Oxygen regulates epithelial stem cell proliferation via RhoA-actomyosin-YAP/TAZ signal in mouse incisor

Keishi Otsu, Hiroko Ida-Yonemochi, Shojiro Ikezaki, Masatsugu Ema, Jiro Hitomi, Hayato Ohshima and Hidemitsu Harada
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MS TITLE: Oxygen regulates epithelial stem cell proliferation via RhoA-actomyosin-YAP/TAZ signal in mouse incisor

AUTHORS: Keishi Otsu, Hiroko Ida-Yonemochi, Shojiro Ikezaki, Masatsugu Ema, Jiro Hitomi, Hayato Ohshima, and Hidemitsu Harada

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested (please also see the Editor's note appended), which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Editors' note
Please address:
- The validity of the findings of in vitro analysis of mHAT9d cell line to the ESC in vivo
- The mechanism of how Hif1Alpha activates RhoA

Reviewer 1

Advance summary and potential significance to field

This paper describes that low oxygen environment restricts proliferation of epithelial stem cells (ESCs) of mouse incisors. Hypoxia activates RhoA signaling leading to stabilization of cortical actin and adherens junctions (AJs). Association of Merlin to AJs suppresses nuclear YAP/TAZ to attenuate cell proliferation. This hypothesis is novel and interesting. However, there are two major concerns. One is that the authors used a cell line for the analyses of mechanisms, but the experimental conditions seems to be inappropriate for some experiments. The other is that it is unclear how much extent the proposed mechanism also operates in vivo, since many experiments are performed only in a cell line. Thus, with current level of analyses, the hypothesis is not clearly supported by the presented data.

Comments for the author

Major comments
1.
Many experiments are performed only in a cell line, and operation of the proposed mechanism in vivo is not clearly shown. Detailed comparison of gene expression patterns in vivo and organ culture are required. For example, relationships among expression of Hif1alpha, active RhoA, pMLC, F-actin, E-cadherin, Merlin, YAP/TAZ and Ki67 should be shown in tissue sections. Patterns of Hif1alpha and active RhoA are similar, but patterns of p-MLC and E-cadherin are slightly different from that of active RhoA. Pattern of YAP is not clearly complementary to active RhoA. Therefore, it is important to compare these patterns in detail, preferably in adjacent sections to clarify how much extent the proposed mechanism is used. In organ cultures, patterns of active RhoA were altered from that of in vivo in both normal and low oxygen conditions. The authors interpreted that the changes observed with normal oxygen condition was caused by the increased oxygen level in OEE and outer SR. If so, these culture conditions could be used to examine whether oxygen levels control cell proliferation through the proposed mechanism. With current results, it is not clear whether the patterns of Ki67 and YAP/TAZ were altered in normoxia conditions correlating with the changes in active RhoA. Again, detailed comparison of expression patterns of the factors/markers described above in serial sections should clarify how much extent the proposed mechanism controls cell proliferation. For detection of nuclear YAP, anti-active YAP antibody (Abcam ab205270) is useful for signal quantification, because it stains only nuclear YAP.

2.
About the experiments using a cell line, experimental designs of some experiments are not appropriate. Authors should briefly describe the identity of the cell line mHAT9d in the main text. In the experiment shown in Figure 4G, authors showed that knockdown of Hif1alpha reduced active RhoA signal. It only showed that basal level RhoA activation is regulated by Hif1alpha. What should be shown in this part is that activation of RhoA by low oxygen is mediated by Hif1 induction. Therefore knockdown should be done under hypoxia condition, and RhoA activation level should be compared with those of normoxia and hypoxia conditions. Similar problem is present with experiments shown in Supplemental Figure 5D. In the experiments shown in Figure 5C, cell densities of the inhibitor treated cells are very sparse although they are described as high-density cells. It is likely that the duration of inhibitor treatment (24 hrs) was too long and most cells were dead or detached from the dishes. Alternatively, the number of plated cells were not enough to make high density culture conditions. Thus, the experimental condition is not optimal.
Similar problems also exist for the experiments shown in Figures 6 B, D, E. and 7F.
The effects of Y27632 and Blebbistatin on F-actin and Hippo signaling/YAP could be observed within an hour or so.

Minor comments
1.
Line 119-120. “GFP fluorescence was diluted twofold at each division, so it is retained preferentially in frequently dividing cells.” Is this correct? The GFP signal will be retained in non-dividing cells.

2.
Line 168. “Together, these results indicated that hypoxia repressed cell proliferation though the inhibition of YAP/TAZ activity in the apical bud.” There is no data or reference showing involvement of YAP/TAZ in regulation of cell proliferation in this cell line or the tissue. Therefore, the results do not “indicate” but “are consistent with the hypothesis”. Demonstration of the involvement of YAP/TAZ in cultured cells should strengthen the story.

3.
Citation of some references are not appropriate. Line 98, regulation of YAP and TAZ by Hippo signal is first shown in Zhao et al doi:10.1101/gad.1602907 and Lei et al doi:10.1128/MCB.01874-07, respectively. Line 162, cell density regulation of YAP in an epithelial cell line is also first shown by Zhao et al doi:10.1101/gad.1602907.

4.
The authors’ name of the reference Csete 2005 is written in all capital letters.

Reviewer 2

Advance summary and potential significance to field

This is an interesting article by Otsu et al. describing how oxygen regulates epithelial stem cell proliferation via RhoA and Yap/Taz in the mouse incisor.
If generalizable to other organ systems this I could have broad implications for organ regeneration and cancer treatment.

Comments for the author

This is an interesting article by Otsu et al. describing how oxygen regulates epithelial stem cell proliferation via RhoA and Yap/Taz in the mouse incisor.

I have only a few comments.

1) It would be interesting to stain for Yap and Taz separately as they do have none redundant functions.
2) How does Hif1alpha increase active RhoA expression? Does it increase RhoA expression in general or does it affect a the expression of a RhoGEF or RhoGAP and if so which one? ChipSeq for Hif1alpha may help clarify this mechanism.

Minor comments:
Line 120 i think should say : "retained preferentially in infrequently dividing cells"
Line 163 : delete "have" and replace "have been" with were Line 173: delete "have"
Line 184: delete "but"

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors show how hypoxia can modulate the behaviour of dental epithelial stem cells (DESCs) and transit amplifying cells (TACs) in the continuously growing mouse incisor. The authors show that hypoxia modulates YAP/TAZ, pathway known to regulate DESCs/TACs, at least partially via the RhoA/ROCK pathway.

The work is of interest, as it is the first report of the effects of hypoxia on DESCs in an important model such as the continuously growing mouse incisor.
The role of hypoxia in stem cell behaviour and its effect on the YAP/TAZ pathway has been already described for other organs; similarly, the connection between RhoA/ROCK/cytoskeletal remodeling and YAP/TAZ is well described.

The article thus conveys important information concerning the modulation of the dental epithelial stem cell niche by oxygen concentration, and provide a first molecular explanation for this effect. The conclusions are overall supported by the results and are in accordance with mechanisms described in other organs / stem cell niches.

Comments for the author

- In the Results section, the authors state that "GFP fluorescence was diluted twofold at each division, so it is retained preferentially in frequently dividing cells". The opposite is true, i.e. the labeling is retained preferentially in slowly dividing cells (as actually shown in the figure 1E).

- The authors show that SCCs reside in areas characterized by lower O2 concentration compared to TACs, and correlate it to the distance from the blood vessels. The hypoxic treatment in vitro leads to an important decrease in cell proliferation and decrease in the size of the labial cervical loop. Can the authors assess if in vitro hypoxia leads to cell death in addition to decreased proliferation?

- In figure 3H: since upon culture in hypoxic conditions the cervical loop is reduced, it would be more accurate to evaluate the proportion of YAP/TAZ+ cells rather than their absolute number.

- In figure S2, it is not clear whether only nuclear localization or overall YAP/TAZ expression is decreased upon culture in hypoxic conditions.
This should be assessed either by Western blot or at least by RT-PCR.

- Figure 4: The pattern of RhoA expression in culture in normoxic conditions (Figure 4C)is very different from that of RhoA expression in WT mice in vivo (Figure 4A). Can the authors comment on this pattern? A first obvious issue is that blood vessels do not delivery oxygen in vitro, thus affecting the relative oxygen pressure in SCCs/TACs observed in vivo. How does this affect their conclusions?

- Figure 5D: The two sections shown are really not comparable The same comment applies to figure 6C

- Figure 6B, D, E: the stainings are convincing, they would be reinforced by a quantification of nuclear/cytoplasmic signal.

- The correlation between distance from blood vessels and hypoxia in the incisor is logically sound, but it is purely correlative (no experiment of deletion of vessels / no direct measurement of oxygen tension). As such it should be discussed.

Minor comments:
- line 51: "microenvironment, containing a heterologous cell population" - the authors probably mean "heterogeneous"
- In the introduction, the authors could indicate in more detail the roles of YAP/TAZ in the regulation of DESCs (lines 102-103).

First revision

Author response to reviewers' comments

Response to the Reviewers
Thank you for your thorough review of our paper and for your suggestions. We have revised

the manuscript accordingly and highlighted the revised part in blue. Below, the reviewers' comments are given in italics.

Editors' note

Please address:
1. The validity of the findings of in vitro analysis of mHAT9d cell line to the ESC in vivo.

Thank you for your suggestion. We have newly performed immunofluorescence and investigated the expression of the suggested molecules in detail using adjacent frontal section in the same mouse incisor (Supplemental Fig. 1). In comparison between SCCs and TACs, the expression of HIF1□, active RhoA, pMLC, F-actin, E-cadherin, and Merlin in SCCs are higher than that in TACs, whereas YAP1, YAP/TAZ, and Ki67 were much lower in SCCs than in TACs. These results are in line with the proposed mechanism. Therefore, we concluded that the mechanism proposed in present study significantly contributes to the regulation of cell proliferation between SCCs and TACs *in vivo*. Please see the response to the first comment of Reviewer #1 for more details.

2. The mechanism of how Hif1Alpha activates RhoA.

We have performed HIF1α siRNA experiments and speculated the mechanism of how HIF1□ regulates RhoA activity. Please see the detail in the response to the second comment of Reviewer #2.

Reviewer 1 Comments for the Author:

Major comments
1. Many experiments are performed only in a cell line, and operation of the proposed mechanism in vivo is not clearly shown. Detailed comparison of gene expression patterns in vivo and organ culture are required. For example, relationships among expression of Hif1alpha, active RhoA, pMLC, F-actin, E-cadherin, Merlin, YAP/TAZ, and Ki67 should be shown in tissue sections. Patterns of Hif1alpha and active RhoA are similar, but patterns of p-MLC and E-cadherin are slightly different from that of active RhoA. Pattern of YAP is not clearly complementary to active RhoA. Therefore, it is important to compare these patterns in detail, preferably in adjacent sections to clarify how much extent the proposed mechanism is used.
In organ cultures, patterns of active RhoA were altered from that of in vivo in both normal and low oxygen conditions. The authors interpreted that the changes observed with normal oxygen condition was caused by the increased oxygen level in OEE and outer SR. If so, these culture conditions could be used to examine whether oxygen levels control cell proliferation through the proposed mechanism. With current results, it is not clear whether the patterns of Ki67 and YAP/TAZ were altered in normoxia conditions correlating with the changes in active RhoA. Again, detailed comparison of expression patterns of the factors/markers described above in serial sections should clarify how much extent the proposed mechanism controls cell proliferation. For detection of nuclear YAP, anti-active YAP antibody (Abcam, ab205270) is useful for signal quantification, because it stains only nuclear YAP.

Thank you for your suggestion. To compare the expression pattern of the suggested molecules *in vivo*, we have conducted an immunofluorescence analysis using adjacent frontal section in the same mouse incisor (Supplemental Fig.1). We found that the expression patterns of HIF1□, active RhoA, F-actin, and Merlin were slightly different from pMLC and E-cadherin in the apical bud. In SR, pMLC and E-cadherin were highly expressed, whereas the expression of Ki67, YAP/TAZ, and YAP1 (Abcam, ab205270) was weak; this was consist with our proposed mechanism. Meanwhile, the expression of HIF1□, active RhoA, F-actin, and Merlin in SR appeared to be relatively weak. This discordance of expression patterns suggests the minor involvement of our proposed mechanism in SCCs within the apical bud, including SR, OEE, and BE. However, in comparison between the apical bud and TACs, the expression of HIF1□, active RhoA, pMLC, F-actin, E-cadherin, and Merlin in the apical bud were higher than that in TACs, whereas the expression of YAP1, YAP/TAZ, and Ki67 were much lower in SCCs than in TACs. These results are in line with the proposed mechanism. Therefore, we

concluded that the mechanism proposed in the present study significantly contributed to the regulation of cell proliferation between SCCs and TACs.

Also, as the reviewer suggested, we have conducted immunostaining for those molecules using adjacent sections in the same cultured incisor under hypoxic condition (Supplemental Fig.2). Similar to the results observed *in vivo*, the expression pattern of the HIF1α, active RhoA, pMLC, F-actin, E-cadherin, and Merlin was slightly different. We speculate the presence of oxygen level gradient within apical bud which may subtly change. However, the experiments clearly showed that hypoxia increased the expressions of those molecules and decreased that of Ki67 and YAP1 in TACs of dental epithelium.

Since the detailed regulatory mechanism within the apical bud *in vivo* remains elusive, we will address this at the single cell level in future studies.
We added these results to Supplemental Figures 1 and 2 and modified the results and discussion (Line 342) accordingly.

2. About the experiments using a cell line, experimental designs of some experiments are not appropriate. Authors should briefly describe the identity of the cell line mHAT9d in the main text.

We described the identity of the cell line and cited the relevant references in the results section of main text in line 164.

In the experiment shown in Figure 4G, authors showed that knockdown of Hif1alpha reduced active RhoA signal. It only showed that basal level RhoA activation is regulated by Hif1alpha. What should be shown in this part is that activation of RhoA by low oxygen is mediated by Hif1 induction. Therefore, knockdown should be done under hypoxia condition, and RhoA activation level should be compared with those of normoxia and hypoxia conditions. Similar problem is present with experiments shown in Supplemental Figure 5D.

We performed the suggested experiment and improved the figures (Fig. 4D-G, J and Fig. S10E) and discussion (line 284) accordingly.

In the experiments shown in Figure 5C, cell densities of the inhibitor treated cells are very sparse, although they are described as high-density cells. It is likely that the duration of inhibitor treatment (24 hrs) was too long and most cells were dead or detached from the dishes. Alternatively, the number of plated cells were not enough to make high density culture conditions. Thus, the experimental condition is not optimal. Similar problems also exist for the experiments shown in Figures 6 B, D, E. and 7F. The effects of Y27632 and Blebbistatin on F-actin and Hippo signaling/YAP could be observed within an hour or so.

Regarding experimental duration, to obtain sufficient effect of Y27632 and Blebbistatin, one hour was found to be too short for our cell line, while 24 h was optimal in which cells were not dead and detached from the dishes. The cells proliferated well as shown in Figure 6a. Thus, we performed the experiments again and improved the figures (Figs. 5C, 6B, D, E and 7F) accordingly.

<u>Minor comments</u>
3. Line 119-120. "GFP fluorescence was diluted twofold at each division, so it is retained preferentially in frequently dividing cells." Is this correct? The GFP signal will be retained in non-dividing cells.

We corrected the sentence accordingly (line 122).

4. Line 168. "Together, these results indicated that hypoxia repressed cell proliferation though the inhibition of YAP/TAZ activity in the apical bud." There is no data or reference showing involvement of YAP/TAZ in regulation of cell proliferation in this cell line or the tissue. Therefore, the results do not "indicate" but "are consistent with the hypothesis". Demonstration of the involvement of YAP/TAZ in cultured cells should strengthen the story.

We have conducted additional experiments and demonstrated YAP1 involvement in cell proliferation in our cell line using YAP1 siRNA and YAP1 localization based on cell density. We added the results to the supplementary Figs. S4 and S5 and revised the text accordingly (in line 167).

5. Citation of some references are not appropriate. Line 98, regulation of YAP and TAZ by Hippo signal is first shown in Zhao et al doi:10.1101/gad.1602907 and Lei et al doi:10.1128/MCB.01874-07, respectively. Line 162, cell density regulation of YAP in an epithelial cell line is also first shown by Zhao et al doi:10.1101/gad.1602907.

We included the relevant references as the reviewer suggested (line 98 and 166).

6. The authors' name of the reference Csete 2005 is written in all capital letters.

We corrected the citation and bibliography accordingly (line 543).

Reviewer 2 Comments for the Author:

1. It would be interesting to stain for Yap and Taz separately as they do have none redundant functions.

Thank you for your suggestion. We separately stained YAP1 and TAZ *in vivo* where YAP1 showed clear differences between SCCs and TACs, while no difference in TAZ was observed. In addition, hypoxia had no effect on TAZ mRNA expression, meanwhile, it reduced YAP1 mRNA expression. These results illustrated the non-redundant functions of YAP and TAZ as the reviewer suggested. We have added the results to the supplementary Figs. S1 and S6 and modified the results and discussion accordingly (line 318).

2. How does Hif1alpha increase active RhoA expression? Does it increase RhoA expression in general or does it affect the expression of a RhoGEF or RhoGAP and if so which one? ChipSeq for Hif1alpha may help clarify this mechanism.

We have removed unpublished data provided for the referees in confidence.

Minor comments:

3. Line 120 i think should say : "retained preferentially in infrequently dividing cells"

We corrected the sentence accordingly (line122).

4. Line 163 : delete "have" and replace "have been" with were

We corrected the sentence as suggested (line 167 and 168).

5. Line 173: delete "have"

We have deleted "have" as suggested (line 180).

6. Line 184: delete"but"

We have deleted "but" as suggested (line 190).

Reviewer 3 Comments for the Author:

1. In the Results section, the authors state that "GFP fluorescence was diluted twofold at each division, so it is retained preferentially in frequently dividing cells". The opposite is true, i.e. the labeling is retained preferentially in slowly dividing cells (as actually shown in the figure 1E).

We corrected the sentence accordingly (line 122).

2. The authors show that SCCs reside in areas characterized by lower O2 concentration compared to TACs, and correlate it to the distance from the blood vessels. The hypoxic treatment in vitro leads to an important decrease in cell proliferation and decrease in the size of the labial cervical loop. Can the authors assess if in vitro hypoxia leads to cell death in addition to decreased proliferation?

Thank you for this great suggestion. To address the reviewer's question, we performed TUNEL staining assay of the cultured incisor and mHAT9d cells. The results showed that hypoxia did not induce cell death. We added the results in Supplementary Fig.3 and the manuscript in line 157, 174.

3. In figure 3H: since upon culture in hypoxic conditions the cervical loop is reduced, it would be more accurate to evaluate the proportion of YAP/TAZ+ cells rather than their absolute number.

We have modified the data analysis from the absolute number to the proportion of YAP/TAZ + cells in Fig.3I.

4. In figure S2, it is not clear whether only nuclear localization or overall YAP/TAZ expression is decreased upon culture in hypoxic conditions. This should be assessed either by Western blot or at least by RT-PCR.

As the reviewer suggested, we performed qPCR experiments and showed that hypoxia reduced not only nuclear localization of YAP/TAZ but also YAP1 mRNA expression. We have added the data to Fig.S6 and modified the manuscript in line 174.

5. Figure 4: The pattern of RhoA expression in culture in normoxic conditions (Figure 4C)is very different from that of RhoA expression in WT mice in vivo (Figure 4A). Can the authors comment on this pattern? A first obvious issue is that blood vessels do not delivery oxygen in vitro, thus affecting the relative oxygen pressure in SCCs/TACs observed in vivo. How does this affect their conclusions?

This is an excellent point. As the reviewer pointed out, because blood vessels do not deliver oxygen to the tissue in vitro, we believed that the relative oxygen pressure in SCCs/TACs in vitro would be totally different from that in vivo. Thus, this may be the reason why in vitro RhoA expression pattern was different from in vivo RhoA expression pattern. Although we have no direct experimental evidence for the correlation between blood vessel distribution and oxygen pressure in vivo and in vitro, we will address the issue by the direct measurement of oxygen tension in the future study.

6. Figure 5D: The two sections shown are really not comparable The same comment applies to figure 6C

We performed the experiments again and improved the figures (Fig.5D, 6C).

7. Figure 6B, D, E: the stainings are convincing, they would be reinforced by a quantification of nuclear/cytoplasmic signal.

We added the quantification of nuclear/cytoplasmic signal in Fig. 6B, D and E as the reviewer suggested.

8. The correlation between distance from blood vessels and hypoxia in the incisor is logically sound, but it is purely correlative (no experiment of deletion of vessels / no direct measurement of oxygen tension). As such it should be discussed.

We discussed the point and revised the manuscript in line 259.

Minor comments:

9. line 51: "microenvironment, containing a heterologous cell population" - the authors probably mean "heterogeneous"

We corrected the word in line 51.

10. In the introduction, the authors could indicate in more detail the roles of YAP/TAZ in the regulation of DESCs (lines 102-103).

We thank the reviewer for this excellent suggestion. We added a more detailed description of the role of YAP/TAZ in DESCs as recommended in line102.

Second decision letter

MS ID#: DEVELOP/2020/194787

MS TITLE: Oxygen regulates epithelial stem cell proliferation via RhoA-actomyosin-YAP/TAZ signal in mouse incisor

AUTHORS: Keishi Otsu, Hiroko Ida-Yonemochi, Shojiro Ikezaki, Masatsugu Ema, Jiro Hitomi, Hayato Ohshima, and Hidemitsu Harada
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This paper describes that low oxygen environment restricts proliferation of epithelial stem cells (ESCs) of mouse incisors. At the molecular level, hypoxia induces HF1alpha and activates RhoA signaling leading to stabilization of cortical actin and adherens junctions (AJs). Association of Merlin to AJs suppresses nuclear YAP/TAZ to attenuate cell proliferation. These findings demonstrated the importance of microenvironmental oxygen to control ESC behavior and also provided a novel molecular mechanism. These are novel and important findings for the scientists working with various types of stem cells.

Comments for the author

In the revised version, authors appropriately responded to the comments made by the reviewers. The model is well supported by the data. This is a nice work.

Reviewer 2

Advance summary and potential significance to field

This is an interesting article by Otsu et al. describing how oxygen regulates epithelial stem cell proliferation via RhoA and Yap/Taz in the mouse incisor.
If generalizable to other organ systems this I could have broad implications for organ regeneration and cancer treatment.

Comments for the author

The authors have addressed all my concerns.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors show how hypoxia can modulate the behaviour of dental epithelial stem cells (DESCs) and transit amplifying cells (TACs) in the continuously growing mouse incisor. The authors show that hypoxia modulates YAP/TAZ, pathway known to regulate DESCs/TACs, at least partially via the RhoA/ROCK pathway.
The work is of interest, as it is the first report of the effects of hypoxia on DESCs in an important model such as the continuously growing mouse incisor. The role of hypoxia in stem cell behaviour and its effect on the YAP/TAZ pathway has been already described for other organs; similarly, the connection between RhoA/ROCK/cytoskeletal remodeling and YAP/TAZ is well described.
The article thus conveys important information concerning the modulation of the dental epithelial stem cell niche by oxygen concentration, and provide a first molecular explanation for this effect.
The conclusions are overall supported by the results and are in accordance with mechanisms described in other organs / stem cell niches.

Comments for the author

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1. In the Results section, the authors state that "GFP fluorescence was diluted twofold at each division, so it is retained preferentially in frequently dividing cells". The opposite is true, i.e. the labeling is retained preferentially in slowly dividing cells (as actually shown in the figure 1E).

We corrected the sentence accordingly (line 122).
-- Properly addressed

2. The authors show that SCCs reside in areas characterized by lower O2 concentration compared to TACs, and correlate it to the distance from the blood vessels. The hypoxic treatment in vitro leads to an important decrease in cell proliferation and decrease in the size of the labial cervical loop. Can the authors assess if in vitro hypoxia leads to cell death in addition to decreased proliferation?

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4. In figure S2, it is not clear whether only nuclear localization or overall YAP/TAZ expression is decreased upon culture in hypoxic conditions. This should be assessed either by Western blot or at least by RT-PCR.

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6. Figure 5D: The two sections shown are really not comparable The same comment applies to figure 6C

We performed the experiments again and improved the figures (Fig.5D, 6C).

--properly addressed

7. Figure 6B, D, E: the stainings are convincing, they would be reinforced by a quantification of nuclear/cytoplasmic signal.

We added the quantification of nuclear/cytoplasmic signal in Fig. 6B, D and E as the reviewer suggested.

--properly addressed

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We discussed the point and revised the manuscript in line 259.

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Minor comments:
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We corrected the word in line 51.

-- properly addressed

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We thank the reviewer for this excellent suggestion. We added a more detailed description of the role of YAP/TAZ in DESCs as recommended in line102.

- properly addressed