A novel non-disruptive and efficient knock-in allows fate tracing of resident osteoblast progenitors during repair of vertebral lesions in medaka

Wen Hui Tan and Christoph Winkler
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First decision letter

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MS TITLE: A novel non-disruptive and efficient knock-in allows fate tracing of resident osteoblast progenitors during repair of vertebral lesions in medaka

AUTHORS: Wen Hui Tan and Christoph Winkler

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 interest in your work and appreciate the quality of the presented data. From reading the manuscript and the reviews, I agree that the Cre-ERT2 knock-in techniques you describe are important for expanding use of the model system. They also have criticisms and recommend a substantial revision of your manuscript before we can consider publication, in particular 1) this approach is demonstrated with only one locus, and thus it is unclear how reliable it is - an additional example(s) targeting another gene(s) should be demonstrated; and 2) it should be demonstrated how well the targeted allele functions at other stages like adults, or when attempting to label many, most, or all cells in a population. I agree with these concerns, whereas I do not think it is necessary to demonstrate rescue of a knock-out allele (Reviewer 3, comment #4). Although I understand that these considerable, further experiments would take time if you have not already initiated them, we would be happy receive a revised version of the manuscript addressing these and the other reviewer comments. 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.

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.

Reviewer 1

Advance summary and potential significance to field

In the manuscript, Hui and Winkler describe a new knock-in line in which P2A-CreER is integrated downstream the Col10a1 coding sequence. This is performed by homologous recombination in medaka, which is interesting as teleost in general do not display high levels of homologous recombination. Transgenesis and knock-in using non-homologous end-repair is easier and more efficient in medaka and zebrafish. Using the knock-in line, the authors demonstrate clonal analysis of Col10a1 cells and perform live-imaging to beautifully display the recruitment of the cells upon neural arch injury.

Comments for the author

I wish to state that the study performed by the authors is meticulous; however, to this reviewer, it lacks in certain critical area.
Firstly, to this reviewer, it is a bit confusing as to which technique is the real focus for the manuscript. Is the knock-in protocol or the availability of a transgenic line to lineage trace Col10a1-expressing cells. It is not obvious as to what takes the center stage for a 'Techniques paper'. (I did not have access to the Cover Letter to the Editor, and from my reading, the manuscript is equally divided between the two techniques).
If it is the knock-in protocol, to this reviewer, the demonstration for one gene is limited. I must commend the authors on the meticulous way they characterize the impact of C-terminal fusion to Col10a1 and characterize the knock-in. However, the technique is similar to GeneWeld in zebrafish (doi: 10.7554/eLife.53968) or p2BaitD in medaka (doi: 10.1111/dgd.12700). In-fact, using p2BaitD, Murakami et al., introduce Luciferase and GFP downstream the pdia6 gene in medaka.
This reviewer does realize that knock-in of CreER is more challenging and the addition of a reporter cassette in the knock-in construct is well appreciated.
There is no doubt that the protocol and plasmids would be requested by teleost researchers. However, the technique needs to be validated for 3 – 4 genes at-least, if not for 5 – 8.
If on the other hand (as I predict from the title and the keywords, but not from the manuscript format), the novel technology is the Col10a1 CreER line, this reviewer is a bit unsatisfied by the absence of explanation as to why this could not be achieved using the 5.8 kb upstream sequence of Col10a1. This sequence was identified and utilized by the same group (doi: 10.1016/j.ydbio.2013.05.030) in a beautiful study of the Col10a1-expressing cells. Further, the Supplementary video is obtained from this line. It is unclear to me as to exactly what was missing within this regulatory sequence that prompted the generation of a knock-in line.
The authors mention in abstract, "However, a confounding factor is the use of transgenic Cre drivers that do not accurately recapitulate endogenous gene expression or knock-in Cre drivers that alter endogenous protein activity or levels.", and in introduction, "In zebrafish and medaka, several transgenic Cre driver lines have been generated using either the Tol2 transposon system or I-SceI meganuclease (Dasyani et al., 2019; Knopf et al., 2011; Lee et al., 2014).
However, these transgenesis approaches are limited as transgenes are often randomly integrated into the genome, potentially resulting in position effects and ectopic transgene expression (Kondrychyn et al., 2009; Thermes et al., 2002). For accurate cell lineage analyses, Cre driver lines are needed that recapitulate endogenous gene expression patterns with high fidelity."
While this is absolutely true, does it apply to Col10a1? This reviewer is left wondering if the 5.8 kb promoter sequence of Col10a1 driving CreER would not achieve the same goals.
Further, the knock-in line was not shown by the author to achieve dramatic level of recombination. This data is missing, in my opinion. It would be worthwhile to show the maximum possible Cre-based recombination that can be achieved. The authors beautifully show single-cell labeling, but is the CreER expression strong enough to label all the Col10a1-positive cells? Fig. 5 F-G and Fig. 6 A is with Col10a1:GFP line. It would be important to provide Cre labeling outcomes that match the

extent of Col10a1 expression cell population. Lack of this might suggest low level expression of CreER, which is normal from knock-ins. Transgenes do drive expression that is many-fold higher than what normal genes do, but there is a reason for “over-expression” of fluorescent proteins and Cre. To this reviewer, it is critical to demonstrate the recombination capacity of the knock-in line. Lastly, the utilization of knock-in Col10a1:CreER to uncover new biology, even to scratch the surface, is not convincingly demonstrated. The recruitment of Col10a1 osteoblast progenitors after bone lesions has been convincingly demonstrated by the authors in previous work. The live imaging can be achieved by Col10a1:GFP line. It would be of interest to demonstrate new biological questions that the newly generated line can answer (even if definitive answers are not provided). For instance, the clonal capacity of individual osteoblast progenitors could be of interest. Overall, the authors have generated a novel tool; however, the utility of the tool to the general research community needs further validation, particularly with respect to the wide readership of Development. Thus, this reviewer cannot suggest publication in the current format.

Reviewer 2

Advance summary and potential significance to field

A manuscript “A novel non-disruptive and efficient knock-in allows fate tracing of resident osteoblast progenitors during repair of vertebral lesions in medaka” described the methods for establishing the CreRET2 drive line with novel CRISPR/Cas9 mediated homology-directed knock-in approach. This method can allow us to tag the CreERT2 and the endogenous protein via P2A peptide. After translation of the CreERT2 tagged protein, P2A peptide cleaved in vivo and de novo protein function is still maintained, thus this approach can allow us to establish the creERT2 driver lines which precisely expressed CreERT2 protein by the de novo promoters and enhancers without disruption of endogenous protein functions. Many people think this approach is possible, but no one has been established such Cre driver lines until now. The method described in this manuscript can be apply to other protein loci. I think this method could open new window for precise lineage tracing of cells in several biological phenomenon and also applicable to the tissue specific gene KO with very precise spatio-temporal manner. Authors established the col10a1^p2a-CreERT2 knock-in medaka using this method and showed that tamoxifen-inducible CreERT2 efficiently recombined loxP sites specifically in col10a1 cells. They also observed that labelled cells (col10a1 expressing cells) at neural arches migrated to the bone degradation sites and re-mineralize and repair them. I have no major comments on this manuscript but have some minor suggestions to improve this manuscript.

Comments for the author

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1. Confirmation of co-expression between col10a1 and CreERT2 genes were based on mRNA in situ hybridization. As for the Cre driver lines, Cre protein synthesis is important. I ask authors to show the results of immuno-staining of Cre protein. Is it same with mRNA expression? Protein expression is sometimes different from mRNA expression.

2. Have you tried to make the medaka line with col10a1 tagged by florescence proteins? If yes, is it possible to visualize the col10a1 expression cells?
3. Have you tried to use other self-cleaving peptide such as T2A. P2A is working for medaka. It is good. I am wondering another self-cleaving peptide.
Because the cleaving efficiencies of self-cleavage peptides are different among species. T2A is mainly used for mouse system. P2A is mainly used for medaka and zebrafish. If T2A is also working in medaka, we can have alternative method.
4. As for the KI experiments, A position of double strand breaks by CRISPR/Cas9 and insertion site of donor DNA fragment is important factor of donor DNA integration efficiency. I suggest to show this information with nucleotide sequence level in Figure 1.
5. A knock-in donor plasmid described in this manuscript can be applicable for establishing other CreERT2 driver lines. Availability this donor plasmid should described in the text. I prefer to deposit this plasmid to addgene.

Reviewer 3

Advance summary and potential significance to field

In this study, the authors present a method to improve efficiency of Crispr/Cas9 mediated knock-in via Homology Directed Repair in medaka, which was used to trace col10a1+ osteoblasts in a model of RANKL-induced osteoporotic bone lesion. The authors suggest to knock-in CreERT2 near the stop codon of coding sequences of a gene, and to include a cassette which allows for screening of F0-injected embryos by looking at the developing heart. They then present data on a CreERT2-knock-in allele of col10a1, which does not interfere with gene function, opposite to previously published knock-in methods in teleost fish. The approach presented is very useful (significant) for researchers working with the teleost animal model (zebrafish, medaka). Aside of the methodological approach, the authors show that genetically labeled col10a1+ migrate to bone lesions in order to support remineralization of damaged bone areas. These data are of high interest to researchers working on the pathogenesis of bone fragility.

Comments for the author

The study is well designed, the shown data are convincing and the manuscript is well written. I would suggest the following experiments to complete the picture regarding the statements of the manuscript:
1) The authors claim that efficiency of the knock-in is increased by their approach. This is misleading, because very likely, not the knock-in rate is increased, but - by including a heart marker for screening in the F0 - chances to identify founders are increased. This should be clarified.
2) The authors present the approach to knock-in a sequence close to the stop-codon as a valuable method. It would be useful to present F0- data on a second example of a knock-in of CreERT2 (i.e. that the method works in another locus as well - the respective gene may be relevant in the bone field or not). F1 data and beyond would not be required.
3) The authors present convincing data on transgene and endogenous gene expression in Figure 3, however only up to 9 dpf. Does CreERT2 expression recapitulate also adult col10a1 gene expression and does it lead to the same fate mapping results in fin regeneration as the authors' previously published col10a1:CreERT2 (classic transgenesis) line? It should be demonstrated that the knock-in is functional at the adult stage.
4) In Figure 4, the authors show that CreERT2 knock-in including knock-in of a reporter does not compromise viability and leaves bone and cartilage formation unaffected. In order to demonstrate the functionality of the knock-in allele I suggest to rescue the col10a1 mutant with the knock-in allele.
5) Recombination events in 4-HT treated larvae occurred in half of the treated embryos. Is this because the other half of the embryos did not carry the CreERT2 transgene? Although the idea of labeling only very few cells makes sense for tracing these individual cells in osteoporotic lesion assays, the authors should present data that would show a higher recombination rate (more cells per embryo, more embryos carrying the transgene). This would be necessary in future settings, in which recombination would be required to overexpress genes that change the col10a1+ osteoblasts phenotype (opposite to lineage tracing studies).

6) The authors argue that RANKL induction generates a chemokine gradient that leads to (directed?) migration of osteoblasts to lesioned bone areas. Is it possible that RANKL induction upregulates osteoblast migration per se? This could potentially be tested by looking at osteoblast migratory tracks at earlier time points post RANKL induction. Also, while the authors present migration tracks for osteoblasts without RANKL overexpression in the Supplement (these are more or less stationary), I am not aware that they have shown migratory tracks for osteoblasts after RANKL induction - is the migration directed or random? This should be tested (also with the help of higher recombination rates, see point 5) and discussed accordingly.

Minor points:
6) The homology arms that were used in the bait are rather short - does the length (according to the authors experience) impact the approach? The knock-in region including the cmlc2 reporter is very long, which is why I think too short homology arm sequences might be problematic.
7) Instead of 'C-terminal' (e.g. line 164), it should be either 'C-terminal end' or 'C-terminus'.
8) Figure 1. Please show the gel for genotyping with FP1 and FP2 - the band sizes would be very different in size depending on whether knock-in occurred, i.e. the band size after successful knock-in would be more than 5kb. How does the PCR approach work here (in terms of elongation time)?
9) Figure 2 (and legend): It is sufficient to name the concentrations of donor plasmid, Cas9 protein etc. in the Materials & Methods section.

First revision

Author response to reviewers' comments

Editor's comments:
As you will see, the referees express interest in your work and appreciate the quality of the presented data. From reading the manuscript and the reviews, I agree that the Cre-ERT2 knock-in techniques you describe are important for expanding use of the model system. They also have criticisms and recommend a substantial revision of your manuscript before we can consider publication, in particular 1) this approach is demonstrated with only one locus, and thus it is unclear how reliable it is - an additional example(s) targeting another gene(s) should be demonstrated; and 2) it should be demonstrated how well the targeted allele functions at other stages like adults, or when attempting to label many, most, or all cells in a population. I agree with these concerns, whereas I do not think it is necessary to demonstrate rescue of a knock-out allele (Reviewer 3, comment #4). Although I understand that these considerable, further experiments would take time if you have not already initiated them, we would be happy receive a revised version of the manuscript addressing these and the other reviewer comments. 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.

Author's response: We have now targeted two additional loci (*osr1*, *col2a1a*) to demonstrate reliability and efficacy of the method. Furthermore, we have labelled cells in the adult fin and used experimental conditions to label a majority of Cre-expressing cells as requested. These new data are now shown in revised Fig. 5 and Supplementary Figs. S2 and S3.

Comment Reviewer 1: Advance Summary and Potential Significance to Field: In the manuscript, Hui and Winkler describe a new knock-in line in which P2A-CreER is integrated downstream the Col10a1 coding sequence. This is performed by homologous recombination in medaka, which is interesting as teleost in general do not display high levels of homologous recombination. Transgenesis and knock-in using non-homologous end-repair is easier and more efficient in medaka and zebrafish. Using the knock-in line, the authors demonstrate clonal analysis of Col10a1 cells and perform live-imaging to beautifully display the recruitment of the cells upon neural arch injury.

I wish to state that the study performed by the authors is meticulous; however, to this reviewer, it lacks in certain critical area.

Author's response: We thank the reviewer for the encouraging comments. We provided additional data in the revised manuscript in order to address the reviewer's concerns.

Comment Reviewer 1: Firstly, to this reviewer, it is a bit confusing as to which technique is the real focus for the manuscript. Is the knock-in protocol or the availability of a transgenic line to lineage trace Col10a1-expressing cells. It is not obvious as to what takes the center stage for a 'Techniques paper'. (I did not have access to the Cover Letter to the Editor, and from my reading, the manuscript is equally divided between the two techniques).

If it is the knock-in protocol, to this reviewer, the demonstration for one gene is limited. I must commend the authors on the meticulous way they characterize the impact of C-terminal fusion to Col10a1 and characterize the knock-in. However, the technique is similar to GeneWeld in zebrafish (doi: 10.7554/eLife.53968) or p2BaitD in medaka (doi: 10.1111/dgd.12700). In-fact, using p2BaitD, Murakami et al., introduce Luciferase and GFP downstream the pdia6 gene in medaka.

This reviewer does realize that knock-in of CreER is more challenging and the addition of a reporter cassette in the knock-in construct is well appreciated. There is no doubt that the protocol and plasmids would be requested by teleost researchers. However, the technique needs to be validated for 3 – 4 genes at- least, if not for 5 – 8.

Author's response: As mentioned in the response below, the main focus of our manuscript is the novel Col10a1 CreER knock-in line to lineage trace osteoblast progenitors. However, to address the reviewer's concerns about efficacy and reliability of the method, we have now also validated our knock-in approach for two other genes (*osr1*, *col2a1a*) in the F0 generation and included the data in new Supplementary Figs. S2 and S3. Similar efficiencies were obtained suggesting that this method can also be used for tagging other medaka proteins with CreER.

Comment Reviewer 1: If on the other hand (as I predict from the title and the keywords, but not from the manuscript format), the novel technology is the Col10a1 CreER line, this reviewer is a bit unsatisfied by the absence of explanation as to why this could not be achieved using the 5.8 kb upstream sequence of Col10a1. This sequence was identified and utilized by the same group (doi: 10.1016/j.ydbio.2013.05.030) in a beautiful study of the Col10a1-expressing cells. Further, the Supplementary video is obtained from this line. It is unclear to me as to exactly what was missing within this regulatory sequence that prompted the generation of a knock-in line.

Author's response: As the reviewer correctly suggests, the main focus in this manuscript is the Col10a1 CreER line. We have previously generated a *col10a1*:CreERT2-p2a-mCherry transgenic line using a 5.8 kb upstream regulatory sequence of Col10a1. However, this transgene is ectopically expressed in spinal cord neurons (new Supplementary Fig. S4) suggesting that the chosen promoter sequence is not complete or that the genomic integration site affected its regulation. Therefore, this transgenic line is not suitable for lineage tracing of *col10a1* osteoblast progenitors in the vertebral column. We have now included the transgenic findings in the manuscript and changes the text accordingly.

Comment Reviewer 1: The authors mention in abstract, "However, a confounding factor is the use of transgenic Cre drivers that do not accurately recapitulate endogenous gene expression or knock-in Cre drivers that alter endogenous protein activity or levels.", and in introduction, "In zebrafish and medaka, several transgenic Cre driver lines have been generated using either the Tol2 transposon system or I-SceI meganuclease (Dasyani et al., 2019; Knopf et al., 2011; Lee et al., 2014). However, these transgenesis approaches are limited as transgenes are often randomly integrated into the genome, potentially resulting in position effects and ectopic transgene expression (Kondrychyn et al., 2009; Thermes et al., 2002). For accurate cell lineage analyses, Cre driver lines are needed that recapitulate endogenous gene expression patterns with high fidelity."
While this is absolutely true, does it apply to Col10a1? This reviewer is left wondering if the 5.8 kb promoter sequence of Col10a1 driving CreER would not achieve the same goals.

Author's response: As mentioned above, for the *col10a1*:CreERT2-p2a-mCherry transgenic line, ectopic transgene expression was observed in spinal cord neurons (new Supplementary Fig. S4). The Col10a1 Cre knock-in line, on the other hand, faithfully recapitulates endogenous *col10a1* expression in the vertebral column (in Fig. 3A-B''' and new Supplementary Fig. S4) and is therefore a useful tool for accurate lineage tracing of *col10a1* cells in the trunk at larval stages.

Comment Reviewer 1: Further, the knock-in line was not shown by the author to achieve dramatic level of recombination. This data is missing, in my opinion. It would be worthwhile to show the maximum possible Cre-based recombination that can be achieved. The authors beautifully show single-cell labeling, but is the CreER expression strong enough to label all the Col10a1-positive cells? Fig. 5 F-G and Fig. 6 A is with Col10a1:GFP line. It would be important to provide Cre labeling outcomes that match the extent of Col10a1 expression cell population. Lack of this might suggest low level expression of CreER, which is normal from knock-ins. Transgenes do drive expression that is many-fold higher than what normal genes do, but there is a reason for "over-expression" of fluorescent proteins and Cre. To this reviewer, it is critical to demonstrate the recombination capacity of the knock-in line.

Author's response: As requested by the reviewer, we have increased the 4-HT treatment duration from 6 to 24 hours to induce higher rates of Cre-based recombination, now shown in new Fig. 5H-K. Under these conditions, Cre/*loxP* recombination was observed in 20 out of analysed 20 larvae (100%) and an average of 57.4% *col10a1* cells were labelled in the larval vertebral column (new Fig. 5H-K).

Comment Reviewer 1: Lastly, the utilization of knock-in Col10a1:CreER to uncover new biology, even to scratch the surface, is not convincingly demonstrated. The recruitment of Col10a1 osteoblast progenitors after bone lesions has been convincingly demonstrated by the authors in previous work. The live imaging can be achieved by Col10a1:GFP line. It would be of interest to demonstrate new biological questions that the newly generated line can answer (even if definitive answers are not provided). For instance, the clonal capacity of individual osteoblast progenitors could be of interest.

Author's response: While we previously reported accumulation of *col10a1* osteoblast progenitors at bone lesion sites, the origin of these cells from where they were recruited from remained unknown. To address that question, live time-lapse imaging could be used. However, there is a limit to the maximum imaging time window in time-lapse imaging as after 12 hours, photobleaching occurs and the survival of larvae is compromised. Consequently, with time-lapse imaging, we were not able to detect any recruitment of *col10a1* cells from neural arches to bone lesions (new Supplementary Fig. 8B). On the other hand, the knock-in Col10a1:CreER line proved as very useful as it allowed to track movement of distinct *col10a1* cells over longer time periods (i.e. 48 hours, Fig. 6D-E''), which could not have been achieved with live time-lapse imaging. We have now discussed this in more detail in the manuscript.

Comment Reviewer 1: Overall, the authors have generated a novel tool; however, the utility of the tool to the general research community needs further validation, particularly with respect to the wide readership of Development. Thus, this reviewer cannot suggest publication in the current format.

Author's response: With the substantial revisions made to the manuscript, we hope that we could convince the reviewer that our protocol is indeed useful for a wider community interested in efficient teleost knock-in and precise cell lineage tracing.

Comment Reviewer 2: Advance Summary and Potential Significance to Field: A manuscript "A novel non-disruptive and efficient knock-in allows fate tracing of resident osteoblast progenitors during repair of vertebral lesions in medaka" described the methods for establishing the CreRET2 drive line with novel CRISPR/Cas9 mediated homology-directed knock-in approach. This method can allow us to tag the CreERT2 and the endogenous protein via P2A peptide. After translation of the CreERT2 tagged protein, P2A peptide cleaved in vivo and de novo protein function is still maintained, thus this approach can allow us to establish the creERT2 driver lines which precisely expressed CreERT2 protein by the de novo promoters and enhancers without disruption of endogenous

protein functions. Many people think this approach is possible, but no one has been established such Cre driver lines until now. The method described in this manuscript can be apply to other protein loci. I think this method could open new window for precise lineage tracing of cells in several biological phenomenon and also applicable to the tissue specific gene KO with very precise spatio- temporal manner. Authors established the col10a1^p2a-CreERT2 knock-in medaka using this method and showed that tamoxifen-inducible CreERT2 efficiently recombined loxP sites specifically in col10a1 cells. They also observed that labelled cells (col10a1 expressing cells) at neural arches migrated to the bone degradation sites and re-mineralize and repair them. I have no major comments on this manuscript but have some minor suggestions to improve this manuscript.

Author's response: We thank the reviewer for the constructive comments and insightful suggestions.

Comment Reviewer 2: 1. Confirmation of co-expression between col10a1 and CreERT2 genes were based on mRNA in situ hybridization. As for the Cre driver lines, Cre protein synthesis is important. I ask authors to show the results of immuno-staining of Cre protein. Is it same with mRNA expression? Protein expression is sometimes different from mRNA expression.

Author's response: As requested by the reviewer, we performed immunostaining of Cre and demonstrate that Cre protein is expressed in *col10a1*-expressing cells (new Fig. 3C-D').

Comment Reviewer 2: 2. Have you tried to make the medaka line with col10a1 tagged by florescence proteins? If yes, is it possible to visualize the col10a1 expression cells?

Author's response: We have not tried making such a line. However, there is no particular reason why our protocol cannot be used to express a functional fluorescent protein in a similar manner as CreER shown in the present study.

Comment Reviewer 2: 3. Have you tried to use other self-cleaving peptide such as T2A. P2A is working for medaka. It is good. I am wondering another self-cleaving peptide. Because the cleaving efficiencies of self-cleavage peptides are different among species. T2A is mainly used for mouse system. P2A is mainly used for medaka and zebrafish. If T2A is also working in medaka, we can have alternative method.

Author's response: We have not tried T2A. We tried E2A and P2A, and found that P2A works more efficiently than E2A in medaka.

Comment Reviewer 2: 4. As for the KI experiments, A position of double strand breaks by CRISPR/Cas9 and insertion site of donor DNA fragment is important factor of donor DNA integration efficiency. I suggest to show this information with nucleotide sequence level in Figure 1.

Author's response: We agree with the reviewer and have included this information in the new Fig. 1.

Comment Reviewer 2: 5. A knock-in donor plasmid described in this manuscript can be applicable for establishing other CreERT2 driver lines. Availability this donor plasmid should described in the text. I prefer to deposit this plasmid to addgene.

Author's response: As suggested, we have deposited our plasmids to Addgene and included the Addgene IDs in the text.

Comment Reviewer 3: Advance Summary and Potential Significance to Field: In this study, the authors present a method to improve efficiency of Crispr/Cas9 mediated knock-in via Homology Directed Repair in medaka, which was used to trace col10a1+ osteoblasts in a model of RANKL-induced osteoporotic bone lesion. The authors suggest to knock-in CreERT2 near the stop codon of coding sequences of a gene, and to include a cassette which allows for screening of F0-injected embryos by looking at the developing heart. They then present data on a CreERT2-knock-in allele of col10a1, which does not interfere with gene function, opposite to previously published knock-in

methods in teleost fish. The approach presented is very useful (significant) for researchers working with the teleost animal model (zebrafish, medaka). Aside of the methodological approach, the authors show that genetically labeled col10a1+ migrate to bone lesions in order to support remineralization of damaged bone areas. These data are of high interest to researchers working on the pathogenesis of bone fragility.

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Comment Reviewer 3: 2) The authors present the approach to knock-in a sequence close to the stop-codon as a valuable method. It would be useful to present F0- data on a second example of a knock-in of CreERT2 (i.e. that the method works in another locus as well - the respective gene may be relevant in the bone field or not). F1 data and beyond would not be required.

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Comment Reviewer 3: 3) The authors present convincing data on transgene and endogenous gene expression in Figure 3, however only up to 9 dpf. Does CreERT2 expression recapitulate also adult col10a1 gene expression and does it lead to the same fate mapping results in fin regeneration as the authors' previously published col10a1:CreERT2 (classic transgenesis) line? It should be demonstrated that the knock-in is functional at the adult stage.

Author's response: As requested, we have now performed experiments at adult stages. In new Fig. 5L, we demonstrate that CreERT2 in the *col10a1* Cre knock-in line is functional at adult stages in the fin. Similar to our earlier findings in a *col10a1* Cre transgenic line, the *col10a1* Cre knock-in line was able to specifically label joint cells and osteoblasts in the adult fin, as well as mark cells which contribute as a cellular source to the regenerating fin blastema (new Fig. 5L, M).

Comment Reviewer 3: 4) In Figure 4, the authors show that CreERT2 knock-in including knock-in of a reporter does not compromise viability and leaves bone and cartilage formation unaffected. In order to demonstrate the functionality of the knock-in allele I suggest to rescue the col10a1 mutant with the knock-in allele.

Author's response: Both CreERT2 knock-in and the mutation in the *col10a1* mutant are at the same position in the endogenous *col10a1* locus. Hence, fish with both knock-in and mutant alleles can only be heterozygous for both alleles. As the mutant phenotype in the *col10a1* mutant results from a dominant negative mechanism, the knock-in allele would not rescue the mutant phenotype.

Comment Reviewer 3: 5) Recombination events in 4-HT treated larvae occurred in half of the treated embryos. Is this because the other half of the embryos did not carry the CreERT2 transgene? Although the idea of labeling only very few cells makes sense for tracing these individual cells in osteoporotic lesion assays, the authors should present data that would show a higher recombination rate (more cells per embryo, more embryos carrying the transgene). This would be necessary in future settings, in which recombination would be required to overexpress genes that change the col10a1+ osteoblasts phenotype (opposite to lineage tracing studies).

Author's response: We agree with the reviewer and now show that higher rates of recombination can be achieved by increasing the duration of 4-HT treatment from 6 hours to 24 hours (new Fig. 5H). Under these conditions, we observed recombination events in 20 out of 20 larvae (100%) and on

average in 57.4% of *col10a1* cells in the vertebral column (new Fig. 5H-K).

Comment Reviewer 3: 6) The authors argue that RANKL induction generates a chemokine gradient that leads to (directed?) migration of osteoblasts to lesioned bone areas. Is it possible that RANKL induction upregulates osteoblast migration per se? This could potentially be tested by looking at osteoblast migratory tracks at earlier time points post RANKL induction. Also, while the authors present migration tracks for osteoblasts without RANKL overexpression in the Supplement (these are more or less stationary), I am not aware that they have shown migratory tracks for osteoblasts after RANKL induction - is the migration directed or random? This should be tested (also with the help of higher recombination rates, see point 5) and discussed accordingly.

Author's response: As requested, migratory tracks for *col10a1* cells after Rankl induction have been added in the new Supplementary Fig. S8B. While *col10a1* cells at the tip of neural arches migrate exclusively dorsally in the absence of Rankl induction (new Supplementary Fig. S8A, red arrows), some *col10a1* cells at the tip of neural arches switched migration pattern and moved ventrally after Rankl induction, towards the vertebral bodies where bone lesions are present.
Also, we want to clarify that the mentioned Rankl-induced chemokine gradient triggers recruitment of osteoclasts (not osteoblasts) to bone matrix. The corresponding chemokine receptor Cxcr3.2 is expressed in osteoclast but not osteoblast progenitors (Phan et al., 2021). Therefore, osteoblast recruitment after Rankl-induction likely occurs indirectly. We think that osteoporotic bone lesions caused by excessive activity of recruited osteoclasts are the trigger for recruitment of osteoblasts to repair lesions, but the underlying molecular mechanisms remain unknown.

Comment Reviewer 3: Minor points:
6) The homology arms that were used in the bait are rather short - does the length (according to the authors experience) impact the approach? The knock-in region including the cmlc2 reporter is very long, which is why I think too short homology arm sequences might be problematic.

Author's response: We have not tried homology arms of other length for knock-in at the *col10a1* locus. However, for knock-in at *osr1* and *col2a1a* loci as shown in new Supplementary Fig. S2 and S3, we used homology arms of similar lengths and obtained knock-ins at the F0 generation with similar efficiencies as with *col10a1*. Hence, we believe that the lengths of homology arms we used, ranging from 198 bp to 487 bp, are sufficient for efficient knock-in.

Comment Reviewer 3: 7) Instead of 'C-terminal' (e.g. line 164), it should be either 'C-terminal end' or 'C-terminus'.

Author's response: 'C-terminal' has been changed to 'C-terminal end'.

Comment Reviewer 3: 8) Figure 1. Please show the gel for genotyping with FP1 and FP2 - the band sizes would be very different in size depending on whether knock-in occurred, i.e. the band size after successful knock-in would be more than 5kb. How does the PCR approach work here (in terms of elongation time)?

Author's response: For genotyping of heterozygous and homozygous carriers, we performed multiplex PCR with three primers as shown in Fig. 1 (FP1, RP1 and RP2). FP1 and RP2 will amplify only the wild-type band (596 bp) but not the knock-in band (i.e. the 5 kb band). Hence, we used an elongation time of 40 seconds, using a Taq polymerase that elongates 1 kb/min. Under these same conditions, the knock-in insert is amplified with primers FP1 and RP1 present in the same mix. We have added the genotyping gel image in new Supplementary Fig. S6.

Comment Reviewer 3: 9) Figure 2 (and legend): It is sufficient to name the concentrations of donor plasmid, Cas9 protein etc. in the Materials & Methods section.

Author's response: Figure 2 and the legend were edited as suggested.

Second decision letter

MS ID#: DEVELOP/2021/200238

MS TITLE: A novel non-disruptive and efficient knock-in allows fate tracing of resident osteoblast progenitors during repair of vertebral lesions in medaka

AUTHORS: Wen Hui Tan and Christoph Winkler
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. I ask that you consider and incorporate the minor additional points by Reviewer #1 intended to improve the manuscript, appended below.

Reviewer 1

Advance summary and potential significance to field

Hui and Winkler have developed a method for knock-in of CreER into medaka locus.
Using this technique they generated a new line for lineage tracing Col10a1 cells.

In the revised manuscipt, I commend the authors for addressing all the concerns raised by Reviewer 1. The technique is important as CreER represents a rather large insertion (~3 kb), which is not yet a standard in the teleost field. The authors perform the knock-in downstream of the gene, thereby avoiding any harmful impact of the loss of gene function. In the revision, the authors show that the knock-in recaptulates endogenous expression better than a short promoter-driven expressoin. Further, the authors show that the Col10a1 CreER knock-in is capable of inducing recombination in a large population of Col10a1-expressing cells.

Finally, the authors show promising, though preliminary data (See Minor Point 1), that the technique works for multiple genomic loci.

Comments for the author

Minor Points:
1. If possible, it would be beneficial to include the percentage of fish that transmit a functional CreER knock-in for osr1 and col2a2. The F0 data presented is very promising, but it would be informational for the readers to have a knowledge of germline transmission rates.
2. Personally, I feel the comparison between the knock-in and promoter-driven line is very important. The expression of promoter-driven transgene in the spinal cord is critical factor to push for a knock-in. I would consider making Supplementary Fig. S4 a part of main figure (Fig. 3, maybe). I feel this aspect should be highlighted.
3. As this is a technique article, I would suggest the authors to deposit the mbait plasmid along with a detailed protocol to Addgene. I could not find a 'Data Availability' section outlining how this would be accomplished. I consider it a neccessity for publication to have the data uploaded to an publicly accessible domain.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript, "A novel non-disruptive and efficient knock-in allows fate tracing of resident osteoblast progenitors during spinal repair in medaka" by Tan Wen Hui and Christoph Winkler discussed establishing the Cre drive line with a novel CRISPR/Cas9 mediated homology-directed knock-in approach using col10a1 gene and the results applied to the other two loci, osr1 and col2a2. They achieved similar knock-in efficiencies for osr1 and col2a2. Based on these data, the method described in this manuscript can be applied to other loci as well.

Furthermore, authors have shown that col10a and CreERT2 have the same protein expression. It is an important result for establishing Cre driver lines using fusion proteins.

Comments for the author

Authors appropriately answered to most of my comments. I have no more comments to this manuscript and this manuscript is an acceptable as is.

Reviewer 3

Advance summary and potential significance to field

In this study, the authors present a method to improve efficiency of Crispr/Cas9 mediated knock-in via Homology Directed Repair in medaka, which was used to trace col10a1+ osteoblasts in a model of RANKL-induced osteoporotic bone lesion. The authors suggest to knock-in CreERT2 near the stop codon of coding sequences of a gene, and to include a cassette which allows for screening of F0-injected embryos by looking at the developing heart. They then present data on a CreERT2-knock-in allele of col10a1, which does not interfere with gene function, opposite to previously published knock-in methods in teleost fish. The approach presented is very useful (significant) for researchers working with the teleost animal model (zebrafish, medaka). Aside of the methodological approach, the authors show that genetically labeled col10a1+ migrate to bone lesions in order to support remineralization of damaged bone areas. These data are of high interest to researchers working on the pathogenesis of bone fragility.

Comments for the author

Thank you very much for addressing my review comments adequately. I have no further comments, congratulations on this work!