in neighboring keratinocytes, it is unclear whether the DDR positive and representative melanocyte shown has TAF. Since arrows point to TAF as per the figure legend, and no arrows are shown in the melanocyte, it would appear that the green DDR foci are non-telomeric. If this is indeed correct, then the conclusions should be altered. If arrows were inadvertently omitted, the authors should revise the image and also show enlarges versions of melanocyte TAF in a column next to enlarged keratinocyte TAF.
Given the concerns raised above, should the x-axis (Figure 2B) instead state "mean gH2AX foci in melanocytes"?
10. Are keratinocytes with TAF senescent; do they express p16? It would be useful to show this given that it is suggested that ABT-737 treatment can reduce the number of p16 positive keratinocytes in tissue (see Figure 5)

11. Also, authors should include a description what "neighboring" keratinocytes means. Are these cells in immediate vicinity to melanocytes or is there a distance threshold where the paracrine effects become less effective? Also, as mentioned above, it is unclear why senescent keratinocytes cause paracrine senescence in other keratinocytes.

12. Figure 2B: how many melanocytes and keratinocytes were analysed for each group?

13. The melanoderm model should be described in greater detail. For example, it is unclear whether these are cross sections, or whether the cells had been imaged from above.
Also, in melanoderms, are the TAF containing keratinocytes directly neighboring the senescent melanocytes, similar to what is observed in old donors (2A)? More details should be described/quantified.

14. Figure 2N-O: The increase of "epidermal" thickness of melanoderms containing proliferating, but not senescent melanocytes is interesting. Is this increase in thickness due to proliferating keratinocytes, melanocytes, or both? Similarly, is the lack of increase in thickness due to a lack of proliferation of melanocytes, keratinocytes, both or due to a lack of extracellular matrix production? The authors should measure percentages of these two cell types and the density (number of each cell type per specific area) in their 6 analyzed groups.

15. The last sentence in this section should be altered as it jumps to the conclusion that paracrine telomere dysfunction causes aging associated epidermal thinning. This was not directly demonstrated (only melanoderm thinning was demonstrated).

16. Figure 3B: What was the stress in "stress induced-senescent melanocytes"? Please provide details.

17. Supp Figure 6E: A quantitation of the immunoblots would be useful since the differences appear to be small.

18. Figure 4: What is the mechanism by which mitochondrial ROS causes TAF in cells? Is this due to damaging shelterin components and thereby "uncapping" telomeres? Is this due to double strand break formation? Does this require cell proliferation?

19. Figure 5: The authors assume that the senolytic agent ABT-737 clears senescent melanocytes from melanoderms, but this is not directly demonstrated. Instead, it is shown that cells containing markers of senescence, such as p16 and TAF, are reduced upon ABT-737 treatment, but this is not necessarily evidence that senescent melanocytes with damaging SASP are eliminated. A way in which to address this could be to demonstrate that there is an increase in apoptotic melanocytes (or cells), a decrease in melanocytes overall, and potentially a decrease in IP10 expressing

cells/melanocytes in ABT-737 treated melanoderms.

20. Figure 5D: Authors show that keratinocytes containing 2 or more TAF show reduced development in ABT-737 treated melanocytes. Why weren't melanocytes containing 1 TAF (which is probably sufficient to cause senescence) included in this group? Are keratinocytes with only 1 TAF functional whereas those with 2 or more undergo senescence?

21. Figure 5H: As above, it is unclear what rescues the thinning of melanoderms generated in the presence of senescent cells. Do the number of melanocytes or keratinocytes increase in the presence of ABT-737? Quantitations of melanocytes/keratinocytes (as described above, #14) might be useful.

22. The sentence "Therefore, these data implicate senescent melanocytes as causal in the induction of paracrine telomere damage and senescence in neighbouring epidermal cells" should be changed to ".....neighboring keratinocytes".

Referee #2:

Victorelli et al report that senescent melanocytes contribute to skin aging by inducing telomeric damage and senescence into neighboring keratinocytes. They provide a mechanism by which this would be executed, and they demonstrate that elimination of senescent melanocytes can be achieved by using a common senolytic and can restore normal epidermal thickness. The concept of accumulation of senescent melanocytes in the aging skin is interesting, but not new. Besides some ex vivo analyses done in Figure 1, the remaining experiments rely on using highly artificial and poorly physiological cell culture models. The absence of fibroblasts from the skin equivalent is also a big limitation. The study is very preliminary, and several characterizations are missing. Moreover, due to absence of in vivo evidence, the results are far from being conclusive.
Additional major points to consider:

*
It is really hard to have conclusive interpretation of Figure 1A based on the images provided. If the point is that only melanocytes enter senescence in aged skin, then additional data should be provided;
*
From the methods section of Figure 1, it is stated that 'data from randomly selected participants were used'. What data? Do they mean samples? Or that the stainings were done somewhere else? This is definitely not clear;
*
While down-regulation of SIRT-1 has been somehow associated to senescence, it can't be considered a senescent marker. If authors are claiming that the p16+ melanocytes are unequivocally senescent, then other markers should be evaluated;
*
In Figure 2, authors that paracrine senescence, particularly form melanocytes to keratinocytes, is a major component to drive aging in the skin. However, in Figure 1 they do not provide any evidence that keratinocytes in aged skin are also senescent;
*
Most keratinocytes do not proliferate, and exposing them to the CM of melanocytes might contribute to their differentiation (instead of senescence). Showing that highly proliferative cells (fibroblasts) accumulate DNA damage upon exposure to a CM rich of pro-inflammatory and pro-mitogenic factors is not surprising and not physiologically relevant;
*
Related to the previous point, the epidermal thinning might be a combination of enhanced differentiation and absence of stem cells from the skin equivalent cultures;
*
Genetic manipulation of cxcr3 and ip-10 should be performed to validate the claim that ip-10 signaling is responsible for paracrine DNA damage and senescence;

*
The experiment of senescent cells clearance in figure 5 suggests that at the end of the treatment there are no p16+ melanocytes. Considering that the majority of the melanocytes are made senescent and then incorporated into melanoderms, most of the embedded melanocytes should be p16+ at the beginning of the culture. Does this mean that after ABT737 there are almost no melanocytes in the melanoderm culture? This would explain the thicker epidermis, as keratinocytes will have more space to grow

Referee #3:

The manuscript studies human skin ageing initially confirming and building on previous observations showing that melanocytes are the only cell type in the epidermis. The authors then show that these melanocytes have a typical Senescence associated secretory phenotype (SASP) and that this SASP suppresses neighoboring keratinocyte proliferation via ROS. Finally, targeting senescence by a proposed senolytic drug ABT737 or by antoxidant suppresses this effect in vitro. This is an interesting manuscript showing a role for senescence in melanocytes in skin ageing with paracrine and autocrine SASP signaling to induce DNA damage at telomeres. Overall the data is of good quality and convincing. A few issues should be addressed prior to publication

- The authors should control the specificity of the SIRT1 staining in Fig1C for example by using another antibody or in situ hybridization. Currently, the signal is very low and seems to be limited to only one of the two melanocytes in the field of view in the young skin, when also the keratinocytes should in fact express SIRT1
- As the DNA damage at telomeres is a critical point of the manuscript, it should be confirmed with another method or marker, eg 53BP1
- It is difficult to assess the quality and power of the quantitative image analyses as it is not clear from the figure dot plots (or materials and methods) how many cells from how many individuals have been analyzed for each assay.
- One is left wondering why the DNA damage in keratinocytes does not trigger senescence in these cells. One possibility is the high turnover rate of keratinocytes in the tissue clears the senescent cells out of the tissue. Are the irradiation doses that trigger melanocyte senescence in principle able to do the same in keratinocytes or are keratinocytes simply resistant?

Additional comments Referee #2:

I am remaining of the opinion that the cellular systems entailed in the study are very superficial and non-physiological. I'm worried this could overinterpret the role of senescent melanocytes during skin aging. I also remain quite doubtful about the CXCR3-ROS axis as the experiments are not technically accurate and sound.

Additional comments Referee #1:

I agree with most of the comments, concerns and criticisms of the other referees, many which are shared by me. All of them could be resolved experimentally. In regards to the concerns that superficial and non-physiological experimental systems are used, I would suggest that the authors emphasize the limitations of the melanoderm model in their discussion. Nonetheless, I believe that this system provides evidence of the contribution of senescent melanocytes to skin aging and should therefore not be dismissed as superficial. Of note, a mouse model system of skin aging would likely not provide any relevant information for this study that characterizes the causes for human skin aging as a) mouse cells are possibly more resistant to developing dysfunctional telomeres damaged by ROS (due to expression of hTERT in all cells), b) paracrine senescence is caused by other SASP factors and other mechanisms that likely do not involve telomeres, and c) skin architecture, aging and healing differs significantly between mice and humans.

Additional comments Referee #3:

I agree with ref#1 that most concerns can be addressed with the experiments that have been outlined in the reviewer reports. Of course, cell culture experiments always have limitations, and as suggested by ref1, the authors should be encouraged to explicitly outline the limitations of the cell culture studies in the discussion section and to avoid over stating the role of melanocyte senescence in skin aging.

1st Revision - authors' response 7th Aug 2019

We would like to thank the reviewers for their time and insightful comments, which we believe, have helped improve the quality of our study.
We have made every effort to address the reviewer's comments; both through the inclusion of new experimental data, additional methodological detail and discussion.
Please see our point-by-point response to referees below.

Referee #1:
Victorelli et al for EMBO J

Cellular senescence has been demonstrated to cause functional decline of mammalian skin during aging, yet details, such as the contribution of specific cell types, the causes of cellular senescence, and the pathways involved in senescence induction are currently unclear. This is an important and novel study as it begins to address these unresolved questions. The authors reveal an aging-associated increase in melanocytes with features of senescence in human skin, demonstrate that senescent melanocytes can induce paracrine telomere dysfunction in fibroblasts and senescence thorough mechanisms involving IP10-CXCR3-ROS signaling, provide evidence that this mechanism of inducing paracrine senescence in keratinocytes is active also in aged skin and melanoderms, and suggest that senescent melanocytes are a cause of aging associated skin thinning using melanoderms as a model system. Finally, the authors demonstrate that senescent melanocyte-induced skin thinning can be rescued using a senolytic and ROS scavenger. The study therefore not only provides further evidence that cellular senescence is causative of aging associated decline of mammalian tissue and organ systems but it also reveals therapeutic opportunities to alleviate such decline. This is a well-executed study, yet some issues should be resolved in order to strengthen it.
We really appreciate the positive comments from the reviewer and are particularly encouraged by the fact that the reviewer finds the study important, novel and well executed.
Major and minor issues are listed below.

1. Figure 1A: The authors state "all p16 positive cells ...were also positive for melanocyte markers (Figure 1A)". However, the Figure shows no p16 positive cells in the young donor and only one p16 positive cell in the old donor. The authors therefore either should show a micrograph in which approximately 5-10% of melanocytes are p16 positive, which is the average percentage based on the graph in Fig 1B, and/or state that "the p16 positive cell ...was also positive for melanocyte marker..."
The reviewer is absolutely correct that the images shown are not a true representation of the data. However, at the magnification we have used for the quantification, we can only visualize on average 3-4 melanocytes (detected by either MelanA or S100)- so it would be impossible to show a truly representative micrograph containing 5-10% p16 positive melanocytes. We should point out that we have in fact quantified the entire skin section for each subject and find that the only p16 positive cells we find in the epidermis are melanocytes (more clarity regarding quantification methodology is now included in methods section).

2. Figure 1B and D (and in general thorough the study where subjective quantitations were performed, the following information should be provided): A description is needed that explains how p16/Sirt1 positive cells were quantified. This should include how many separate individuals performed the analysis, whether this was conducted in a blinded manner, etc.... Also, since Sirt1 and/or p16 levels are not always detectable (positive) or not (negative), but also likely sometimes contain a faint, intermediate signal, a description is needed that states what criteria were used to determine whether the cells were considered p16/Sirt1 positive and negative?
We appreciate the comments and now provide a much more detailed description of how the quantifications were performed in the methods section. In summary, all quantifications were done in

a blinded manner and in most instances quantified independently by at least 3 different analysts. In the case of p16 positivity- the staining intensity was particularly strong for melanocytes and this allowed us to clearly distinguish between positive and negative cells. In the case of SIRT1- we noticed that while it was expressed in most cells throughout the epidermis, the fluorescence intensity was the strongest in melanocytes than keratinocytes from young subjects and decreased with age (by thresholding the fluorescence intensity in the same manner in all micrographs- we could easily distinguish between SIRT1 positive and negative melanocytes). We have now included other potentially more specific senescent markers such as absence of HMGB1 (Davalos et al. 2013) and expression of p21.

3. An aging-associated increase in 53BP1 positive melanocytes in skin of human donors has previously been demonstrated and should thus be cited here (Suram et al., 2012 EMBO J. 31 2839)
We apologize for not having cited this paper. It is now included in the discussion.

4. It seems that not all DDR or TAF positive cells are also positive for p16 since the percentages of DDR positive cells (7% young/10% old) are greater compared to p16 positive cells (0% young/7% old). What is the correlation between the induction of the DDR/TAF and the senescence status in melanocytes? Is the induction of DDR/TAF independent of p16 and senescence in dermal melanocytes? One way to address this is to co-immunostain tissue section with MelanA/H2AX (or TAF)/p16 and determine whether DDR positive melanocytes are also p16 positive.
This is an excellent suggestion by the reviewer- particularly it has been shown that senescent cells are heterogeneous and may be dependent on the p16 or p21 pathway depending on the stimuli (Herbig 2004). Since receiving the comments we have spent a considerable amount of time/effort in our laboratory optimizing co-immuno FISH for TAF, MelanA and p16- however, for technical reasons we have never managed to obtain good enough quality of images that allowed us to perform accurate quantifications. Generally, when we combined p16 antibody together with gH2A.X or 53BP1 - we get much less defined DDR foci. We do find however, that *in vitro*- senescent melanocytes (both induced by X-ray, UVA+B irradiation or replicative senescence) show similar % of TAF and p16 (however, we appreciate that this is not indicative of what happens *in vivo*). We have also analyzed p21 expression in melanocytes (See revised Figure 1D) and in contrast to p16 or TAF we do not find an age-dependent increase, so it would appear that melanocyte senescence may be more dependent on p16 than p21.

5. Telomeric signals in the micrograph from the old donor (Figure 1E old) appear to cluster into very large foci. Is this a common feature of melanocytes in old donors? If so, what is the significance of this? Does this clustering potentially create such large telomeric foci that the overlap with DDR foci becomes coincidental? How did the authors control for this? Does this clustering affect q-FISH analysis? If not, how can the authors be certain of that?

We carefully analyzed most of our images and realized that the micrograph shown was not truly representative and that clustering of telomeres is a relatively rare event in most melanocytes. However, the reviewer is absolutely correct that this may be a confounding factor and affect our analysis. We have now included a sentence in the discussion acknowledging the possibility. We have also included in revised Figure 1 A a more representative image.

6. Figure 1K-M: The authors should rephrase the two sentences describing telomere lengths in TAF as their statements suggest that telomeres in TAF are longer than any other telomere in a melanocyte. See: "...by quantifying telomere FISH intensities of telomeres co-localising with γH2AX (TAF) and those that did not co-localise with γH2AX (non-TAF) in melanocytes, we found that telomeres associated with γH2AX were significantly longer than those not co-localising with γH2AX (Figure 1J-L)". This is an incorrect statement since the authors demonstrate in Figure 1M that many TAF have signal intensities less than non-TAF telomeres.

We have rephrased the sentences so that it becomes clearer. We do observe a slight shift towards stronger telomere FISH signals in DDR positive telomeres, however, we agree with the reviewer that this cannot be interpreted as longer telomeres being specifically targeted. Our overall interpretation of the results is that telomeres co-localizing with γH2AX occur irrespective of length and the data suggests that these are not preferentially short (similarly to what was observed by D'adda di Fagagna and Herbig's labs amongst others).

7. Figure 2: The authors suggest that melanocytes and keratinocytes containing TAF are senescent. This has not been directly demonstrated. It is therefore unclear whether cells with H2AX foci (or potentially TAF) in tissue (2A) or in melanoderms (2I) have the ability to cause paracrine senescence. Can the authors show that TAF containing melanocytes express SA-betaGal activity/p16/low laminB1 etc?
We understand the concerns of the reviewer and appreciate the suggestions. However, as previously mentioned we have failed for technical reasons to conduct quadruple staining for TAF, melanocyte and other senescent markers.

8. The authors show that melanocytes with H2AX foci (or TAF) have the ability to cause paracrine TAF/senescence. It is curious why TAF containing senescent keratinocytes don't also cause paracrine TAF and senescence in neighboring keratinocytes. Do the authors observe larger clusters of TAF containing keratinocytes that spread beyond just once cell distance from a TAF containing melanocyte? Why or why not?
This is an excellent point. We do not observe clusters of TAF in keratinocytes- in fact, we observe higher TAF in keratinocytes in the epidermal layers immediately adjacent to melanocytes, however, if we analyze more distal layers we observed that TAF decreases (we have now included this new analysis in Appendix Figure S1C). One interpretation is that upon keratinocyte cell division, TAF gets diluted or repaired. Another possibility is that TAF positive keratinocytes at upper layers are shed off from the epidermis (as suggested by reviewer 3) and this may explain why we also do not see any keratinocytes positive for senescent marker p16. It is possible that the faster turnover of keratinocytes (as opposed to melanocytes) precludes their ability to induce paracrine effects.

9. Figure 2A (and 2I): Although the authors suggest that TAF in melanocytes causes paracrine TAF in neighboring keratinocytes, it is unclear whether the DDR positive and representative melanocyte shown has TAF. Since arrows point to TAF as per the figure legend, and no arrows are shown in the melanocyte, it would appear that the green DDR foci are non-telomeric. If this is indeed correct, then the conclusions should be altered. If arrows were inadvertently omitted, the authors should revise the image and also show enlarges versions of melanocyte TAF in a column next to enlarged keratinocyte TAF.
Given the concerns raised above, should the x-axis (Figure 2B) instead state "mean gH2AX foci in melanocytes"?
We apologize that this was not clear. These images are of 1 single plane from a Z-stack so it was difficult to show a plane where TAF were present in both melanocytes and keratinocytes. We now include Z-projections where several TAF can be visible in both cell types (on the side we include some examples of magnifications of individual TAF from single Z-planes).

10. Are keratinocytes with TAF senescent; do they express p16? It would be useful to show this given that it is suggested that ABT-737 treatment can reduce the number of p16 positive keratinocytes in tissue (see Figure 5)

We do not see p16 positive keratinocytes *in vivo* whereas we do see TAF keratinocytes so believe it is unlikely that these co-express *in vivo*. However, as we see them in the melanoderms it indicates that they could co-occur even if not so evident *in vivo* (possible through epidermal shedding as highlighted by Reviewer 3). Unfortunately, we have struggled for technical reasons to conduct quadruple co-staining for gH2A.X/53BP1, telomeres, MelanA and p16, so unfortunately we cannot answer this particular question. Since we see a however, we cannot be sure. We now include in the discussion this as a limitation.
11. Also, authors should include a description what "neighboring" keratinocytes means. Are these cells in immediate vicinity to melanocytes or is there a distance threshold where the paracrine effects become less effective? Also, as mentioned above, it is unclear why senescent keratinocytes cause paracrine senescence in other keratinocytes.

We have now quantified TAF in keratinocytes based on the distance (mm) to melanocytes (See new Appendix FigureS1C).

12. Figure 2B: how many melanocytes and keratinocytes were analysed for each group?
We now provide detailed information in the methods section.

13. The melanoderm model should be described in greater detail. For example, it is unclear whether these are cross sections, or whether the cells had been imaged from above.
Also, in melanoderms, are the TAF containing keratinocytes directly neighboring the senescent melanocytes, similar to what is observed in old donors (2A)? More details should be described/quantified.
We now provide detailed information in the methods section. Regarding the specific questions from the reviewer- these are cross sections and we observed that most TAF were located within the 2 epidermal layers immediately adjacent to melanocytes (similarly to what we observed in human skin sections from older individuals).

14. Figure 2N-O: The increase of "epidermal" thickness of melanoderms containing proliferating, but not senescent melanocytes is interesting. Is this increase in thickness due to proliferating keratinocytes, melanocytes, or both? Similarly, is the lack of increase in thickness due to a lack of proliferation of melanocytes, keratinocytes, both or due to a lack of extracellular matrix production? The authors should measure percentages of these two cell types and the density (number of each cell type per specific area) in their 6 analyzed groups.

We have quantified total number of melanocytes and keratinocytes and present the information in the revised version. While numbers of senescent melanocytes remained unchanged, the total number of young melanocytes present in melanoderms did increase 21 days post-epidermal development (from 1 to 2 melanocytes per field of view). However, given the relatively low number of melanocytes present in melanoderms we believe this cannot *per se* explain the differences in epidermal thickness (see Appendix FigS3B). Consistent with an impact on proliferation of keratinocytes, we observed decreased % of Ki67 positive keratinocytes (Figure 2M) and reduced total number of keratinocytes (Figure 2L) in melanoderms with senescent melanocytes. We also analyzed expression of keratinocyte differentiation marker keratin 10 and found that K10 positive epidermal layers were reduced in the presence of senescent melanocytes which would be a result of reduced proliferation (see new Appendix Figure S3A). Finally, we also analyzed expression of collagen XVII since it was recently shown to be decreased during aging and that its increased expression impacted positively on skin regeneration (Liu et al. 2019). We found reduced collagen XVII expression in the presence of senescent melanocytes (see new Appendix Figure S3C and D)..

15. The last sentence in this section should be altered as it jumps to the conclusion that paracrine telomere dysfunction causes aging associated epidermal thinning. This was not directly demonstrated (only melanoderm thinning was demonstrated).

We have rephrased it and toned down our conclusions.

16. Figure 3B: What was the stress in "stress induced-senescent melanocytes"? Please provide details.
Stress-induced senescence in this instance was achieved by exposure to 10Gy X-ray irradiation (characterization of senescence is provided in Appendix FigS2). We made sure it was clarified in the text.

17. Supp Figure 6E: A quantitation of the immunoblots would be useful since the differences appear to be small.
Quantification is provided. The reviewer is correct that differences are small- however reproducible in separate experiments and consistent with published data.
18. Figure 4: What is the mechanism by which mitochondrial ROS causes TAF in cells? Is this due to damaging shelterin components and thereby "uncapping" telomeres? Is this due to double strand break formation? Does this require cell proliferation?
We appreciate these questions and agree that they are important. We are currently pursuing these questions in our laboratory and hope to publish them as a separate investigation. We have however extended our discussion to suggest possible mechanisms by which this may occur: Mild ROS exposure has been shown to accelerate the rate of telomere shortening, potentially by inducing single-stranded breaks at telomere regions in dividing cells (Petersen 1998, von Zglinicki 2002). Acute exposure to ROS or genotoxic stresses have been shown to induce DNA double stranded breaks at telomeres independently from telomere length, expression of shelterin component TRF2, telomerase expression or cell division (Hewitt et al. 2012; Fumagalli et al. 2012; Anderson et al.

2019). In post-mitotic cardiomyocytes, we have observed that oxidative stress caused by mitochondrial dysfunction or impairment in antioxidant defense systems in different mice models was able to induce TAF irrespectively of length or cell division (Anderson et al. 2019). Probably more relevant was the recent finding by the group of Utz Herbig that TGF-b1 (a component of the SASP) caused rapid telomere erosion and telomere dysfunction in fibroblasts also via a ROS dependent pathway and that this could be suppressed by expression of hTERT (Razdan et al. 2018)- it is possible that a similar mechanism occurs in this instance.

19. Figure 5: The authors assume that the senolytic agent ABT-737 clears senescent melanocytes from melanoderms, but this is not directly demonstrated. Instead, it is shown that cells containing markers of senescence, such as p16 and TAF, are reduced upon ABT-737 treatment, but this is not necessarily evidence that senescent melanocytes with damaging SASP are eliminated. A way in which to address this could be to demonstrate that there is an increase in apoptotic melanocytes (or cells), a decrease in melanocytes overall, and potentially a decrease in IP10 expressing cells/melanocytes in ABT-737 treated melanoderms.
We now show quantification of positive melanocytes and that these are absent following ABT-737 treatment (See appendix Figure S5)

20. Figure 5D: Authors show that keratinocytes containing 2 or more TAF show reduced development in ABT-737 treated melanocytes. Why weren't melanocytes containing 1 TAF (which is probably sufficient to cause senescence) included in this group? Are keratinocytes with only 1 TAF functional whereas those with 2 or more undergo senescence?
We have now included the quantification.
Regarding the specific question- we are simply not certain how many TAF are necessary to induce senescence. This may be cell-type specific. While early studies have suggested that 1 dysfunctional telomere may be sufficient to induce senescence (di Leonardo et al. 1994), other more recent work has suggested that 5 dysfunctional telomeres may be required to induce senescence (Kaul et al. 2012). In mice, our group has previously presented data as % of cells with more than 3 TAF as a potential marker of senescence since it was closely quantitatively to other senescent markers (Ogrodnik et al. 2017; Anderson et al. 2019) and that is the rationale why we analyzed in Figure 1 % of melanocytes containing more than 3 TAF.

21. Figure 5H: As above, it is unclear what rescues the thinning of melanoderms generated in the presence of senescent cells. Do the number of melanocytes or keratinocytes increase in the presence of ABT-737? Quantitations of melanocytes/keratinocytes (as described above, #14) might be useful.
It is now provided see Appendix Figure S5. ABT737 eliminates all melanocytes in melanoderms and restores keratinocyte numbers.

22. The sentence "Therefore, these data implicate senescent melanocytes as causal in the induction of paracrine telomere damage and senescence in neighbouring epidermal cells" should be changed to ".....neighboring keratinocytes".

It has been corrected.

Referee #2:

Victorelli et al report that senescent melanocytes contribute to skin aging by inducing telomeric damage and senescence into neighboring keratinocytes. They provide a mechanism by which this would be executed, and they demonstrate that elimination of senescent melanocytes can be achieved by using a common senolytic and can restore normal epidermal thickness. The concept of accumulation of senescent melanocytes in the aging skin is interesting, but not new. Besides some ex vivo analyses done in Figure 1, the remaining experiments rely on using highly artificial and poorly physiological cell culture models. The absence of fibroblasts from the skin equivalent is also a big limitation. The study is very preliminary, and several characterizations are missing. Moreover, due to absence of in vivo evidence, the results are far from being conclusive.

We are aware that there are limitations to our study and based on the reviewer's comments have expanded the discussion to highlight some of them. However, we also realize that there are no optimal models to investigate some of the hypotheses in the context of human skin aging and that mice differ considerably from humans in several aspects of their skin physiology (as pointed out by reviewer 1) and, crucially, do not normally contain inter-follicle melanocytes bar possibly in their tails. The rationale behind using melanoderms containing only melanocytes and keratinocytes (without the dermal component) was an attempt to conduct a proof-of-concept experiment to test the specific hypothesis that senescent melanocytes (which accumulate during skin aging) may contribute to aging of the epidermis (without other confounding factors). Our data suggest that melanocyte senescence is likely to be physiologically relevant since TAF positive melanocytes correlate with features of skin aging such as flattening of the epidermal-dermal junction (See Figure EV2). Previous work has shown that p16 positive cells in the epidermis correlated with more facial wrinkles and higher perceived age (Waaijer et al. 2016) and importantly was associated with age-related pathologies (Waaijer et al. 2012).
Based on the reviewer's and editor's suggestions we have expanded our characterization of senescence in the skin and melanoderms and highlighted the limitations of our study- we hope this addresses some of the concerns from the reviewer.

Additional major points to consider:

*
It is really hard to have conclusive interpretation of Figure 1A based on the images provided. If the point is that only melanocytes enter senescence in aged skin, then additional data should be provided;

We do not claim that melanocytes are the only cells that become senescent in the epidermis- however, they are the ones that express senescent marker p16. Telomere-initiated senescence has been shown to strongly rely on p53 and is usually accompanied by increased p21 expression. P16 is thought to be induced at later time points and provide a second barrier to prevent the growth of cells with dysfunctional telomeres (Beasejour et al 2003). We have now extended our characterization of melanocyte senescence in human skin: we found that melanocytes show a decrease in HMGB1. We also evaluated p21 (but found no age-dependent increase).
We have also conducted a similar analysis specifically in keratinocytes, we evaluated markers of senescence p16, p21, TAF and absence of HMGB1 specifically in keratinocytes from young and old subjects. We did not detect any p16 positivity in keratinocytes from any age group; however, found an age-dependent increase in p21-positive cells located predominantly in the granular layer of the epidermis (see new Figure EV3A and B). We also found a significant decrease in expression of HMGB1 (see new Figure EV3C and D). Finally, we observed that the both the mean number and % of keratinocytes positive for TAF increased in older subjects (see new Figure EV3E-G). Whilst this suggests senescent keratinocytes might be present through a p21 senescence check-point pathway, it also indicates that p16 is not the primary senescence marker for keratinocytes in-vivo.

From the methods section of Figure 1, it is stated that 'data from randomly selected participants were used'. What data? Do they mean samples? Or that the staining were done somewhere else? This is definitely not clear;
We apologize for the lack of clarity. We have now provided a more detailed explanation of how the samples were obtained and analyzed.

While down-regulation of SIRT-1 has been somehow associated to senescence, it can't be considered a senescent marker. If authors are claiming that the p16+ melanocytes are unequivocally senescent, then other markers should be evaluated;

We are in agreement that SIRT1 loss may not be a specific marker of senescent cells. We have now conducted analysis of Alarmin HMGB1 which has been shown to be released from the nucleus in senescent cells (Davalos et al. 2013) and p21 expression.

In Figure 2, authors that paracrine senescence, particularly form melanocytes to keratinocytes, is a major component to drive aging in the skin. However, in Figure 1 they do not provide any evidence that keratinocytes in aged skin are also senescent;

We do not propose that the paracrine senescence solely drives senescence in surrounding cells but cellular damage which can lead to senescence. This is supported by the in-vitro experiments demonstrating DNA damage by the SASP on keratinocytes and fibroblasts, DNA damage in the melanoderms as well as in surrounding cells in-vivo. Together, these data point to the evidence that senescent melanocytes damage surrounding cells.

Most keratinocytes do not proliferate, and exposing them to the CM of melanocytes might contribute to their differentiation (instead of senescence). Showing that highly proliferative cells (fibroblasts) accumulate DNA damage upon exposure to a CM rich of pro-inflammatory and pro-mitogenic factors is not surprising and not physiologically relevant;

Regarding physiological relevance- keratinocytes divide along the basal layer to then differentiate and form the stratum corneum whilst leaving parity of basal cell numbers to continue the process. We observed in human skin sections that keratinocytes in close proximity to melanocytes contain telomere damage and, hence, overtime likely leads to intra-cellular aging affecting the ability of basal keratinocytes to proliferate and differentiate at an optimal rate, possibly contributing to epidermal atrophy. In addition, to our knowledge, only 1 previous study has suggested that SASP factors can induce telomere dysfunction in neighboring cells (Razdan 2018).

Related to the previous point, the epidermal thinning might be a combination of enhanced differentiation and absence of stem cells from the skin equivalent cultures;

We thank the reviewer for this excellent suggestion. The epidermis is regenerated by the germinative layer of basal keratinocytes. These epidermal progenitor cells retain a dedifferentiated status by anchoring to the basement membrane (Dev Cell. 2016 Sep 26;38(6):601-9). Forced differentiation of basal keratinocytes – for example by genetic deletion of integrin adhesion receptors (EMBO J. 2000 Aug 1; 19(15): 3990–4003) – induces epidermal hyperplasia, a compensatory hyperproliferation of basal keratinocytes (increased Ki67 expression), and acanthosis (thickening of the keratin 10-positive stratum spinosum). Interestingly, the opposite is observed here with senescence, where we observe a decrease in keratin 10-positive layers (see new data Appendix Figure S3) and Ki67-positive cells in melanoderms. Our results suggest that epidermal turnover is reduced in senescent epidermis as was reported for aged human skin (Adv Wound Care (New Rochelle). 2013 Feb; 2(1): 5–10).

Genetic manipulation of cxcr3 and ip-10 should be performed to validate the claim that ip-10 signaling is responsible for paracrine DNA damage and senescence;

We thank the reviewer for the helpful suggestion. As suggested by the reviewer, we have knock-down CXCR3 using 2 independent siRNAs and found similar results as with AMG487 (see revised Figure 4)- which validated this claim.

The experiment of senescent cells clearance in figure 5 suggests that at the end of the treatment there are no p16+ melanocytes. Considering that the majority of the melanocytes are made senescent and then incorporated into melanoderms, most of the embedded melanocytes should be p16+ at the beginning of the culture. Does this mean that after ABT737 there are almost no melanocytes in the melanoderm culture? This would explain the thicker epidermis, as keratinocytes will have more space to grow

The reviewer has made an important point. With ABT737 treatment we have effectively eliminated all melanocytes from the melanoderm (see quantifications of MelanA in Appendix Figure 5A), however, given the overall low number of melanocytes present in the melanoderm it is an unlikely scenario that this may somehow facility proliferation of keratinocytes. Accordingly, in melanoderms containing senescent melanocytes, numbers of melanocytes are also significantly reduced (which mimics what has been reported during skin aging) and this does not result in increased proliferation of keratinocytes due to more space availability. We have now discussed this further in the paper.

Referee #3:

The manuscript studies human skin ageing initially confirming and building on previous observations showing that melanocytes are the only cell type in the epidermis. The authors then show that these melanocytes have a typical Senescence associated secretory phenotype (SASP) and

that this SASP suppresses neighoboring keratinocyte proliferation via ROS. Finally, targeting senescence by a proposed senolytic drug ABT737 or by antoxidant suppresses this effect in vitro. This is an interesting manuscript showing a role for senescence in melanocytes in skin ageing with paracrine and autocrine SASP signaling to induce DNA damage at telomeres. Overall the data is of good quality and convincing. A few issues should be addressed prior to publication

We really appreciate the positive comments from the reviewer and are particularly encouraged by the fact that the reviewer finds the study of good quality and convincing.

- The authors should control the specificity of the SIRT1 staining in Fig1C for example by using another antibody or in situ hybridization. Currently, the signal is very low and seems to be limited to only one of the two melanocytes in the field of view in the young skin, when also the keratinocytes should in fact express SIRT1
We agree that SIRT1 may not be the best marker for senescence as we discussed above and have now expanded our analysis by detecting additional senescent markers such as absence of HMGB1 and p21 in the manuscript (see revised Figure 1). As the reviewer pointed out we have noticed that SIRT1 was expressed ubiquitously in most cells in the epidermis; however, it was stronger in melanocytes from young individuals. When thresholding the fluorescence intensity (applying always the same parameters) we could see SIRT1 positivity in melanocytes from young subjects but not from older and this allowed us to score SIRT1 positive or negative melanocytes (SIRT1 intensity in keratinocytes was also weaker in both young and old skin using the same parameters).

- As the DNA damage at telomeres is a critical point of the manuscript, it should be confirmed with another method or marker, eg 53BP1
We thank the reviewer for the suggestion. We have analyzed TAF using 53BP1 and obtained similar results as with gH2A.X- see new Figure EV1.

- It is difficult to assess the quality and power of the quantitative image analyses as it is not clear from the figure dot plots (or materials and methods) how many cells from how many individuals have been analyzed for each assay.
We have now provided detailed information in the methods section.

- One is left wondering why the DNA damage in keratinocytes does not trigger senescence in these cells. One possibility is the high turnover rate of keratinocytes in the tissue clears the senescent cells out of the tissue. Are the irradiation doses that trigger melanocyte senescence in principle able to do the same in keratinocytes or are keratinocytes simply resistant?

Our new data indicates that senescence may indeed be triggered since we observed increased TAF, loss of HMGB1 and p21 in older individuals in keratinocytes. P16 positivity was not observed. It is possible that p21-mediated senescence occurs in these cells (we observed that it was expressed predominantly in the granular layer of the epidermis) however; we speculate they may be shed off before p16 can be expressed (which has been shown to be activated at a later stage of the senescence arrest Beausejour et al. 2003).

Additional comments Referee #2:

I am remaining of the opinion that the cellular systems entailed in the study are very superficial and non-physiological. I'm worried this could overinterpret the role of senescent melanocytes during skin aging. I also remain quite doubtful about the CXCR3-ROS axis as the experiments are not technically accurate and sound

We have discussed further the limitations of the models used. We have also performed additional experiments to strengthen the role of CXCR3 in paracrine TAF induction. We made sure to tone down some of our conclusions to avoid over interpretation. There is however, several limitations in using mouse models to investigate human skin aging. Hence, human skin models that recapitulate the human epidermal barrier and skin pigmentation presented alongside *in vivo* human skin data provides valuable insights nonetheless.

Additional comments Referee #1:

I agree with most of the comments, concerns and criticisms of the other referees, many which are shared by me. All of them could be resolved experimentally. In regards to the concerns that superficial and non-physiological experimental systems are used, I would suggest that the authors emphasize the limitations of the melanoderm model in their discussion. Nonetheless, I believe that this system provides evidence of the contribution of senescent melanocytes to skin aging and should therefore not be dismissed as superficial. Of note, a mouse model system of skin aging would likely not provide any relevant information for this study that characterizes the causes for human skin aging as a) mouse cells are possibly more resistant to developing dysfunctional telomeres damaged by ROS (due to expression of hTERT in all cells), b) paracrine senescence is caused by other SASP factors and other mechanisms that likely do not involve telomeres, and c) skin architecture, aging and healing differs significantly between mice and humans.

We thank the reviewer for the positive comments. We acknowledge and discuss the limitations of the models used in our study but similarly to the reviewer see limitations in conclusions derived from the use of mice (where melanocyte biology and functionality is quite different from humans).

Additional comments Referee #3:

I agree with ref#1 that most concerns can be addressed with the experiments that have been outlined in the reviewer reports. Of course, cell culture experiments always have limitations, and as suggested by ref1, the authors should be encouraged to explicitly outline the limitations of the cell culture studies in the discussion section and to avoid over stating the role of melanocyte senescence in skin aging.
We thank the reviewer for the constructive comments. We have now made sure limitations of our study are discussed and toned down some of our conclusions.

2nd Editorial Decision 6th Sep 2019

Thank you for re-submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the original referees whose comments are enclosed below. As you will see, the referees state that your manuscript has substantially improved during the revision and they are in favour of publication. While referee # 2 remains somewhat more hesitant we have - in light of the strong support of the other two referees, decided to conclude that the manuscript is suitable for publication at EMBO Journal, pending satisfactory minor adjustments.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending the remaining concerns of referee #2 are addressed in a minor revision by additional analyses or complementary discussion of the findings and introducing caveats where appropriate. Also, we need you to consider a number of points related to formatting and data representation as outlined below.

Referee #1:

The authors have significantly revised the original submission and have adequately addressed all concerns and suggestions from this reviewer.

Referee #2:

Victorelli et al revised an interesting manuscript studying senescent melanocytes in the aging skin. Compared to the first submission, they added a number of experiments and certainly strengthen the quality of the paper. However, this reviewer remains of the idea that there is no sufficient experimental evidence to claim that 1) melanocytes are the only p16+ cells in aging skin; 2) senescent melanocytes promote DNA damage via the SASP (and, particularly, via one single factor/pathway)

Referee #3:

The authors have addressed all my concerns and the manuscript has been substantially improved

2nd Revision - authors' response 14th Sep 2019

The authors performed the requested editorial changes.

3rd Editorial Decision 18th Sep 2019

The authors performed the requested editorial changes.

