Author's response to reviews

Title: Small intestinal mucosa expression of putative chaperone fls485

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Version: 2 Date: 25 October 2009

Author's response to reviews: see over
Dear Professor M. Norton, Editor-in-Chief,

Dear Professor J. Ann Le Good, Scientific Editor,

please find attached the revised version of our manuscript “Small intestinal mucosa expression of putative chaperone fls485” (Ms. No. 1050521622591076) that we wish to re-submit for consideration for publication in *BMC Gastroenterology*.

We have addressed the Editor’s and Reviewer’s comments, and performed additional experiments including a functional test. A detailed point-by-point response to the comments is provided. In the revised version of the manuscript all revisions are marked by underlining.

Please do not hesitate to contact us if any further information is required.

Sincerely,

Nikolaus Gassler
We thank the Editors and the Reviewers for the thoughtful comments and suggestions for improvement of the manuscript. In the following lines we specify – point by point – how we revised the manuscript according to the remarks of the reviewers.

Reviewer: Michael George
Reviewer's report:
The authors present a study of fls485 mRNA and protein localization in human small intestinal tissue with the goal of increasing understanding of its potential to function in differentiation of epithelial cells along the crypt-villus axis. While such information would be of interest in the field, there are concerns with this set of data that will need to be addressed. No functional information was presented and the localization analysis with regard to the CVA did not supply any relevant new information. Improvements in the experiments and data presentation will help to more clearly address the questions of fls485 localization and function.

Thank you very much for your comments and suggestions for improvement of the manuscript.

In response to your criticism, functional data were now included in the body of the manuscript. For this purpose, the CaCo2 maturation model was used as suggested by Reviewer Mark Musch. In this established model important functional aspects of cellular maturation along the crypt-villus axis are displayed. The experiments were performed by the co-author Ursula Schneider and the new co-author Christina Klaus. The authorship approved inclusion of Christina Klaus as a new co-author of the manuscript.

We feel that the reviewer statement ‘the localization analysis with regard to the CVA did not supply any relevant new information’ is not correct. The presence of fls485 protein in the CVA is not published up to now. All relevant studies refer to FLS485 gene expression, but fls485 protein synthesis is not addressed. The phenomenon of fls485 protein synthesis and the protein distribution along the CVA are important new data given in the present version of the manuscript.

**Major Compulsory Revisions:**
1. No molecular weight markers were run with the Western blots gels, thus detection of fls485 cannot be confirmed. The Western blot analysis in Figure 2C-D showing significantly different staining patterns in tissues and a loss of the double band at 50-55 KDa detected by Ab #7 in the cell lines in Figure 1 adds further concerns to the protein analyses. Improvements may require a better antibody. It would be very interesting to see fluorescent images of GFP tagged fls485 in healthy and diseased tissues, and may add considerable insight into both localization and function.

Ad 1. In all Western blot experiments, the molecular weight marker Rainbow Marker (Amersham) was used. This point is now clearly addressed in the section Material and Methods.

We agree with the referee that additional anti-fls485 antibodies are useful for the in-detail analysis of the fls485 protein. As stated in the section Discussion, however, the establishment of anti-fls485 antibodies was already performed in a multi-step procedure. In a first attempt two peptides were synthesized including the most immunogenic fls485 sequences (EKKLLHFIQLV and KRKAKQSRR). Unfortunately, the resulting antibodies were not able to recognize the protein in Western blotting and immunohistochemistry. In a second
approach, the fls485 core protein was synthesized and successfully used for mice experiments. It has to be stressed that the resulting antibodies were from mice immunization with the core protein and not from immunization experiments with short peptides. Consequently, the location of antibody-antigen (fls485) interaction is not clear and different recognition patterns are possible. The phenomenon is demonstrated in different Figures of the manuscript, e.g. Figure 1 and Figure 2C-D. Following your comments, we re-phrased our Discussion regarding this special issue.

The observation of GFP-tagged fls485 is a powerful tool to analyze the protein in healthy and diseased conditions. However, the manuscript presented here was strongly focused on human tissues where the functional use of expression constructs is limited. Unfortunately at present we are not able to analyze fls485 mice models. Therefore we think that your interesting point of criticism could be behind the scope of our present work. However, we will regard this interesting approach for functional application in mice.

2. The data in Figure 3A-B are confusing. Because the control data is plotted, it is unclear what the “fold change” is in comparison to. Figure 3C-D does not supply any information that supports the graphs shown in A-B. The authors should show expression of fls485 in celiac diseased tissue versus healthy control tissue and demonstrate a reduction.

Ad 2. We apologize for the confusion due to the misleading labels in Figure 3A-B, where fls485 expression profiles with different fls485 primer sets are demonstrated. The fold change reflects the difference in fls485 expression between celiac-diseased samples Marsh IIIa-c (expression level 1) and celiac-diseased samples lower than Marsh IIIa or normal tissues (control). To clarify this issue, the labels as well as the Figure legend 3 were changed. In the Figure 3 legend the term ‘controls’ was explained with ‘normal mucosal specimens’.

Anti-fls485 immunostaining of healthy control tissue is already demonstrated in Figure 2E. In our opinion, a comparison of Figure 2E and Figure 3C makes the difference in anti-fls485 immunostaining clear. In this context we would not prefer an additional Figure demonstrating anti-fls485 immunostaining of healthy control tissue, because redundancy is given. In the present Figure setting, Figure 3D is essential to demonstrate our negative control. In response to your criticism Figure 2E is additionally cited in the paragraph entitled ‘Impaired expression of fls483 in celiac disease’.

Minor Essential Revisions:
1. The authors should provide references for numerous descriptions of “standard procedures”, “standard protocols”, “conventional clinical and histological criteria”, etc. described in the Methods section.

The references requested were included.

2. Lines should be numbered for review.

To our knowledge, numbering of lines is not requested by the Journal and it could be the case that this contradicts the Journal style. Therefore lines were not numbered in the revised version of the manuscript. Thank you for your understanding.
3. The Discussion should focus less on restating previous findings and discuss the implications of the results obtained by the authors. This section is expected to improve substantially once the suggested experimental improvements have been made.

As suggested by the Referee, the section Discussion was re-written and improved. Implications of additional experiments were included.
Reviewer: Mark Musch
Reviewer's report:
The manuscript of Reinartz et al investigates the expression of a chaperone protein fls485 in intestinal epithelia. This gene product has received little attention, however, it possesses many domains that suggest it could be an important regulator of cell growth and or differentiation. An important facet of the studies is the generation of antibodies that allow assessment of protein expression. The authors determine that this gene product is expressed in intestinal epithelial cells in both crypt and villus enterocytes, but the expression is higher in the villus cells, consistent with a potential role in differentiation. Fls485 expression was also found in some cells of the lamina propria, supporting this its expression is not confined to epithelial cells. The investigators also demonstrate expression in a number of cultured intestinal epithelial cell lines. Fls485 is determined to be a cytosolic protein and the expression is decreased in celiac disease. The studies are potentially interesting and fls485 has the probability of being an important regulatory protein in the epithelial cells, however, at present the impact of the studies is limited since a functional role has not been established.

Thank you very much for your comments and suggestions for improvement of the manuscript.

We do absolutely agree with you that the impact of the study could be essentially improved by functional studies. As stated below, functional studies were now successfully performed using the proposed CaCo2 cell culture system.

Major Compulsory Revisions
(1) The investigators have the opportunity to determine in fls485 may have a role in differentiation using an established cultured in vitro cell line, Caco2. This cell line differentiates post-confluence in culture. Expression of villin, cysteine-sensitive alkaline phosphatase, and sucrase increase after Caco2 cells come to contact inhibition of growth. While it may be presumptive to state that this is precisely representative of maturation of villus small intestinal enterocytes, it is a model that is well accepted. It is stated that this cell line expresses fls485 and the investigators have the expertise to determine if fls485 expression increases as these cells mature. This investigation should also include studies of the maturation to determine if one or more of the differentiated characteristics are regulated by fls485. The investigators could extend these studies using RNA silencing technologies to determine whether fls485 is necessary for maturation or simply expression parallels maturation of other markers.

Following your recommendations, the proposed CaCo2 cell culture system was established and cellular maturation was controlled by villin expression. Interestingly, the maturation process of CaCo2 cells was clearly paralleled by an increase in fls485 expression. These functional data reflect the descriptive in situ findings in human intestinal tissues very well. In the revised version of the manuscript, the new functional findings very included and illustrated in Figure 3.

The additional experiments were performed by the co-author Ursula Schneider and the new co-author Christina Klaus. The authorship approved inclusion of Christina Klaus as a new co-author of the manuscript.

Your idea to investigate consequences of fls485 silencing in the CaCo2 model is excellent. Although we have spent much effort to establish the technique, the fls485 knockdown was not significant. Unfortunately, we have no chance to include these interesting experiments in the revised version of the manuscript, because time is running out for manuscript revision.
However, we will further investigate this issue and include the data in a forthcoming manuscript. In the revised version of the manuscript the interesting point of fls485 silencing was already addressed in the section Discussion.

(2) Another point that could be presented more clearly is the reason for investigations of celiac disease and not other intestinal pathologies. Fls485 expression might be anticipated to be up or downregulated by a number of conditions such as bacterial infections by Salmonella or viral infection by rotavirus. These two conditions may be difficult to obtain, however, through the connection to the Pathology department, the investigators may be able to obtain sections of inflammatory bowel diseases, both pediatric and adult. It is appreciated that immunohistochemistry is a poorly quantitative technology however, to obtain fresh tissues for Western blotting and better quantitation would be excellent, but perhaps beyond the scope of the present studies. Another condition that might regulate fls485 expression is colon cancer where the importance of other chaperone proteins is being established as biomarkers as well as in the pathogenesis of the disease. Sections from a Pathology bank could well provide important information that allow further investigation of the roles of this protein.

Our intention was to analyse fls485 expression in the crypt-villus/plateau axis of human intestine. For this purpose a panel of anti-fls485 antibodies was synthesized and used for immunohistochemical studies of normal intestinal mucosa. In our opinion these studies are best completed by investigation of tissue specimens with celiac disease, especially tissue damage Marsh III. Marsh III conditions are characterized by villi damage, elongation of crypts, and disturbed epithelial function. These are very useful prerequisites to study expression of molecules with a crypt-villus/plateau axis gradient, such as fls485. The gradual mucosal damage is exclusively found in celiac disease and not in the other condition (i.e. inflammatory bowel disease, colon cancer). In summary, we think that celiac diseased mucosal probes are the best in vivo model to analyse the fls485 tissue gradient expression along crypt-villus/plateau axis.

We do absolutely agree with you that conditions like bacterial infections, inflammatory bowel disease, and colon cancer are very interesting candidates for additional analyses of mucosal fls485 expression. In cooperation with our clinicians, surgical specimens appropriate for fresh tissue preparations and subsequent molecular analyses should be available. However, the present manuscript was intended to analyse the fls485 gradient in human intestinal mucosa. We think that the aim of this original study could be hampered by inclusion of pathological conditions which are not mainly characterized by disturbed crypt-villus/plateau axis. Therefore we would prefer not to include these data, because we feel that the conditions are beyond the scope of the present studies.

Minor Essential Revisions
(1) For the studies of intestine, it would be good to also include a longitudinal study of fls485 expression. Sections of stomach, duodenum, jejunum, ileum, cecum, proximal and distal colon are readily obtained from animal models and perhaps the investigators have access to a pathology bank of preserved tissues. For these sections, it would be interesting and potentially important to establish which epithelial cells in each segment express fls485. By itself this survey is not sufficient for publication, but it would add to the initial investigations of this gene product.
We do absolutely agree with you. New experiments were performed and the additional data were addressed in the revised version of the manuscript, section Results and Figure 2C,D.
Reviewer: Dan Hershko

Reviewer's report:

In the present manuscript, the authors describe the expression of fls485 in the small mucosa of normal subjects and patients with celiac disease. This study was followed previous findings by the group that the fls485 gene was 1 among 415 genes expressed in enterocytes. The expression and function of here they show that fls485 protein in human tissues is unknown.

The authors have found, as previously reported, that fls485 is found with increasing gradient from crypt to villi of normal mucosa and that the expression of the protein is impaired in celiac disease Marsh IIIa-c.

Thank you very much for your comments and suggestions for improvement of the manuscript.

In the present manuscript we want to show the in situ mucosal distribution of fls485 expression and synthesis in normal and diseased human intestinal tissues. These points are not addressed in the Gut-paper, where expression profiling of enterocytes isolated from the upper parts of small intestinal crypts or villi was performed and fls485 was identified. The present manuscript includes several new data which were not addressed in previous work. We think that your statement ‘the authors have found, as previously reported, that fls485 is found with increasing gradient…’ does not reflect the bi-modal experimental approach to investigate fls485 expression and synthesis.

Major concerns:

1. The antibodies generated expressed two distinct bands, one of the expected size and another unexpected band. This may lead to false interpretation of the results observed by immunohistochemistry.

   We do agree with you that this point is of high importance. We think our observation that fls485 mRNA in situ hybridization data parallel the immunohistochemical findings points to valid antibody function in immunohistochemistry. As stated in the manuscript, the immunohistochemical findings with antibodies #7 and #10 did not differ. Therefore some evidence is given for valid antibody function in immunohistochemistry.

2. Although the authors claim that an agreement between mRNA exists, they do not provide the results of all experiments and the distribution of the results in normal mucosa. Attempts should also be made to reproduce the results of in-situ hybridization.

   As stated in the section Methods, the expression of fls485 mRNA was analysed with two primer pairs. This procedure was necessary to make sure that the overwhelming number of fls485 mRNA species was detected. Figures 3A and 3B illustrate the findings from all experiments. The n-number of mucosal probes is clearly stated in the section Results including normal intestinal mucosa: n=28; Marsh I and II specimens: n=6; Marsh IIIa-c specimens: n=8. The experiments were repeated three-times and then summarized in the Figures 3A and 3B. In this setting distribution of mRNA in normal mucosa was investigated and illustrated in the Figure.

   In situ hybridization experiments were performed on different human intestinal tissues and sense controls were used in parallel. As stated in the manuscript, the in situ distribution of fls485 mRNA displayed not an increasing gradient which was in contrast to the protein and array findings. However, the in situ mRNA results were repeatedly found and reproduced...
using non-radioactive mRNA in situ hybridization. At present, the scientific reasons for the missing mRNA gradient in in situ mRNA hybridization experiments are not clear. However, the issue was already addressed in the section Discussion of the manuscript. It is assumed that existence of several fls485 mRNA species with differences in hybridization affinity to our anti-fls485 riboprobe could be the most important reason. As proposed in the manuscript, tissue dissection along the crypt-villus axis and subsequent qRT-PCR analysis could be one approach to further investigate fls485 mRNA tissue distribution. In the time period for manuscript revision, however, it was not feasible to perform these additional experiments.

3. Since the function and meaning of these results are still unclear the importance of the findings are of limited interest.

We do agree with you that function and meaning of the findings are still unclear. However, we wish to stress that the present study is the first one that gives evidence for existence of fls485 protein in the intestine. In addition, the tools to perform functional analyses in human tissues are limited and this manuscript exclusively presents data of human tissues/ cells. We think that generation and analysis of the fls485 mice is evidently behind the scope of the manuscript. In response to recommendations of referee 2, a CaCo2 model was used to further elucidate functional aspects of fls485 expression. In this setting the maturation process of CaCo2 cells was clearly paralleled by an increase in fls485 expression. These data are now included in the body of the revised manuscript and are promising for additional fls485 silencing experiments. The functional experiments were performed by the co-author Ursula Schneider and the new co-author Christina Klaus. The authorship approved inclusion of Christina Klaus as a new co-author of the manuscript.