Foxn1 overexpression promotes thymic epithelial progenitor cell proliferation and mTEC maintenance, but does not prevent thymic involution

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MS TITLE: Foxn1 overexpression promotes thymic epithelial progenitor cell proliferation and mTEC maintenance, but does not prevent thymic involution

AUTHORS: Jie Li, Lucas P Wachsmuth, Shiyun Xiao, Brian Condie, and Nancy Manley

Many apologies for the extended amount of time its taken to have your paper reviewed - unfortunately I had difficulty finding appropriate reviewers. 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, 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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

It was previously reported that in Foxn1-overexpressing mouse strains, in which Foxn1 is more than 20-fold overexpressed induced by the K14 or Foxn1 promoter, the thymus is enlarged and age-dependent thymic involution is attenuated. This manuscript describes the phenotypes of the thymus in a K5-promoter-driven Foxn1 transgenic mice. The results show that Foxn1 expression in TECs is approximately 5-fold elevated in the K5.Foxn1-transgenic mice, and the K5.Foxn1-transgene partially restores premature TEC defects in Foxn1-lacZ-knockin mice, in which Foxn1 expression is reduced to approximately 35% of normal expression levels. Interestingly, the development and aging-dependent involution of the thymus are largely unaffected in the K5.Foxn1-transgenic mice, although the density of Plet1-expressing TECs, which resemble TEC progenitors, is elevated in the thymus of K5.Foxn1-transgenic mice. These phenotypes are distinct from, and milder than, the previously reported phenotypes in Foxn1-overexpressing mouse strains, indicating that a modest elevation of Foxn1 expression levels mildly affects TEC development and maintenance. These results are important in view of the highly susceptible nature of the thymus and thymic involution to the dosage and the context of Foxn1 expression in TECs.

Comments for the author

The partial rescue of defective TECs in Foxn1-lacZ-knockin mice by the K5.Foxn1 transgene is interesting. Can you estimate the Foxn1 expression levels in K5.Foxn1-tg Foxn1Z/Z TECs? Also, is the loss of aberrant DN1 population (Figure 4C) associated with restoration of DLL4 and/or IL-7 expression in TECs?

It was previously reported that K5.Foxn1 transgenic mice sometimes exhibit flaky skin and sparse hair (Weiner, et al. 2007). Since it is well known that the thymus is highly susceptible to systemic stress, it should be useful to comment on how the skin is affected and whether the stress is elevated in the K5.Foxn1 transgenic mice examined in this study.

Figure 5E: Plet1+ cells appear dendritic, which is unexpected as immature progenitor cells. Can you comment on this morphology for TEC progenitors?

Figure S1B: please comment on the significant decrease in the frequency of DN2 cells.

Please double-check the order of figure panels according to the order in the text.

Reviewer 2

Advance summary and potential significance to field

The formation of mature thymus epithelial cells (TEC) is a key event in the development of the thymus, and its persistence at post-birth stages. As such, thymic epithelial cell biology represents an important aspect of the developmental biology of the immune system. The transcription factor Foxn1 is known to play a key role in thymus organogenesis and development, yet many aspects of its importance remain poorly understood.

These include patterns of expression during TEC development, regulation of TEC progenitors, and regulation of thymus atrophy. The Manley and Condie labs have made important contributions to understanding thymus development, including the role of Foxn1 in this process. Here, they further examine the role of Foxn1 in thymus microenvironments, using K5.Foxn1 mice to alter Foxn levels in TEC. There are several important advances here. First, they show that increasing levels of Foxn1 have a separate impact on TEC proliferation and differentiation, which advances our understanding of control of TEC development, and the role of Foxn1 availability in this process. Second, they show that reintroduction of Foxn1 into nude mice via K5 driven expression is sufficient to generate functional thymus tissue containing cortex and medulla areas. This is important as it clearly demonstrates the ability of K5 expressing cells to generate a complete thymus. Overall the experiments and clear, well thought out, and will advance the field

Comments for the author

There are several places were the manuscript would benefit from further analysis in order to strengthen the conclusions that are drawn. Frequently, data is from experiments involving confocal microscopy to compare TEC populations in control mice and mice expressing K5.Foxn1. The inclusion of additional flow cytometric data would be helpful to provide clear quantitative and qualitative comparisons.

1. In K5.Foxn1 mice, what frequency of TEC, including cTEC and mTEC subsets, express Foxn1 compared to WT mice? Can this be assessed by flow cytometry, or perhaps even confocal?

2. When analysing the impact of K5.Foxn1 expression on Aire expression in mTEC (Figure 3), can flow cytometric analysis of Aire+ mTEC numbers and percentages be shown? This would enhance the microscopy data already shown.

3. For analysis of Treg in Figure 4, Foxp3 alone does not distinguish between Foxp3+CD25- Treg precursors and mature Foxp3+CD25+ Treg. Can the authors include analysis of CD25 to strengthen their claims on Treg frequency? Or indicate that current data encompasses Treg and Treg precursors.

4. The rescue of the nude phenotype via K5Foxn1 is very interesting. Again, the inclusion of quantitative flow cytometric data to show the numbers and frequencies of cTEC and mTEC populations is important here. It is also important to provide analysis of thymocyte development in K5Foxn1 nude mice - are CD4/CD8 thymocytes distributed normally? Do the mice have peripheral CD4 and CD8 T-cells ?

Reviewer 3

Advance summary and potential significance to field

In their manuscript, Li et al characterize the existing K5:Foxn1 transgene in context of endogenous Foxn1 wild type and two Foxn1 deficiency models, namely the Foxn1:lacZ (moderate reduction of Foxn1) and the Nude (complete reduction of Foxn1) mouse models.

The authors determine that the K5:Foxn1 transgene does not grossly impact thymus development and conventional T cell thymopoeisis on Foxn1 wild type background although in older mice (10 month) an increased mTEC maintenance is observed.

A modest but significant rescue effect is seen in the moderate Foxn1 reduction model, which is accompanied with an interesting observation that regulatory T cells may be proportionally expanded. The rescue of the thymic epithelium is attributed to an expansion of putative progenitor cells as identified by Plet1 and Cldn3 positivity.

Lastly, the authors investigate the rescue of the Nude mutation mouse model, and observe modest rescue of thymus formation toward cortex and medulla formation.

The manuscript adds to ongoing discussions on the role of Foxn1 in thymus development and function, and how Foxn1 levels are involved in different developmental aspects. The phenotypic characterization also sets aside the K5:Foxn1 transgene from other Foxn1 overexpression systems such as K14:Foxn1 and Rosa26/CAG:Foxn1 mouse models. From a readers perspective, the manuscript is well written, clear and well prepared figures.

Unfortunately, the manuscript is missing the opportunity to interrogate the Krt5-Foxn1 transgene in context of the latest literature. The latest citation I found in the manuscript is from 2017, and the field has since moved from the traditional mTEC/cTEC distinction to sophisticated resolution of TEC subsets, including higher resolution cTEC and mTEC subsets, intertypical cells, mimetics, TUFT cells and different age-associated TEC progenitors. In addition, mechanistic insights that could add to the understanding of Foxn1 expression patterns and impact of subTEC type-specific Foxn1 levels are limited.

Overall, the authors have presented us with a series of rescue experiments that are successful at varying levels. This will contribute to the ongoing discussions among specialists in this area. Beyond that, it seems that limitations on resolution and mechanistic insights will limit the impact on a larger audience in the area of developmental biology.

Comments for the author

Major
1. Overall, the manuscript seems to ignore recent advances on TEC subset discovery in recent years. A more thorough characterization of different TEC subsets as described in the literature 2018-2022 seems relevant.
2. It is not clear what mechanistic insights the manuscript adds to the current view on Foxn1 function, beyond an outlining phenotypic characterization of a transgene effect. In my opinion, the phenotypic characterization does not reveal new insights on Foxn1 function nor the definitive role of Foxn1 levels. Also, the by the author mentioned differences observed in phenotypes between different transgenics can simply be due to expression patterns rather than Foxn1 levels, or a combination of transgene promoter level and expression pattern.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

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Relative Foxn1 and Dll4 data are now provided in Fig 2 and Fig 4 respectively. Indeed, Dll4 levels are rescued to roughly normal levels in these mice.

It was previously reported that K5.Foxn1 transgenic mice sometimes exhibit flaky skin and sparse hair (Weiner, et al. 2007). Since it is well known that the thymus is highly susceptible to systemic stress, it should be useful to comment on how the skin is affected and whether the stress is elevated in the K5.Foxn1 transgenic mice examined in this study.

While it is true that the thymus is susceptible to systemic stress, it is also true that the degree of affect is strain-specific. Both published data and our own unpublished data show that C57Bl/6 mice are resistant to stress-induced thymus phenotypes (for example, Toxicological Sciences 62, 250-

256, 2001). Regardless, we did not see obvious skin or sparse hair phenotypes in our colonies, which could be due to differences in the genetic background in our colony relative to the original paper.

Figure 5E: Plet1+ cells appear dendritic, which is unexpected as immature progenitor cells. Can you comment on this morphology for TEC progenitors?

In general, TEC have quite unique morphologies, and so they do not look like 'typical' progenitor cells. This is also affected by IHC methods. Plet 1 antibody is sensitive for different staining methods. We used paraffin treated by HCl for Figure 5E to be compatible with BrdU, so that shows the cell morphology in more detail than frozen sections used in other figures.

Figure S1B: please comment on the significant decrease in the frequency of DN2 cells.

The decline in DN2 cells is unusual, as DN1a,b cells are not obviously affected (Fig. 4E,F), and we did not see a similar dip in DN2 cells either at 6 months (SFig. 1D, E) or at 1 month in the +/Z;Tg+ mice (SFig. 3). We repeated the experiment, and after increasing n numbers, the decline is not statistically different. We have updated this figure.

Please double-check the order of figure panels according to the order in the text.

We have either edited the text or rearranged the order of figure panels to match the text for all figures.

Reviewer 2 Advance Summary and Potential Significance to Field:
The formation of mature thymus epithelial cells (TEC) is a key event in the development of the thymus, and its persistence at post-birth stages. As such, thymic epithelial cell biology represents an important aspect of the developmental biology of the immune system. The transcription factor Foxn1 is known to play a key role in thymus organogenesis and development, yet many aspects of its importance remain poorly understood. These include patterns of expression during TEC development, regulation of TEC progenitors, and regulation of thymus atrophy. The Manley and Condie labs have made important contributions to understanding thymus development, including the role of Foxn1 in this process. Here, they further examine the role of Foxn1 in thymus microenvironments, using K5.Foxn1 mice to alter Foxn1 levels in TEC. There are several important advances here. First, they show that increasing levels of Foxn1 have a separate impact on TEC proliferation and differentiation, which advances our understanding of control of TEC development, and the role of Foxn1 availability in this process. Second, they show that reintroduction of Foxn1 into nude mice via K5 driven expression is sufficient to generate functional thymus tissue containing cortex and medulla areas. This is important as it clearly demonstrates the ability of K5 expressing cells to generate a complete thymus. Overall, the experiments and clear, well thought out, and will advance the field

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1. In K5.Foxn1 mice, what frequency of TEC, including cTEC and mTEC subsets, express Foxn1 compared to WT mice? Can this be assessed by flow cytometry, or perhaps even confocal?

This is a limitation of the system unfortunately. Currently, there is no method to evaluate Foxn1 expression by flow cytometry. While we can image Foxn1 expression by confocal, there is not an easy way to estimate percentages in specific subsets by this method. The imaging data provided in Figure 1 indicate relatively uniform increases in Foxn1 expression across both medulla and cortex, which indicates broad expression of the transgene.

2. When analysing the impact of K5.Foxn1 expression on Aire expression in mTEC (Figure 3), can flow cytometric analysis of Aire+ mTEC numbers and percentages be shown? This would enhance the microscopy data already shown.

Yes, it was shown in Supplemental Figure 3. We have now moved these data to Figure 3.

3. For analysis of Treg in Figure 4, Foxp3 alone does not distinguish between Foxp3+CD25- Treg precursors and mature Foxp3+CD25+ Treg. Can the authors include analysis of CD25 to strengthen their claims on Treg frequency? Or indicate that current data encompasses Treg and Treg precursors.

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K5.Foxn1tg;nude mice have very small thymi, and recovery of sufficient numbers of TECs for the subset analysis requested was not feasible. Thymocyte data including DN1 subsets, DN subsets, and CD4/8 subsets, as well as peripheral T cells (splenocytes) are now included in Supplementary Figure 7.

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Reviewer 3 Comments for the Author:
Major

1.Overall, the manuscript seems to ignore recent advances on TEC subset discovery in recent years. A more thorough characterization of different TEC subsets as described in the literature 2018-2022 seems relevant.

We apologize for not including more recent references which have now been updated. This manuscript was originally written several years ago and we did not adequately update this aspect of the paper.

The higher resolution TEC subsets that the reviewer is asking for were all identified by scRNA-seq, which we clearly are reluctant to embark upon for these transgenic animals, especially as we have no funding to do so. Indeed, there have been several such studies, all of which define variations on these subsets. While some of these cell types can be identified by IHC and FACS there are not standard protocols for doing so. And, given the various studies out there to draw upon, the best way to do it would indeed by scRNA-seq. While we agree that it would provide a more detailed analysis of the TEC phenotypes, it would require substantial work, time, and money to repeat all these experiments with those tools, and believe it is not reasonable to be expected to do this analysis for this paper.

[2.It]2.It is not clear what mechanistic insights the manuscript adds to the current view on Foxn1 function, beyond an outlining phenotypic characterization of a transgene effect. In my opinion, the phenotypic characterization does not reveal new insights on Foxn1 function nor the definitive role of Foxn1 levels. Also, the by the author mentioned differences observed in phenotypes between different transgenics can simply be due to expression patterns rather than Foxn1 levels, or a combination of transgene promoter level and expression pattern.

The other two reviewers did not share this opinion. And of course, neither do we.

Second decision letter

MS ID#: DEVELOP/2022/200995

MS TITLE: Foxn1 overexpression promotes thymic epithelial progenitor cell proliferation and mTEC maintenance, but does not prevent thymic involution

AUTHORS: Jie Li, Lucas P Wachsmuth, Shiyun Xiao, Brian Condie, and Nancy Manley

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

I think the authors adequately responded to all of my previous comments.

Comments for the author

I have no more suggestions.

Reviewer 2

Advance summary and potential significance to field

Studies that examine the regulation of thymic epithelial cells by Foxn1 are important in understanding both thymus organogenesis and the role of this transcription factor in. thymic epithelial cell biology. The authors describe interesting new findings by analysis of new transgenic mouse strains. Of particular interest is regulation of thymus development when Foxn1 is controlled

via a K5 transgene. This is interesting as is perhaps indicates expression of K5 in a TEC progenitor that can generate both cortex and medulla environments. While this was suggested by previous phenotyping data, these functional experiments provide strong evidence for this.

Comments for the author

This manuscript is well performed and of interest.

Reviewer 3

Advance summary and potential significance to field

since the last submission, the authors have added references to address some of my comments but decided to not provide new data along the lines of suggested revisions, which would clarify the transgene behavior and consequences.

Comments for the author

suggestions and major comments from the first review cycle remain.