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# Nrf2 links epidermal barrier function with antioxidant defense

Matthias Schäfer, Hany Farwanah, Ann-Helen Willrodt, Aaron J. Huebner, Konrad Sandhoff, Dennis Roop, Daniel Hohl, Wilhelm Bloch, and Sabine Werner

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**Review timeline:**

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| Submission date: | 01 October 2011 |
| Editorial Decision: | 07 November 2011 |
| Revision received: | 20 December 2011 |
| Editorial Decision: | 16 January 2012 |
| Revision received: | 17 January 2012 |
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 November 2011

Thank you for the submission of your manuscript "Nrf2 links epidermal barrier function with antioxidant defense" to EMBO Molecular Medicine. We have now received the enclosed reports from the reviewers that were asked to assess it.

You will be glad to see that the reviewers are positive about your manuscript and recommend publication of the paper pending some revisions. Specifically, the reviewers highlight that the link between the dysregulation of SPRRs and the phenotype caused by Nrf2 overexpression should be further strengthened. In addition, reviewers 2 and 3 note that the statistical analyses should be extended to all qRT-PCR data.

Given the balance of the evaluations, we would be willing to consider a revised manuscript with the understanding that the reviewers' concerns must be convincingly addressed within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless discussed otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

 

***** Reviewer's comments *****

Referee #1:

This is a nice paper that marries complementary genetic and pharmacological approaches to address what are the beneficial and detrimental effects of longterm nrf2 activation in skin keratinocytes in vivo and in vitro. Such a study is timely because nrf2 is a clear therapeutic target to aid cytoprotection and yet may have negative consequences as suggested by it being upregulated in several cancers.

This work follows on from a study in which K5 cre was used to drive constitutively active NRF2 in the basal keratinocytes of transgenic mice. In the current study, addition of an MMV enhancer quadruples expression of the caNRF2, and similarly high levels of activity are achieved by topical application of sulphorafane or tBHQ to skin of wild type mice. In both instances the skin becomes hyperproliferative and thickened with associated inflammation and resembles a human clinical condition, ichthyosis, or scaley skin.

The authors show that hyperproiferation is not a direct, cell autonomous function of NRF2 activation because keratinocytes isolated in vitro from these mice proliferate no faster than wild type keratinocytes in culture. Rather it appears that a breakdown in barrier function may be the initiating defect which in turn leads to an influx of immune cells and associated induction of pro-inflammatory cytokines. And they go on to show that the barrier function defect is not because of tight junction disruption, but rather to alterations in lipid metabolism and lamellar body formation in terminal keratinocytes.

To address what might be the molecular changes leading to these defects, the authors undertake a microarray comparison that reveals 80+ genes including several well established nrf 2 targets which nicely validates this assay and their approach. Several of the novel targets, particularly the sprrs and slpi, are prime candidates for "disruptors" of normal keratinocyte function and differentiation and the authors present some evidence that this is the case eg they show by ultrasound treatment of isolated corneocytes that overexpression of sprrs has made them more fragile. The authors report in the text and even in the legend of Fig 7 that increased slpi levels leads to inhibition of klk7 from data in previous literature, and they presume that this is what leads to reduced shedding of the cornified cells, but I think it would be good to show direct evidence of reduced klk7 in their own model.

Overall an important paper that warns caution with regard the likely downstream consequences of nrf2 activation in skin keratinocytes, beyond the obviously beneficial effects of protection via antioxidants and antimcrobials.

Referee #2:

This manuscript describes an interesting, well designed and carefully performed study investigating the role of Nrf2 in epidermal homeostasis. The findings report a new role of Nrf2 in epidermal barrier function, which might have consequences for the pharmacological use of Nrf2 activators. The authors describe that morphological and functional alterations in the epidermis of Nrf2 transgenic mice resemble those of the human skin disease ichthyosis. It would be interesting to know whether expression and function of Nrf2 and the newly identified Nrf2 target genes Slpi and Sprr2 are also deregulated in human ichthyosis. Would it be possible to test this in human diseased tissue samples? Furthermore, it would be helpful to block the dysregulated Nrf2 target genes Slpi and Sprr2d to corroborate their causative role in the phenotype of Nrf2 transgenic mice?

Specific comments:
1. Throughout the manuscript error bars on the qRT-PCR analysis are missing. How many samples were tested in each analysis?
2. Fig. 5A, it is quite difficult for the reader to identify the structures described. A photograph with better quality should be included.
3. Fig. 6F, it is difficult for the reader to identify increased numbers of destroyed corneocytes of

transgenic mice. A photograph with better quality should be included.

Referee #3 (Comments on Novelty/Model System):

Error bars are missing from several figures comparing mRNA expression by qRTPCR

Referee #3 (Other Remarks):

This is a well written manuscript which contains novel and interesting data: Overexpression of NRF2 in keratinocytes under a CMV promoter results in hyperkeratotic skin phenotype. Small proline-rich proteins and the secretory leukocyte peptidase inhibitor are identified as novel targets for NRF2 and are described to be associated with increased corneocyte fragility. The fact that the phenotype can be induced also by application of pharmacologic stimulators of NRF2 corroborates the findings in the transgenic model
Comments:
1. The association between overexpression of SPRRs and corneocyte fragility might be causative but this is not formally demonstrated therefore the discussion of this association should be toned down.
2. Ichthyoses are a heterogeneous group diseases with the common feature of hyperkeratosis. If the authors want to suggest a resemblance with "ichthyosis" they should be more specific and try to establish the (histopathologic) relationship to a particular type of this disease
3. The reasoning that the epidermal barrier defect is causative for the inflammation observed is likely, however, the fact that IL-1beta is strongly increased (figure 4) already at the earliest timepoint shown hints that also other mechanisms could be involved. The authors should comment on that.
4. One of the unexplained findings in this manuscript is the amelioration of the phenotype with age. The authors should discuss possible mechanisms which might explain this phenomenon. Only the persistent elevation of Slpi is shown at 6 months. What are expression levels of NRF2 at age of 6 months? Is the CMV promoter silenced? Are other - compensatory mechanisms - activated ? In this context I recommend to show also a comparative photograph of wt and tg mice at age 6 months in the supplementary information.

1st Revision - Authors' Response 20 December 2011

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*We agree that we have not experimentally proven that the enhanced expression of Slpi is at least in part responsible for the phenotype. To test this hypothesis, we would have to mate our transgenic mice with Slpi knockout mice. We have initiated such a project, but this will of course take a long time and cannot be done within three months. Nevertheless, we have added new data that strengthen our hypothesis:*

*We stained the skin of K5cre-CMVcaNrf2 and control mice with an antibody against desmoplakin, which shows the presence of corneodesmosomes in the most upper layers of the hyperthickend stratum corneum of K5cre-CMVcaNrf2 mice. By electron microscopy we identified reduced degradation of corneodesmosomes in transgenic mice, which provides indirect evidence for reduced Klk7 activity. These new data are now shown in Suppl. Fig. S2B and Fig. 6D.*

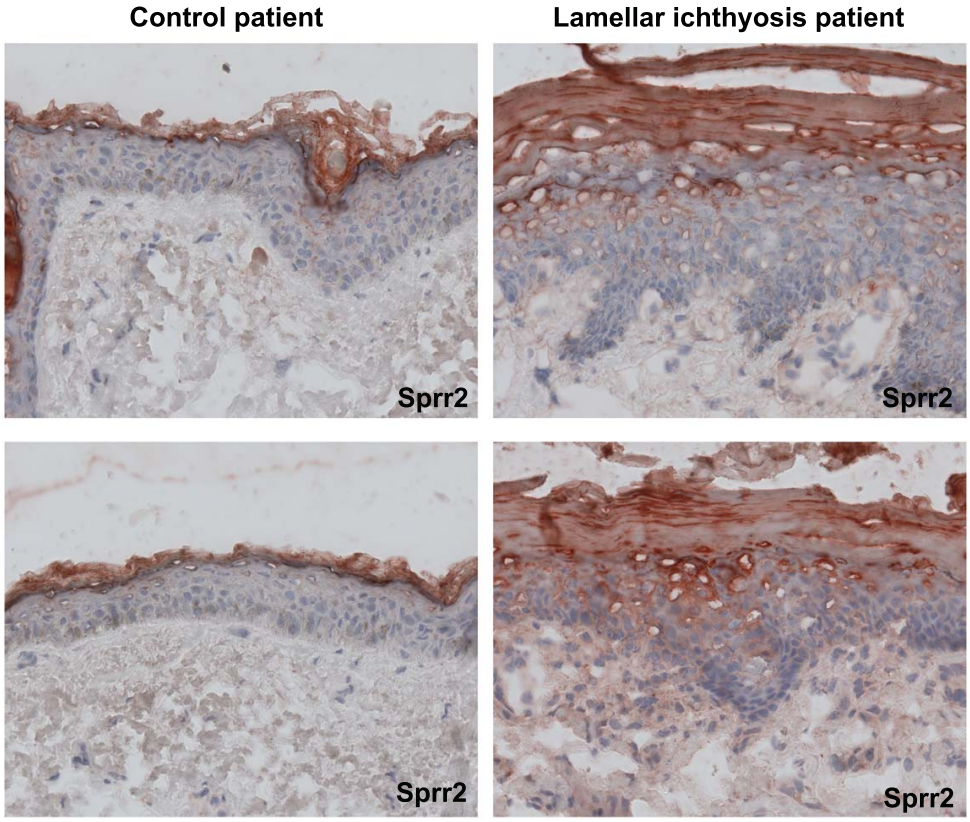
Overall an important paper that warns caution with regard the likely downstream consequences of nrf2 activation in skin keratinocytes, beyond the obviously beneficial effects of protection via antioxidants and antimcrobials.

Referee #2:

This manuscript describes an interesting, well designed and carefully performed study investigating the role of Nrf2 in epidermal homeostasis. The findings report a new role of Nrf2 in epidermal barrier function, which might have consequences for the pharmacological use of Nrf2 activators. The authors describe that morphological and functional alterations in the epidermis of Nrf2 transgenic mice resemble those of the human skin disease ichthyosis. It would be interesting to know whether expression and function of Nrf2 and the newly identified Nrf2 target genes Slpi and Sprr2 are also deregulated in human ichthyosis. Would it be possible to test this in human diseased tissue samples?

*This is indeed a very interesting question that should be addressed. Ideally, one should stain sections from normal and diseased skin with an Nrf2 antibody to determine if Nrf2 is in the nucleus and therefore active in affected skin of ichthyosis patients. Unfortunately, the available antibodies are not suitable for detection of Nrf2 in tissue sections. Therefore, it will be necessary to obtain RNA from normal and diseased skin to look for Nrf2 target genes. It is very difficult to obtain such material, but we have initiated this study in collaboration with clinical colleagues. Due to the heterogeneity of ichthyoses we will have to analyze tissue from a large number of patients and this will take a rather long time. We therefore believe that this is beyond the scope of this study, but*

*should be done in the future. Nevertheless, we have performed some preliminary experiments to look for Sprr2 expression in patients with ichthyosis. Upon staining of a few available sections with an Sprr2 antibody we found that Sprr2 proteins are indeed strongly expressed in these patients. Since this result is still preliminary we prefer not to show it in the manuscript, but we have included this result as additional information for the reviewer.*




Furthermore, it would be helpful to block the dysregulated Nrf2 target genes Slpi and Sprr2d to corroborate their causative role in the phenotype of Nrf2 transgenic mice?

*Please see our response to reviewer 1 regarding this issue. The question can only be addressed by generation of double mutant mice, since there are no specific inhibitors for Slpi and Sprr2d.*

Specific comments:

1.) Throughout the manuscript error bars on the qRT-PCR analysis are missing. How many samples were tested in each analysis?

*We apologize for not having described this in sufficient detail and we have now extended this information in Materials and Methods. Furthermore, we have included the number of mice or cell cultures used for each experiments in the legends. Instead of using individual mice we pooled RNAs from 2-3 mice for each experiment. Therefore, there are no error bars. However, we have repeated each experiment with pooled RNA from independent mice and we mention that all results were reproduced with RNA from an independent pool. We would be happy to send these data to the reviewer upon request. In Fig. S2A we have added the error bars in the graph.*

2.) Fig. 5A, it is quite difficult for the reader to identify the structures described. A photograph with better quality should be included.

*Unfortunately, the arrows had been moved when we generated the PDF and they therefore did not point to the structures that should have been highlighted. We have now corrected the position of the arrows. In addition, we have increased the magnification of the pictures in Fig. 5A and rearranged the figure accordingly.*

3.) Fig. 6F, it is difficult for the reader to identify increased numbers of destroyed corneocytes of transgenic mice. A photograph with better quality should be included.

*We apologize for not having clarified this issue in the initial version of our manuscript. The cells that are detected are all intact cells. As soon as they are destroyed there are only debris detectable. To clarify this issue we now indicate intact corneocytes with an arrow and cell debris with an arrowhead.*

Referee #3 (Comments on Novelty/Model System):

Error bars are missing from several figures comparing mRNA expression by qRTPCR

*Please see our reply to comment 1 of reviewer No.2*

Referee #3 (Other Remarks):

This is a well written manuscript which contains novel and interesting data: Overexpression of NRF2 in keratinocytes under a CMV promoter results in hyperkeratotic skin phenotype. Small proline-rich proteins and the secretory leukocyte peptidase inhibitor are identified as novel targets for NRF2 and are described to be associated with increased corneocyte fragility. The fact that the phenotype can be induced also by application of pharmacologic stimulators of NRF2 corroborates the findings in the transgenic model

Comments:

1. The association between overexpression of SPRRs and corneocyte fragility might be causative but this is not formally demonstrated therefore the discussion of this association should be toned down.

*We agree with the reviewer and therefore, we have discussed this more carefully (Discussion, page 18).*

2. Ichthyoses are a heterogeneous group diseases with the common feature of hyperkeratosis. If the authors want to suggest a resemblance with "ichthyosis" they should be more specific and try to establish the (histopathologic) relationship to a particular type of this disease

*As suggested by the reviewer, we now mention that the phenotype is most closely related to the phenotype of patients with Lamellar Ichthyosis, and we list the criteria on which we base this conclusion (Discussion, page 16). We have also included a figure that shows the macroscopic and histological phenotype of our mice in comparison to a patient with Lamellar Ichthyosis (Supporting information Fig. S.3)*

3. The reasoning that the epidermal barrier defect is causative for the inflammation observed is likely, however, the fact that IL-1beta is strongly increased (figure 4) already at the earliest timepoint shown hints that also other mechanisms could be involved. The authors should comment on that.

*We agree with the reviewer that there is already an increase in IL-1beta at P2.5. However, we could not detect upregulation of any other pro-inflammatory cytokine at this stage and we also could not detect any neutrophils. In a replicate experiment, the increase in IL-1beta was lower at this stage (see additional information for reviewers). Therefore, it seems most likely that the inflammatory phenotype is locally initiated at this time point, but not fully developed yet. We have tried to clarify this issue in the text (page 9), and we also discuss this more carefully (Discussion, page 16).*

4. One of the unexplained findings in this manuscript is the amelioration of the phenotype with age. The authors should discuss possible mechanisms, which might explain this phenomenon. Only the persistent elevation of Slpi is shown at 6 months. What are expression levels of NRF2 at age of 6 months? Is the CMV promoter silenced? Are other - compensatory mechanisms - activated? In this context I recommend to show also a comparative photograph of wt and tg mice at age 6 months in the supplementary information.

*We had already shown a comparative photograph of wt and tg mice at the age of 6 months in Fig. 3A of the the original version. We have now modified the figure to allow a direct comparison of the phenotype at P32 and 6 months.*

In addition, we analyzed the expression of caNrf2 and of the classical Nrf2 target genes Nqo1, Gclc, Gclm and Srxn in P2.5, P32 and 6m old K5cre-CMVcaNrf2 mice by qRT-PCR. We observed an increase in expression of caNrf2 and some classical targets at P32 and a decrease at 6m in two independent experiments (please see additional information for the reviewers). However, the effect was quite variable and therefore, we did not make a strong point of this observation. Nevertheless, we now show the caNrf2 expression in Fig. S1B and describe this observation in the Results (page 8).

2nd Editorial Decision 16 January 2012

Thank you for the submission of your revised manuscript "Nrf2 links epidermal barrier function with antioxidant defense" to EMBO Molecular Medicine. We have now received the reports from the reviewers who were asked to re-review your manuscript.

You will be glad to see that the reviewers are now globally supportive and we can proceed with

official acceptance of your manuscript pending the minor changes detailed below:

- Please provide a Table of Contents on the first page of the Supplementary Material.

- Data of gene expression experiments described in submitted manuscripts should be deposited in a MIAME-compliant format with one of the public databases. We would therefore ask you to submit your microarray data to the ArrayExpress database maintained by the European Bioinformatics Institute for example. ArrayExpress allows authors to submit their data to a confidential section of the database, where they can be put on hold until the time of publication of the corresponding manuscript. Please see http:www.ebi.ac.uk/arrayexpress/Submissions/ or contact the support team at arrayexpress@ebi.ac.uk for further information.

- In addition, we noted that your point-by-point response contains a figure. Since this would be published in the Peer Review Process File, please let us know whether you agree with its publication, if you would like to delete the figure or whether you would like to opt out of publishing the PRPF.

I look forward to seeing a revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

I've now read through the revised manuscript and the author's rebuttal letter. Looks like they have done a good job at revising this MS and have adequately dealt with the minor concerns/suggestions I raised with the first draft. Similarly they appear to have responded well to other reviewers' comments. I vote that this paper is now ready for publication in EMBO Mol Med.

Referee #3 (Comments on Novelty/Model System):

Manuscript contains interesting novel data and reports a potential downside of the activation of the NRF2 pathway which is commonly associated with protective functions.

Referee #3 (Other Remarks):

The authors responded satisfyingly to my questions and criticisms.