Author's response to reviews

Title: Identification of Achaete-scute complex-like 1 (ASCL1) target genes and evaluation of DKK1 and TPH1 expression in pancreatic endocrine tumours

Authors:

Terese A Johansson (terese.johansson@medsci.uu.se)
Gunnar Westin (gunnar.westin@surgsci.uu.se)
Britt Skogseid (britt.skogseid@medsci.uu.se)

Version: 2 Date: 5 June 2009

Author's response to reviews: see over
Uppsala May 20, 2009

To the BioMed Central Editorial Team

Revision of the manuscript no. 5187977625827869 - Identification of Achaete-scute complex-like 1 (ASCL1) target genes and evaluation of DKK1 and TPH1 expression in pancreatic endocrine tumours

We have now revised the manuscript according to the referee - and editorial requests. We have performed the requested qRT-PCR experiment on TPH1 expression in ASCL1 siRNA transfected cells (New Figure 4). Here we also included DKK1. In concordance with the microarray results, both TPH1 and DKK1 clearly showed increased expression, i.e. ASCL1 negatively regulates expression of these genes in BON cells.

We have now included “Authors’ contributions” (line 359).

The microarray data will be deposit on one of the public repositories, as requested.

To clarify, we have made the following changes to the Abstract:

Line 54 (Background)

“Reduced expression of ASCL1 decreases expression of TPH1, the rate…”

has been changed to

“Notch1 signalling regulates expression of TPH1, the rate…”

Line 60 (Results)

“158 annotated ASCL1 target genes were identified in BON1 cells, among them DKK1 and TPH1.”

has been changed to

“158 annotated ASCL1 target genes were identified in BON1 cells, among them DKK1 and TPH1 that were negatively regulated by ASCL1.”

Line 61 (Results)

“An inverse relation of ASCL1 to DKK1 expression was observed for 15 out of 22 tumours (68%).”

has been changed to

“An inverse relation of ASCL1 to DKK1 protein expression was observed for 15 out of 22 tumours (68%).”
We hope that you will find our revised manuscript of sufficient quality and interest for publication in BMC Cancer.

Yours sincerely,

Terese Johansson, Ph.D, and Britt Skogseid, Ph.D., M.D.

Reply to reviewers comments
Manuscript 5187977625827869

Reviewer 1: Herbert Chen

1. We have no data on the roles of Neurogenin-3 in relation to ASCL1 in pancreatic endocrine tumours. This should be addressed in future experiments.

2. The reviewer has demonstrated reduced expression of ASCL1, at the mRNA and protein level, in adenovirus infected BON cells overexpressing Notch1. TPH1 mRNA expression was also reduced (Nakakura et al., ref. no. 12). By directly reducing the expression of ASCL1 by siRNA during transient transfection we demonstrated increased expression of TPH1 in BON cells. This suggests to us that the reduced expression of TPH1 during Notch1 overexpression must be attributed to Notch1 signalling pathway regulatory factor(s) other than ASCL1, thereby explaining the seemingly contradictory results of the two experiments. Perhaps one should bear in mind that heavy overexpression of Notch1 may cause unwanted effects in cell culture.

To clarify, the following sentence has been introduced in the “Discussion” section after the sentence “ASCL1 was found to negatively regulate DKK1 and TPH1 expression in BON1 cells” (line 310): “This may suggest that Notch1 signalling pathway regulatory factor(s) other than ASCL1 is involved in the reduced expression of TPH1 observed in Notch1 overexpressing BON cells (12).”

3. We agree that the technical quality could be improved, but nevertheless the results clearly show reduced ASCL1 protein expression level in siRNA-transfected cells compared to the control. RNA from transfections with ASCL1 siRNA/B was not used in the microarray expression analysis. For clarity, we have removed all information regarding ASCL1 siRNA/B in the “Methods” and “Results” sections.

4. To assess expected effects of reducing the ASCL1 expression level of previously known target genes we only measured transcription, the primary target of siRNA. To determine “if the degree of ASCL1 reduction protein by siRNA corresponds to direct suppression of these factors” is out of scope of this report.

5. No, this has not been addressed.
Reviewer 2: Satyanrayana Rao

We would like to remind the reviewer that PETs are rarely occurring, and that 23 specimens were analysed.

We agree that the obtained results regarding TPH1 protein expression are nonconclusive regarding the relation to ASCL1, and we have not stated otherwise. Evaluation of TPH1 protein expression in PETs has not been published previously and we think that the observed heterogenous expression patterns are of interest.

TPH1 was included in the title also because the results showed that TPH1 constitutes a ASCL1 target gene, which has not been directly demonstrated previously.

Generally, binding sites for ASCL1 have not been determined/identified. Clearly, it is not relevant just to search for putative binding sites without experimental verification. Whether ASCL1 has direct or indirect effects on expression remains to be determined for individual genes of interest.

It is not clear why one should expect an ASCL1 binding site in the TCF3 gene. It does not constitute a target gene (Fig. 2C). To clarify our intention the sentence “Furthermore, we also assessed expression of the transcription factor TCF3 (E12/E47), a putative dimerization partner of ASCL1 that is required for transcription activation (21, 22).” has been changed to (line 243)"

"As a putative negative control we also assessed expression of the transcription factor TCF3 (E12/E47); a recognised dimerisation partner of ASCL1 that is required for transcription activation of ASCL1 target genes (21, 22)."

1. We think that the results are more clearly visualised in this way.

2. The findings are explained in the paper.

See also # 3 of Reviewer 1:

“3. We agree that the technical quality could be improved, but nevertheless the results clearly show reduced ASCL1 protein expression level in siRNA-transfected cells compared to the control.”
Reviewer 3: Eric K Nakakura

1. Dr. Nakakura is the first author of the referenced JCEM paper (ref. no. 12).

See comments to Reviewer 1 (# 2):

“2. The reviewer has demonstrated reduced expression of ASCL1, at the mRNA and protein level, in adenovirus infected BON cells overexpressing Notch1. TPH1 mRNA expression was also reduced (Nakakura et al., ref. no. 12). By directly reducing the expression of ASCL1 by siRNA during transient transfection we demonstrated increased expression of TPH1 in BON cells. This suggests to us that the reduced expression of TPH1 during Notch1 overexpression must be attributed to Notch1 signalling pathway regulatory factor(s) other than ASCL1, thereby explaining the seemingly contradictory results of the two experiments. Perhaps one should bear in mind that heavy overexpression of Notch1 may cause unwanted effects in cell culture. To clarify, the following sentence has been introduced in the “DISCUSSION” section after the sentence “ASCL1 was found to negatively regulate DKK1 and TPH1 expression in BON1 cells” (line 310): “This may suggest that Notch1 signalling pathway regulatory factor(s) other than ASCL1 is involved in the reduced expression of TPH1 observed in Notch1 overexpressing BON cells (12).”

As requested, the microarray data for TPH1 have been verified by quantitative RT-PCR analysis (new Fig. 4). Line 258: “Expression of both DKK1 and TPH1 were found to be increased in ASCL1 siRNA transfected cells (Table 1; Figure 4). Thus, ASCL1 negatively regulates DKK1 and TPH1 in BON1 pancreatic endocrine tumour cells.”

Like Nakakura et al., we have not performed analysis at the protein level.

2. Quantitative RT-PCR analysis showed a small reduction of synaptophysin expression (≤ 2-fold) in the presence of ASCL1 siRNA (Fig. 2A). It is therefore quite possible that this small difference was excluded from the microarray analysis, since we selected probe sets with an adjusted p-value < 0.01 and an abs (log2 ratio) equal to or larger than 1 (which corresponds to a two-fold change in expression).

3. In the new Fig. 4 we also show increased expression of DKK1 in ASCL1 siRNA transfected cells, by quantitative RT-PCR analysis, consistent with the microarray result. The quality of the microarray analysis was also ascertained by the fact that reduced expression of ASCL1 was clearly detected (Table 2).

4. We did see an inversed relation of ASCL1 and DKK1 protein expression in 15 out of the 22 analysed tumours. This is more informative than determining the mRNA expression levels. We also like to point out that evaluation of TPH1 protein expression in PETs has not been published previously, and we think that the observed heterogenous expression patterns are of interest. This particular result cannot be studied/verified by using qRT-PCR.

Regarding sample size, PETs are rarely occurring.

5. We agree, of course, but think that these experiments belong to future explorations.