Placental gene editing via trophectoderm-specific Tat-Cre/loxP recombination

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AUTHORS: Hatice O. Ozguldez and Ivan Bedzhov

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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.

Reviewer 1

Advance summary and potential significance to field

This manuscript by Ozguldez and Bedzhov is a technical report describing the use of Tat-Cre on mouse blastocysts for TE-specific gene deletion. The tissue-specific restriction of Cre depends upon the single-layered epithelial property of the TE, a property that has been widely exploited for many years for immunosurgery, by which the TE layer can be lysed and eliminated, leaving an intact ICM. The idea presented here is simple and elegant and will enable production of mouse models in which any gene of interest can be deleted only in the placental lineage. Since immunosurgery is also used

successfully in blastocysts of other mammals, including human Tat-Cre gene deletion may be used to study the process of implantation/placentation, in vivo or ex vivo. This system neatly overcomes the problem that, to date, no gene has been identified with expression restricted to, but ubiquitous to the entire embryo-derived placental lineage whose promoter could be used to drive lineage-specific gene deletion. The authors used embryos from mice harbouring ubiquitously expressed mT/mG, in which mT is flanked by LoxP sites. When these are recombined, tomato fluorescence is lost and replaced by green expressed from the previously silent mG. The authors titrated the concentration of Cre to achieve ubiquitous GFP expression in the TE. They tested the tissue-specific extent of recombination by deriving and expanding stem cell lines from both TE and ICM. More importantly, optimally treated embryos were transferred to recipients and dissected at various post-implantation stages. In addition to its simplicity, this method removes the risk of leaky Cre the potential requirement for injection of pregnant dams with tamoxifen to induce recombination and the need for breeding the Cre into mice carrying the floxed gene of interest. Thus, this method represents a significant advance in refinement and reduction of animal use.

Comments for the author

1. A table or statement should be provided dividing the total number of embryos (35) into those allocated to each treatment during the titration of Tat-Cre (Fig. 1D).
2. The embryos used to generate the data in Fig. 1D should be analysed in more detail. The total cell numbers and their allocation to TE or ICM should be presented for embryos in all treatments.
This is important to determine at which concentration the Tat-Cre may become toxic.
3. How many ESC and TSC lines were derived and tested for fluorescent label purity from each of the treatments used?
4. Please provide a statement or table documenting the number Tat-Cre treated embryos transferred to recipients, how many were recovered and how many were normal (Fig. 2C and D)? It is not sufficient just to state that all were normal.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Ozguldez and Bedzhov test the ability of cell permeable Tat-Cre to induce recombination of floxed alleles within the trophectoderm of blastocysts. The significance of this work is that this method provides a new way to study gene function within the trophectoderm lineage – a critical, but understudied tissue. The results are convincing and nicely presented, so I recommend for publication as a technical report with only minor suggestions:

Comments for the author

1. The authors should emphasize how their approach provides advantages over other methods for achieving deletion within the trophectoderm lineage. For example, tetraploid complementation affects all extraembryonic tissues, and not just the trophectoderm and lentiviral delivery presents undesirable safety challenges.
2. In describing the derivation of ESC and TSC lines, this would be more convincing if the authors could provide evidence that Tat-Cre is not lethal to ESC lines.
3. In Fig. 1E, the authors should more clearly label the TSC experiments – presumably one column was treated and the other was untreated. But the authors have labeled each row as if the entire row were treated.
4. The citations in paragraph 1 of the Results section are not adequate or accurate. Primary literature rather than a review, should be cited.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

This manuscript by Ozguldez and Bedzhov is a technical report describing the use of Tat-Cre on mouse blastocysts for TE-specific gene deletion. The tissue-specific restriction of Cre depends upon the single-layered epithelial property of the TE, a property that has been widely exploited for many years for immunosurgery, by which the TE layer can be lysed and eliminated, leaving an intact ICM. The idea presented here is simple and elegant and will enable production of mouse models in which any gene of interest can be deleted only in the placental lineage. Since immunosurgery is also used successfully in blastocysts of other mammals, including human, Tat-Cre gene deletion may be used to study the process of implantation/placentation, in vivo or ex vivo. This system neatly overcomes the problem that, to date, no gene has been identified with expression restricted to, but ubiquitous to the entire embryo-derived placental lineage whose promoter could be used to drive lineage-specific gene deletion. The authors used embryos from mice harbouring ubiquitously expressed mT/mG, in which mT is flanked by LoxP sites. When these are recombined, tomato fluorescence is lost and replaced by green, expressed from the previously silent mG. The authors titrated the concentration of Cre to achieve ubiquitous GFP expression in the TE. They tested the tissue-specific extent of recombination by deriving and expanding stem cell lines from both TE and ICM. More importantly, optimally treated embryos were transferred to recipients and dissected at various post-implantation stages. In addition to its simplicity, this method removes the risk of leaky Cre, the potential requirement for injection of pregnant dams with tamoxifen to induce recombination and the need for breeding the Cre into mice carrying the floxed gene of interest. Thus, this method represents a significant advance in refinement and reduction of animal use.

We thank the reviewer for his/her positive comments. We are grateful for his/her suggestions that helped to improve the manuscript. We have addressed all his/her comments in full below.

Reviewer 1 Comments for the Author:

1. A table or statement should be provided dividing the total number of embryos (35) into those allocated to each treatment during the titration of Tat-Cre (Fig. 1D).

The statement distributing the total 35 embryos into groups - *control (n=5), 0.75 µM Tat-Cre (n=10), 1.5 µM Tat-Cre (n=10) and 3 µM Tat-Cre (n=10),* is provided in the figure legend.

2. The embryos used to generate the data in Fig. 1D should be analysed in more detail. The total cell numbers and their allocation to TE or ICM should be presented for embryos in all treatments. This is important to determine at which concentration the Tat-Cre may become toxic.

Following the Reviewer's request, we examined the number of TE and ICM cells to analyse whether the Tat-Cre doses required for the efficient mT/mG recombination are toxic, compared to control (untreated) embryos. The new data is presented as a graph in Figure S1A and the number of embryos used (5 blastocysts per treatment) is stated in the figure legend. We found that "*the number of TE and ICM cells in the 1.5 µM and 3 µM Tat-Cre treated blastocysts was similar to the control embryos (Figure S1A)*", which is stated in the Results section of the main text.

3. How many ESC and TSC lines were derived and tested for fluorescent label purity from each of the treatments used?

We set an arbitrary number of 5 lines (ESC n=5 and TSC n=5) for each dose (0.75 µM, 1.5 µM and 3 µM) of Tat-Cre treatment, which we considered sufficient to examine the inheritance of the mT/mG allele in stem cells derivation. These numbers are now indicated in the figure legend. As shown in the figure and stated in the text, we did not observed leakiness into the ICM, based on the mT expression in the ESC, and blastocyst treatment with 1.5 µM or 3 µM Tat-Cre resulted in the derivation of homogenous mG-expressing TSC, indicating successful recombination in the TE.

4. Please provide a statement or table documenting the number Tat-Cre treated embryos

transferred to recipients, how many were recovered and how many were normal (Fig. 2C and D)? It is not sufficient just to state that all were normal.

This is a very important point that Reviewer 1 raises. It is essential to mention that embryo transfers (at least in our hands) never work with 100% efficiency. So, to be objective, we compared the percentage of post-implantation embryos recovered after control (untreated) and 1.5 μM Tat-Cre treated blastocysts were transferred into recipient mothers. We transferred 42 control and 38 Tat-Cre treated embryos and we found similar rate of embryo recovery after implantation. This data is represented in Figure S1B. Compared to controls (based on the morphology), we did not observe abnormalities in the Tat-Cre embryos. The representative images are placed in Figures 2B, 2C and 2D.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this manuscript, Ozguldez and Bedzhov test the ability of cell permeable Tat-Cre to induce recombination of floxed alleles within the trophectoderm of blastocysts. The significance of this work is that this method provides a new way to study gene function within the trophectoderm lineage – a critical, but understudied tissue. The results are convincing and nicely presented, so I recommend for publication as a technical report with only minor suggestions:

We are pleased that the reviewer considers that our work addresses a significant technical aspect in the field and we are grateful for his / her constructive suggestions.

Reviewer 2 Comments for the Author:

1. The authors should emphasize how their approach provides advantages over other methods for achieving deletion within the trophectoderm lineage. For example, tetraploid complementation affects all extraembryonic tissues, and not just the trophectoderm and lentiviral delivery presents undesirable safety challenges.

Following Reviewer 2 recommendation we added this paragraph in the Discussion:

The Tat-Cre mediated recombination also circumvents undesirable safety challenges that can be encountered using lentiviral delivery of transgenes in the TE (Georgiades et al., 2007). As it targets only the trophoblast lineage, the Tat-Cre approach also has an advantage over the tetraploid complementation assay, where donor ESC can compensate for the endogenous epiblast cells, but still both the TE and the primitive endoderm are host derived (Nagy et al., 1993). Thus, any genetic modification in the host embryo is maintained not only in the trophoblast, but also in the primitive endoderm lineage.

2. In describing the derivation of ESC and TSC lines, this would be more convincing if the authors could provide evidence that Tat-Cre is not lethal to ESC lines.

Our initial labelling of the Tat-Cre concentrations next to the ESC and TSC could have been misleading. Therefore, we re-arranged Figure 1D to make the readout clearer. We did not treated ESC and TSC with Tat-Cre. We only give a 2h pulse of different doses of Tat-Cre to blastocysts and after that the embryos were washed and used for ESC and TSC derivation (indicated with arrows). So, during the derivation procedure the blastocyst outgrowths and subsequently the ESC and TSC were not exposed to Tat-Cre. The ESC in particular (derived from control or Tat-Cre treated blastocysts) were passaged up to 5 times before being frozen down, but to stress again, they were not cultured in medium supplemented with Tat-Cre.

3. In Fig. 1E, the authors should more clearly label the TSC experiments – presumably one column was treated and the other was untreated. But the authors have labeled each row as if the entire row were treated.

Following up from the previous comment of Reviewer 2, we apologize that the initial arrangement of the figure was misleading. The figure is now updated and hopefully the readout is clearer. As we mentioned above, the TSC (and ESC) were not treated with Tat-Cre. They were derived from embryos exposed only for 2h to various concentrations of Tat-Cre.

4. The citations in paragraph 1 of the Results section are not adequate or accurate. Primary literature, rather than a review, should be cited.

References to primary literature are provided and the review articles are replaced, as requested by Reviewer 2.

Strumpf, D., Mao, C. A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F. and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132, 2093-2102.

Russ, A. P., Wattler, S., Colledge, W. H., Aparicio, S. A., Carlton, M. B., Pearce, J. J., Barton, S. C., Surani, M. A., Ryan, K., Nehls, M. C., et al. (2000). Eomesodermin is required for mouse trophoblast development and mesoderm formation. Nature 404, 95-99.

*Baribault, H., Penner, J., Iozzo, R. V. and Wilson-Heiner, M. (1994). Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. Genes Dev **8**, 2964-2973.*

Odaka, C., Loranger, A., Takizawa, K., Ouellet, M., Tremblay, M. J., Murata, S., Inoko, A., Inagaki, M. and Marceau, N. (2013). Keratin 8 is required for the maintenance of architectural structure in thymus epithelium. PLoS One 8, e75101.

Second decision letter

MS ID#: DEVELOP/2020/190371

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AUTHORS: Hatice O. Ozguldez, Rui Fan, and Ivan Bedzhov
ARTICLE TYPE: Techniques and Resources Report

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

Please see previous review.

Comments for the author

The authors have addressed my concerns and I am happy with the revised manuscript.

Reviewer 2

Advance summary and potential significance to field

I am happy with the authors' revision.

Comments for the author

I have no further suggestions.