Author's response to reviews

Title: Upregulation of CRABP1 in human neuroblastoma cells overproducing the Alzheimer-typical Abeta42 reduces their differentiation potential

Authors:

Markus Uhrig (markus.uhrig@web.de)
Peter Brechlin (pbrech@gwdg.de)
Olaf Jahn (jahn@em.mpg.de)
Yuri Knyazev (yknyazev@yahoo.com)
Annette Weninger (a.weninger@dkfz.de)
Laura Busia (laura_busia@yahoo.it)
Kamran Honarnejad (khonarnejad@yahoo.com)
Markus Otto (markus.otto@uni-ulm.de)
Tobias Hartmann (Tobias.Hartmann@Uniklinikum-Saarland.de)

Version: 4 Date: 10 November 2008

Author's response to reviews: see over
Dear Dr. Melissa Norton,

We send you our revised manuscript for publication in *BMC Medicine*. Previously, we had submitted it to *BMC Medical Genomics* where it had been, in principal, accepted. However, we have been offered (by Dr. Andrea Bucceri) publication in your flagship journal *BMC Medicine*. The new title is:

“Upregulation of CRABP1 in human neuroblastoma cells overproducing the Alzheimer-typical Aβ42 reduces their differentiation potential”

Our comments to the reviewers are highlighted in yellow.

We look forward to hearing from you.

Sincerely,

Tobias Hartmann/Markus Uhrig

**Reviewer's report**

**Title:** Genome and proteome-wide analyses of human neuroblastoma cells overproducing the Alzheimer-typical Abeta42 reveal CRABP1 upregulation reducing their differentiation potential

**Version:** 1 **Date:** 17 June 2008

**Reviewer:** Paolo Iadarola
Reviewer’s report:
In their manuscript the authors have investigated the transcriptomic and proteomic responses to increased/decreased ABeta42 and ABeta40 levels generated in human SH-SY5Y cells. By using a combination of genomic and proteomic analyses, the authors have obtained interesting results which demonstrate that an increased ABeta42/ABeta40 ratio up-regulates the cellular retinoic acid binding protein 1 (CRABP1). This finding is of particular importance given that CRABP1 up-regulation increases SH-SY5Y cell line proliferation and reduces its differentiation potential. In my opinion this study was fairly well-done and data carefully interpreted.
In conclusion these results are relevant and robust and might provide a tool for improving our understanding of Alzheimer's disease neurogenesis. I believe that this paper is most worthy of publication and I recommend with high priority.
Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests

Comments to reviewer Paolo Iadarola

Dear Dr. Iadarola,

Thank you for your revision of our manuscript. We do not have any comments.

Reviewer’s report
Title: Genome and proteome-wide analyses of human neuroblastoma cells overproducing the Alzheimer-typical Abeta42 reveal CRABP1 upregulation reducing their differentiation potential
Version: 1 Date: 20 June 2008
Reviewer: Rukhsana Sultana
Reviewer’s report:
This paper is well written.
Minor suggestion:
use Italics wherever there is name of gene.
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.

Comments to reviewer Rukhsana Sultana

Dear Dr. Sultana,
Thank you for your revision of our manuscript. We changed the names into *Italics* where appropriate. According to [http://www.ssr.org/NomenBullets.html](http://www.ssr.org/NomenBullets.html) we followed these guidelines:

- **Full gene names are not in italics and Greek symbols are NEVER used**
  - eg: insulin-like growth factor 1
- **Gene symbols**
  - Greek symbols are never used
  - hyphens are almost never used
  - gene symbols are italicized, first letter upper case all the rest lower case
    - eg: *Igf1* (italicized)
- **Proteins designations**
  - same as the gene symbol, but not italicized and all upper case
    - eg: *IGF1*
- mRNA and cDNA use the gene symbol and formatting conventions
  - eg: "... levels of *Igf1* (italicized) mRNA increased when..."

It has to be taken into consideration that we performed a combined transcriptomics/proteomics approach, so in many cases we intended to refer to both, gene *and* protein-names, and finally decided for the nomenclature of protein names.

**Reviewer's report**

**Title:** Genome and proteome-wide analyses of human neuroblastoma cells overproducing the Alzheimer-typical Abeta42 reveal CRABP1 upregulation reducing their differentiation potential

**Version:** 1  **Date:** 27 June 2008

**Reviewer:** Piruz Nahreini

**Reviewer's report:**

Re: 'Genome and proteome-wide analyses of human neuroblastoma cells overproducing the Alzheimer-typical Abeta42 reveal CRABP1 upregulation reducing their differentiation potential'

Markus Uhrig, Peter Brechlin, Olaf Jahn, Yuri Knyazev, Annette Weninger, Laura Busia, Kamran Honarnejad, Markus Otto and Tobias Hartmann

The authors investigated whether altered ratio of Ab42/Ab40 synthesis may mediate changes in the expression of genes, which in turn may account for neurogenesis documented in Alzheimer’s disease. To do this, they stably expressed the membrane-associated domain of APP (i.e. C99) in a human neuroblastoma cell line and isolated clones expressing different ratios of Ab42/Ab40. Using transcriptomic and proteomic techniques, they identified CRABP1 gene, whose expression augmented in response to increased Ab42/Ab40 ratio. Since CRABP1 was shown to dampen RA-mediated differentiation effects, the authors concluded that the up-regulation of CRABP1 expression in response to increased Ab42/ab40 may partly account for observed pathological neurogenesis in AD patients.

Critiques:

1. Based on the experimental design, one expects to obtain similar effects, specifically altered CRABP1 expression, if different ratios of Ab42/Ab40 are added exogenously into the medium of cultured neuroblastoma cells. Although the authors logically are not in favor of this experiment (discussion section), it is, nevertheless, critical to know the outcome in the context of this study and their conclusion. What if exogenously added Ab42/Ab40 (> 4-6 fold) shows no effect
on the expression of CRABP1? Is it really the Ab42/Ab40 ratio that dictates the up-regulation of CRABP1 or other smaller fragments of C99?

2. To further validate the conclusion of this study and exclude other possibilities, one should also investigate the effect of RNAi-mediated knockdown of C99 or gamma-secretase in the C99145F clones. If the authors’ conclusion is correct, then reduced expression of C99 or gamma-secretase should decrease CRABP1 expression and increase RA-mediated differentiation in the C99145F cells.

3. What is the experimental evidence for enhanced cell proliferation due to increased Ab42/Ab40 ratio? Are there more cells in the S-phase of cell cycle in the C99145F versus C99V50F clones? Have they performed growth or cell cycle analysis?

4. On page 7, top paragraph, it reads “CRABP1 was only up-regulated 1.7 fold in C99V50F compared to C99WT.” Why is it up-regulated if the Ab42/Ab40 ratio is higher in C99WT as compared with C99V50F (page 5, second paragraph, relative ration ~ 0.3)?

5. They should have used inactive RNAi instead of nonsense sequence for the CRABP1 knockdown experiment (page 8, middle of the first paragraph).

6. The current title is fine and comprehensive, but shorter titles generally register better with most readers. Something like: Increased Alzheimer’s Ab42 peptide up regulates CRABP1 and reduces differentiation potential

Final Remarks: Overall, this study contains informative and valuable observations for the neurodegeneration field. The paper and its conclusion would probably be more convincing if the authors address some of the above comments, especially 1 and 2.

Comments to reviewer Piruz Nahreini

Dear Dr. Nahreini,

Thank you for your revision of our manuscript. We changed some of the issues you recommended and included some comments below:

1. We do not expect similar effects (CRABP1 upregulation) when increased Abeta42 levels are added exogenously to the cells for the following reasons: Cleavage of C99 is not only produced at the plasma membrane but also intracellularly at the membrane of the endoplasmic reticulum (ER) resulting also in a small fraction of intracellular Abeta42 (even if much Abeta42 leaves the cell via the secretory pathway). It cannot be excluded that intracellular Abeta42 exerts effects on gene expression (indeed, there is a long lasting debate about this issue in the Alzheimer field [1-3]). Intracellular Abeta42 might thus exert effects alone or synergistically with extracellular Abeta42. We regard our approach to be more physiological than just adding exogenous Abeta42, because we mimic the expected physiological state of the cells by overexpressing the naturally occurring Abeta-precursor C99. By doing this, we expect to produce all possibly necessary cleavage products (to induce or repress transcription), which would be missing by merely adding exogenous Abeta42. Furthermore, there is only very poor agreement in the identified genes in consequence of exogenous Aβ42–treatment by other working groups: Microarray
experiments result in strong variation in the genes identified. There is hardly any overlap of the found genes. Even if this can be partly explained by the use of different cell types and normalisation strategies, we rather believe that these results are the consequence of the unphysiological \textit{exogenous} Aβ-supply. Moreover, the aggregation status and/or conformation of Aβ_{42} is critical for its biological properties. This is highly dependent on the cellular environment. Cellularly produced Aβ provides the best experimental strategy to ensure a native status.

2. Knocking down C99 exclusively is not possible because C99 is a cleavage product of the amyloid precursor protein (APP) (cleaved on the protein level by β-secretase), so only m-RNA for the complete APP but not for C99 is present in the cells. However, knocking down the complete APP cannot be regarded as a proof/disproof of our observations because APP cleavage by β-secretase was not investigated in our approach in which we intentionally overexpressed C99 (the fragment of APP left over after cleavage by β-secretase). So we were able to exclude any effects triggered by β-secretase.

Knocking down the gamma-secretase complex is not easy to accomplish because it consists of several proteins which work synergistically together. A possible way to knockdown its function is to knockdown presenilin1 (PS1), one of its components. However, this is accompanied by detrimental effects: it has been shown that its homolog presenilin2 (PS2) can take over the functions of PS1 [4, 5]. Moreover, \textit{in vivo}, knock-out of PS has led to developmental problems and prenatal death [6].

3. The experimental evidence for enhanced cell proliferation is the number of cells generated per time. The cells were plated on cell culture dishes at comparable densities. At the beginning of the experiment the cells were equally confluent (approximately 30-50% confluent) and the number of cells for clone C99I45F and C99V50F were approximately equal. Cells (in presence of retinoic acid) were checked each day by light microscopy and it was clearly visible already at the first day that the cells overexpressing CRABP1 and an increased Aβ_{42}/Aβ_{40} ratio (clone C99I45F) divided much faster than the cells for clone C99V50F (decreased Aβ_{42}/Aβ_{40} ratio). This was observed for all three independent C99I45F clones and for all three independent C99V50F clones: Starting from 30-50% confluency for both clones, only C99I45F reached 100% confluency after 4-6 days. In contrast to this, for C99V50F, 100% confluency was never reached (observation time approximately 12 days; after 12 days confluency was never >70%). A further sign of enhanced cell proliferation for C99I45F was that the C99I45F cells looked morphologically unchanged (undifferentiated) whereas the non- or slow-proliferating C99V50F cells looked morphologically differentiated. Increased differentiation is generally accompanied by a decreased tendency to proliferate. We followed established protocols in which the dependence of the cell number on retinoic acid was demonstrated [7, 8].

4. As defined in the manuscript we regard a differential expression of fold changes greater than 1.9 fold (cut off ≥1.9) as reliably differentially expressed. The measured differential expression of 1.7 fold is below this cut-off and a real up-regulation can thus not be expected (here, data were shown only for completeness of our results, but might be misleading). The Aβ_{42}/Aβ_{40} ratio in C99WT is only 3 fold higher compared to the ratio in C99V50F. This might be not sufficient to see a strong effect (in comparison to this the Aβ_{42}/Aβ_{40} ratio in C99I45F is more than 20 fold higher
compared to the ratio in C99WT and thus this comparison provides more useful information).

5. Indeed, using inactive RNAi as negative control would have been an alternative approach. However, nonsense sequences (scrambled sequences) are a gold standard as negative controls for knockdown experiments and they are the recommended method by the manufacturer Ambion, one of the leading companies for RNA-interference materials. Furthermore they are ubiquitously used by many researchers.

6. We modified the title into the following more straightforward version: “Upregulation of CRABP1 in human neuroblastoma cells overproducing the Alzheimer-typical Aβ_{42} reduces their differentiation potential”

References:


Reviewer's report

Title: Genome and proteome-wide analyses of human neuroblastoma cells overproducing the Alzheimer-typical Abeta42 reveal CRABP1 upregulation
reducing their differentiation potential

**Version:** 2  **Date:** 21 October 2008  
**Reviewer:** Piruz Nahreini

**Reviewer's report:**  
The authors provided some reasonable explanations in response to my critiques. I believe this paper has some interesting and informative observations and I therefore now favor its acceptance for publication.

1. Is the question posed by the authors well defined? Yes
2. Are the methods appropriate and well described? Yes
3. Are the data sound? Yes
4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes
5. Are the discussion and conclusions well balanced and adequately supported by the data? Yes
6. Are limitations of the work clearly stated? Yes
7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes
8. Do the title and abstract accurately convey what has been found? The modified version does! Yes
9. Is the writing acceptable? Yes