Plap-1 lineage tracing and single-cell transcriptomics reveals cellular dynamics in the periodontal ligament

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DOI: 10.1242/dev.201203

Editor: James Briscoe

Review timeline
Submission to Review Commons: 18 April 2022
Submission to Development: 9 August 2022
Editorial Decision: 5 September 2022

Reviewer 1

Evidence, reproducibility and clarity

Comments:

* In the presented study the authors attempt to perform an in vivo characterisation of the cellular hierarchies and cellular dynamics of the periodontal ligament (PDL), a largely unknown compartment of the periodontal tissue which function is to support mammalian teeth. Periodontal diseases are one of the major causes of adult tooth loss, hence understanding the cellular dynamics of this compartment is of particular interest. To do this, the authors develop a novel traceable mouse line based on the Plap-1 marker, which they identify specifically labels periodontal fibroblasts. The developed Plap-1-GFP-2A-CreER mice are then crossed onto an inducible Rosa26-tdTomato to enable in vivo tracing of Plap-1 PDL fibroblasts. This tool is of significant relevance as it allows for the first time to analyse the cellular fate of the elusive populations that constitute PDL under normal homeostatic and regenerative conditions. This mouse model also enables the authors to sort the cells of interest (as marked by GFP) to perform single-cell RNA sequencing analysis, providing further knowledge on the cellular heterogeneity of PDL cells.

* The work presented in this article is of interest for the periodontal stem cell field and more generally the mesenchymal stem cell field. In particular, through the development of new tools, including a novel lineage traceable mouse line amenable for lineage tracing studies, the authors provide knew knowledge advancing our understanding on the populations and hierarchies that constitute the PDL. Having said this, I find this study rather descriptive and, in certain cases, the significance of the results are somewhat overinterpreted. For instance, lineage tracing studies are rather vague... based on the colocalization of a widely induced traceable fluorophore and markers present in the relevant cell populations, or even just histological positioning of cells; something that entails numerous technical implications and potential artefacts. It would be more convincing to titrate down the tamoxifen levels used to induce Plap-1 traceable mice, in order to track how single-cell derived clones actually contribute to the formation of other PDL populations, and validate this using the relevant markers at critical time points. Quantification of clonal distribution would also provide a deeper understanding of the process.

* Another rather technical, but critical, aspect is the need for further validation of their new mouse model. Particularly, in order to interpret any prospective data on clonal dynamics, it is first important to know whether their new Cre system is tightly regulated, or whether there is leakage

in the absence of Tamoxifen induction. Imaging of aged un-induced animals would help clarify this point.

* Finally, the scRNA-seq is rather superficial, a more in-depth analysis would be required to support the statements based on hierarchies and trajectories proposed by the authors.

* Despite all this, I believe the authors have the tools and data to address most of the aspects discussed below, which would make the study sound and result of advancement in the relevant field.

Significance

See above

Reviewer 2

Evidence, reproducibility and clarity

* The authors describe in a richly illustrated manuscript periodontal ligament associated protein-1 Plap-1 as a periodontal fibroblast (PDLC) associated molecule that has the possibility to differentiate both into cementoblasts lining the tooth surface and into osteoblasts lining the alveolar bone.
* In the introduction, please not only refer to higher expression of Plap-1 in certain tissues, but also refer to the function, as revealed by Sakashita et al (also on the periodontium/susceptibility to periodontitis). Apart from the fact that it is a 43 kDa ECM protein, subtype of the leucine-rich etc., it is also important to briefly sum-up -if scientific data allow - the function of the protein.

- Page 5, line 2: have the authors investigated Plap-1 in tissues other than the periodontal ligament? These experiments seem essential to demonstrate the uniqueness of Plap-1, possibly as a confirmation of the Sakashita et al. paper of 2021. It is always a good habit to confirm previous work in a next study.
- Page 5 on lineage tracing with Tomato: please spend a few lines on the essence of the experiment, either on page 5 or in the legend of Fig. 2, or both. You will thus keep the readers involved who are not familiar with lineage tracing.
- Page 6 and 7: the description of the protocol is very valuable. It is also important the cell numbers of the various cell types were described in great detail (Fig 3). So, authors have now used cells derived from extracted teeth, which is world-wide a sample of convenience. However, after extraction, half of the PDLCs are likely attached to the alveolar bone of the tooth socket. Have the authors ever considered to harvest these cells? In principle, and biologically, PDLC cells at the site of the alveolar bone could be the more osteogenic cells. The PDLC that are attached to tooth could in principle be quite different, being anti-osteogenic and anti-osteoclastogenesis-stimulating.
- Page 6, line 8: indicate what CD51+ cells are. In corresponding figure 3, explain the abbreviations in the X-axis in the legend.
- Page 7 line 1: The proerythroblasts in the PDL are a surprise to me! I assumed that the bone marrow would be the natural niche. Authors are also encouraged to highlight plasma cell specific RNAs in their atlas, since these are quite abundant in periodontitis lesions.
- In figure 4, its seems to me that the stromal cells in 4C are scattered more or less in 3 domains. This idea is strengthened when interpreting 4E Plap-1 and Ibsp. Could the authors specify these domains?
- On Page 7: again for the not-so-informed reader: briefly, in the first sentence, describe the phenomenon of RNA velocity.
- Page 7, line 20: delete "were".
- In figure 5A it could be helpful to put the numbers in the figure as well.
- In figure 6G, it seems like that some osteocytes are positive, which means that they were derived from the TomatoRed cells within 7 days. That is quite remarkable and should gain some attention. Probably use a white arrow and specific mentioning in the legend.
- In the discussion, I miss a clear link and comparison with human periodontal ligament. Is all this mouse specific or are some of these aspects also present in the human periodontal ligament? One study comes to mind that has actually studied gene expression of Plap-1 etc. in PDLC and in alveolar bone derived cells: Loo-Kirana R, et al., Frontiers in Cell and Developmental Biology, 2021:

DOI: 10.3389/fcell.2021.709408. But there is bound to be other studies as well. A brief mirroring of these findings with other studies would be in place.

CROSS-CONSULTATION COMMENTS

I have read and seen the comments of the other reviewers. They are more or less in line with mine, and I have nothing to add.

Significance

Authors identify Flap-1 postive cells as key cells contributing to stem cell ness of the periodontium. With advanced techniques using GFP and Tomato Rd mice they are able to show a kind of hierarchy in cell differentiation. They also describe the presence of all kind of cells in the peridoontal ligament as well as the capacity of the Plap-1 positive cells to contribute to regeneration. It is a very valuable addition to existing literature.

Audience: those, basic scientist but also dentists in general for whom the biology of the periodontal ligament is crucial.

My expertise: periodontal ligament specialist, but more the human part. I use PDL to study osteogenesis and osteoclastogenesis, in presence of bacterial products, inflammatory and anti-inflammatory reagents.

Reviewer 3

Evidence, reproducibility and clarity

This study aims to gain a better understanding of PDLCs and their associated cementum and alveolar bone. The study provides a very clear results for differential expression of Plap-1 and IBSP the periodontal fibroblast and associated cementoblasts and osteoblasts.

The most infesting is the generation of reporter mice for identification of Plap-1+ cells. The generation of this mice lined allowed then to gain insight to the regeneration of periodontium as well as heterogeneity in of Plap-1+ cells.

Minor issues:

1. Many abbreviation in the papers have to be better defined. (Spp1, Bgn, Sparc, Col1a. also DN and DP in legends to Figure 3.
2. Legends to all figure can be written more clearly.
3. Statement in the result (line 27 and 28) cement oblasts and osteoblasts were aligned ..... should be eliminated as the figure 1A does not allow appreciation of such features. Also, the statement does not add anything to the manuscript and its results.
4. The statement on Page 5 (line 1, 2) the protein distribution of Plap-1 needs to be described.
5. Line 14 and 15 on page 5. It should be noted that very few/if any cells are co-expressing Ibsp and td-tomato. The number is so few that brings questions to the conclusion.

Major/important issues to be addressed:

1. The authors have very nicely and clearly shown that Ibsp is expressed by cementoblasts and osteoblasts but not by PDL fibroblasts. Therefore, the lineage tracing experiments after PDL injury should be followed by examination of Ibsp in cementoblasts and osteoblasts originating from the Plap-1+ cells.
2. It is also important to know what is the percentage of Plap-1+/Ly6a+ cells.
3. The author should include a stronger statement for the possible role of Plap-1+/Ly6a+ cells (not Plap-1+ alone) as a source pf progenitors for periodontium.

Significance

by providing new markers and new transgenic animal model, the paper makes an important and significant contribution to the field

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Author response to reviewers' comments

1. General Statements [optional]

We thank the reviewers for their careful reading of the manuscript and for providing thoughtful suggestions to improve it. We have revised the manuscript to address all of the comments carefully. Our edits to the main text are highlighted in yellow.

2. Point-by-point description of the revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Comments:
In the presented study the authors attempt to perform an in vivo characterisation of the cellular hierarchies and cellular dynamics of the periodontal ligament (PDL), a largely unknown compartment of the periodontal tissue which function is to support mammalian teeth. Periodontal diseases are one of the major causes of adult tooth loss, hence understanding the cellular dynamics of this compartment is of particular interest. To do this, the authors develop a novel traceable mouse line based on the Plap-1 marker, which they identify specifically labels periodontal fibroblasts. The developed Plap-1-GFP-2A-CreER mice are then crossed onto an inducible Rosa26-tdTomato to enable in vivo tracing of Plap-1 PDL fibroblasts. This tool is of significant relevance as it allows for the first time to analyse the cellular fate of the elusive populations that constitute PDL under normal homeostatic and regenerative conditions. This mouse model also enables the authors to sort the cells of interest (as marked by GFP) to perform single-cell RNA sequencing analysis, providing further knowledge on the cellular heterogeneity of PDL cells.
Response: We sincerely appreciate your critical reading of the manuscript.

The work presented in this article is of interest for the periodontal stem cell field and more generally the mesenchymal stem cell field. In particular, through the development of new tools, including a novel lineage traceable mouse line amenable for lineage tracing studies, the authors provide knew knowledge advancing our understanding on the populations and hierarchies that constitute the PDL. Having said this, I find this study rather descriptive and, in certain cases, the significance of the results are somewhat overinterpreted. For instance, lineage tracing studies are rather vague... based on the colocalization of a widely induced traceable fluorophore and markers present in the relevant cell populations, or even just histological positioning of cells; something that entails numerous technical implications and potential artefacts. It would be more convincing to titrate down the tamoxifen levels used to induce Plap-1 traceable mice, in order to track how single-cell derived clones actually contribute to the formation of other PDL populations, and validate this using the relevant markers at critical time points. Quantification of clonal distribution would also provide a deeper understanding of the process.
Response: Thank you for your thoughtful suggestions. We found they are critical comments and performed the following additional analyses. First, we have used definitive markers (αSMA for myofibroblasts and *Ibsp* mRNA for cemento-/osteoblast) to clarify what cell type the Plap1 lineage cells differentiated during the wound healing process in a quantitative manner (p.9 line 21 and new Fig. 6J). Second, as suggested, we have performed a clonal analysis using 1/10 dose of Tamoxifen and found the part of clones differentiated into *Ibsp*+ cementoblasts and osteoblasts after 2 weeks (Fig. S1H).

Another rather technical, but critical, aspect is the need for further validation of their new mouse model. Particularly, in order to interpret any prospective data on clonal dynamics, it is first important to know whether their new Cre system is tightly regulated, or whether there is leakage

in the absence of Tamoxifen induction. Imaging of aged un-induced animals would help clarify this point.
Response: We found this comment also very important. First, we have examined the leak in the absence of Tamoxifen. Adult Plap1-CreER; R26tdTomato mice without Tamoxifen have been analyzed in detail, and no tdTomato expression was observed (new Fig. S1G). This has also been confirmed using flow cytometry. We believe that these results will be useful in interpreting future analyses of cellular dynamics.

Finally, the scRNA-seq is rather superficial, a more in-depth analysis would be required to support the statements based on hierarchies and trajectories proposed by the authors.
Response: Thank you for your thoughtful comment. In addition to the existing analysis, pseudotime analyses have been performed to determine what gene expression changes occur during each differentiation (new Fig. S6A-F). We believe that these analyses allowed us to evaluate the molecular state of the cell differentiation process in more detail.

Despite all this, I believe the authors have the tools and data to address most of the aspects discussed below, which would make the study sound and result of advancement in the relevant field.
Response: Thank you for your interest in our manuscript.

Reviewer #1 (Significance (Required)):
See above

Reviewer #2 (Evidence, reproducibility and clarity (Required)):
The authors describe in a richly illustrated manuscript periodontal ligament associated protein-1 Plap-1 as a periodontal fibroblast (PDLC) associated molecule that has the possibility to differentiate both into cementoblasts lining the tooth surface and into osteoblasts lining the alveolar bone.
In the introduction, please not only refer to higher expression of Plap-1 in certain tissues, but also refer to the function, as revealed by Sakashita et al (also on the periodontium/susceptibility to periodontitis). Apart from the fact that it is a 43 kDa ECM protein, subtype of the leucine-rich etc., it is also important to briefly sum-up -if scientific data allow - the function of the protein.
Response: Thank you for your suggestion. We have summarized the functional roles of Plap-1 in the main text (p.4, line 5).

Page 5, line 2: have the authors investigated Plap-1 in tissues other than the periodontal ligament? These experiments seem essential to demonstrate the uniqueness of Plap-1, possibly as a confirmation of the Sakashita et al. paper of 2021. It is always a good habit to confirm previous work in a next study.
Response: According to the suggestion, we have examined Plap1-GFP cells in all tissues whose mRNA expression level was examined in the previous paper (new Fig. S2A-L). We have added the description in the main text (p.5, line 29).

Page 5 on lineage tracing with Tomato: please spend a few lines on the essence of the experiment, either on page 5 or in the legend of Fig. 2, or both. You will thus keep the readers involved who are not familiar with lineage tracing.
Response: Thank you for pointing this out. We have added the statements both in the main text (p.5, line 17) and the legend of Fig. 2.

Page 6 and 7: the description of the protocol is very valuable. It is also important the cell numbers of the various cell types were described in great detail (Fig 3). So, authors have now used cells derived from extracted teeth, which is world-wide a sample of convenience. However, after extraction, half of the PDLCs are likely attached to the alveolar bone of the tooth socket. Have the authors ever considered to harvest these cells? In principle, and biologically, PDLC cells at the site of the alveolar bone could be the more osteogenic cells. The PDLC that are attached to tooth could in principle be quite different, being anti-osteogenic and anti-osteoclastogenesis-stimulating.
Response: We agree that this comparison would be fascinating. Although an efficient method for isolating PDL cells was developed in this study, it is still technically challenging to isolate only the cells closest to the alveolar bone. We have added the analysis of the tooth socket after the extraction with HE staining (new Fig. S3D). There have been some areas where PDL tissue remained

on the alveolar bone surface, but cells in the furcation area close to the alveolar crest have been mostly removed. We have also added the statements in the main text (p.6, line 12) and the legend of Fig. S2.

Page 6, line 8: indicate what CD51+ cells are. In corresponding figure 3, explain the abbreviations in the X-axis in the legend.
Response: According to the reviewer's comment, we have added the statements both in the main text (p.6, line 25) and the legend of Fig.3.

Page 7 line 1: The proerythroblasts in the PDL are a surprise to me! I assumed that the bone marrow would be the natural niche. Authors are also encouraged to highlight plasma cell specific RNAs in their atlas, since these are quite abundant in periodontitis lesions.
Response: Thank you for your interest in the manuscript. As plasma cells play important roles in periodontitis, we have checked their marker RNA expressions (Prdm1, Cd27, and Cxcr4) in our dataset (added to Fig. S4C). However, we did not find the distinct expression pattern within the B cell cluster, possibly because the plasma cells could be differentiated upon inflammation or the depth of this analysis would not be enough to detect a rare cell type. We have also added the statement in the main text (p.11, line 13).

In figure 4, its seems to me that the stromal cells in 4C are scattered more or less in 3 domains. This idea is strengthened when interpreting 4E Plap-1 and Ibsp. Could the authors specify these domains?
Response: Thank you very much for pointing out the importance of heterogeneity in stromal cells. We agree with the reviewer's suggestion. Thus, we performed in-depth analyses on the stromal cells and found many distinct cell types, including PDLSCs, cementoblasts, osteoblasts, fibroblasts, and other transitional cells. Although the results are already described in Figure 5, it was not clear in the previous manuscript. We have revised the manuscript so that readers can easily understand that figure 5 is an expansion of figure 4 focusing on the stromal cells (p.7, line 15).

On Page 7: again for the not-so-informed reader: briefly, in the first sentence, describe the phenomenon of RNA velocity.
Response: Thank you for the suggestion. We have added the statements both in the main text (p.8, line 14) and the legend of Fig. 5.

Page 7, line 20: delete "were".
In figure 5A it could be helpful to put the numbers in the figure as well.
In figure 6G, it seems like that some osteocytes are positive, which means that they were derived from the TomatoRed cells within 7 days. That is quite remarkable and should gain some attention. Probably use a white arrow and specific mentioning in the legend.
Response: Thank you for pointing them out. We have modified them accordingly.

In the discussion, I miss a clear link and comparison with human periodontal ligament. Is all this mouse specific or are some of these aspects also present in the human periodontal ligament? One study comes to mind that has actually studied gene expression of Plap-1 etc. in PDLC and in alveolar bone derived cells: Loo-Kirana R, et al., Frontiers in Cell and Developmental Biology, 2021: DOI: 10.3389/fcell.2021.709408. But there is bound to be other studies as well. A brief mirroring of these findings with other studies would be in place.
Response: According to the reviewer's comment, we added the reference together with other human studies and discussed it in the main text (p.10, line 26).

CROSS-CONSULTATION COMMENTS
I have read and seen the comments of the other reviewers. They are more or less in line with mine, and I have nothing to add.

Reviewer #2 (Significance (Required)):

Authors identify Flap-1 postive cells as key cells contributing to stem cell ness of the periodontium. With advanced techniques using GFP and Tomato Rd mice they are able to show a kind of hierarchy in cell differentiation. They also describe the presence of all kind of cells in the peridoontal

ligament as well as the capacity of the Plap-1 positive cells to contribute to regeneration. It is a very valuable addition to existing literature.
Audience: those, basic scientist but also dentists in general for whom the biology of the periodontal ligament is crucial.
My expertise: periodontal ligament specialist, but more the human part. I use PDL to study osteogenesis and osteoclastogenesis, in presence of bacterial products, inflammatory and anti-inflammatory reagents.
Response: Thank you for your critical reading of the manuscript. We sincerely appreciate your comments.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):
This study aims to gain a better understanding of PDLCs and their associated cementum and alveolar bone. The study provides a very clear results for differential expression of Plap-1 and IBSP the periodontal fibroblast and associated cementoblasts and osteoblasts.
The most infesting is the generation of reporter mice for identification of Plap-1+ cells. The generation of this mice lined allowed then to gain insight to the regeneration of periodontium as well as heterogeneity in of Plap-1+ cells. .

Minor issues:
1. Many abbreviation in the papers have to be better defined. (Spp1, Bgn, Sparc, Col1a. also DN and DP in legends to Figure 3.
Response: Thank you for pointing this out. We have checked through the manuscript and added the statements both in the main text and the legends (highlighted in yellow). We used full gene names retrieved from the National Center for Biotechnology Information website.

2. Legends to all figure can be written more clearly.
Response: According to the reviewer's comment, we have tried to make the legends clear so that the readers can easily follow the content of the figure (highlighted in yellow).

3. Statement in the result (line 27 and 28) cement oblasts and osteoblasts were aligned ..... should be eliminated as the figure 1A does not allow appreciation of such features. Also, the statement does not add anything to the manuscript and its results.
Response: We agree with the reviewer's comments. We have eliminated the statement accordingly.

4. The statement on Page 5 (line 1, 2) the protein distribution of Plap-1 needs to be described.
Response: Thank you for pointing this out. We have added the statements both in the main text (p.5, line 7).

5. Line 14 and 15 on page 5. It should be noted that very few/if any cells are co-expressing Ibsp and td-tomato. The number is so few that brings questions to the conclusion.
Response: Thank you for the important comments. Since some cementoblasts are quiescent (doi.org/10.1016/B978-0-12-802818-6.00002-8), 90-day lineage tracing may not have been sufficient to examine precise cell origin. Thus, we have conducted a new quantitative analysis in a wound healing setting and revealed that more than half of the *Ibsp*-positive cells were of PDLC origin (new Fig. 6I and J). Given that this study has identified the definitive cementoblast marker gene, Sparcl1, future studies will reveal detailed cellular dynamics of the cementoblasts. We have added this discussion in the main text (p. 11, line 22).

Major/important issues to be addressed

1. The authors have very nicely and clearly shown that Ibsp is expressed by cementoblasts and osteoblasts but not by PDL fibroblasts. Therefore, the lineage tracing experiments after PDL injury should be followed by examination of Ibsp in cementoblasts and osteoblasts originating from the Plap-1+ cells.
Response: We totally agreed with the reviewer. We have performed histological analysis and added the new data (new Fig.6G, I, and J and main text p.9 line 25). We hope the new data has improved the manuscript. Thank you for your helpful suggestions.

2. It is also important to know what is the percentage of Plap-1+/Ly6a+ cells.
Response: Thank you for the critical comment. We have performed quantitative FACS analysis and found the population was 5.4 % of the PDL live singlets (new Fig. S6D and main text p.8 line 25).

3. The author should include a stronger statement for the possible role of Plap-1+/Ly6a+ cells (not Plap-1+ alone) as a source pf progenitors for periodontium.
Response: Following your comment, we have added the statement to the main text (p.10, line 31).

Reviewer #3 (Significance (Required)):
by providing new markers and new transgenic animal model, the paper makes an important and significant contribution to the field
Response: Thank you for your critical reading of the manuscript. We sincerely appreciate your comments.

Original submission

First decision letter

MS ID#: DEVELOP/2022/201203

MS TITLE: Plap-1/Aspn lineage tracing and single-cell transcriptomics reveals cellular dynamics in the periodontal ligament

AUTHORS: Tomoaki Iwayama, Mizuho Iwashita, Kazuya Miyashita, Hiromi Sakashita, Shuji Matsumoto, Kiwako Tomita, Phan Bhongsatiern, Tomomi Kitayama, Kentaro Ikegami, Takashi Shimbo, Katsuto Tamai, Masanori A Murayama, Shuhei Ogawa, Yoichiro Iwakura, Satoru Yamada, Lorin Olson, Masahide Takedachi, and Shinya Murakami
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Development through Review Commons.

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

The authors have identified, using state-of-the-art technology, stem cells of the periodontal ligament. Wonderful display of single cell RNA sequencing and superb imaging techniques. For those who are interested in the anatomical and histological build-up of the periodontium, this is a must-read. Whether it can all be translated to the human periodontium, would be an important follow-up step albeit that some suggestions arrize from the literature (Loo-Kirana et al., 2021).

Comments for the author

I would absolutely endorse publication. I have seen this ms previously and to be honest, I think that it has been transferred to Development as a possibility provided by the same publisher, since some of the changes have been highlighted.
As a minor issue: please make sure that the references are OK and in the style mandatory for the journal. I have noticed that the above mentioned Loo-Kirana is with full (first) names included, please turn to surnames and initials. So, Loo-Kirana R., ... de Vries T.J.

Reviewer 2

Advance summary and potential significance to field

This study aims to gain a better understanding of PDLCs and their associated cementum and alveolar bone.

The study provides a very clear results for differential expression of Plap-1 and IBSP the periodontal fibroblast and associated cementoblasts and osteoblasts.

The most infesting is the generation of reporter mice for identification of Plap-1+ cells. The generation of this mice lined allowed then to gain insight to the regeneration of periodontium as well as heterogeneity in of Plap-1+ cells.

Comments for the author

Theses authors have carefully responded to previous concerns. Manuscript has significantly improved