**Department of Molecular Genetics, Biochemistry and Microbiology**
University of Cincinnati Medical enter
PO Box 670524
Cincinnati OH 45267-0524

3110 Medical Sciences Building
231 Albert Sabin Way
Phone (513) 558-5324
Fax (513) 558-8474

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Dear Editor,

I am submitting the revised version of our invited Basic Science Study (ID: 00037529; **ESPS Manuscript NO: 21138**), which has been tentatively accepted for publication in the World Journal of Gastrointestinal Pathophysiology.

We have updated the title, abstract and other sections of the manuscript according to the Guidelines and Requirements for Manuscript Revision-Basic Study. The revised title is "Differential Expression of Pancreatic Protein and Chemosensing Receptor mRNAs in NKCC1-null Intestine", which meets the 12 word limit.

In addition to these revisions we have responded to all of the suggestions of the reviewers and made the necessary corrections as described below.

**Reviewer 1 (00126196)** The core of this manuscript is a rigorous, well-controlled microarray study which revealed interesting gene expression changes in the small intestine of the Nkcc1 KO mice. The authors have carefully controlled for the genetic background of the KO mice, and chose to focus their attention on mRNA species showing reasonably robust levels in either control or KO samples. They also validated the gene expression changes found in the microarray data set with other methods such as RT-PCR. Their finding that pancreatic genes and chemosensory genes are ectopically expressed suggests that cell fate specification defects may exist in the KO intestine. The manuscript is well-written and easy to understand. I have nothing to add except for the following comments/questions, which I hope the authors will address in their discussion:

1. The microarray analysis was performed on 8-week old mice, so the differential gene expression which the authors observed could be due to either defects in cell differentiation that are still ongoing from the regular turnover of intestinal cells or developmental defects that occurred earlier in life. The authors could perhaps acknowledge and discuss these two possibilities.

*Thank you for these suggestions. We have included a paragraph near the end of the Discussion (page 17) in which we discuss in more detail the possibility of defects in cell differentiation and/or development. We cite previous studies in which detailed histological and morphometric analyses of the intestinal tract of 8-week old mice (the same age used in the current study) revealed no differences between WT and KO mice. This argues against major defects in cell differentiation and/or development, however, we note that subtle changes in differentiation, including transient changes in key cell fate regulators that have not been detected by our analyses cannot be ruled out (also, see response to points 2 and 4).*

2. The authors discussed two transcription factors involved in pancreatic and intestinal development, but did not find profound changes in either genes. Could it be possible that changes in expression levels of key cell fate regulators have occurred transiently or only in highly restricted population of cells, thus evading detection in the current analysis?

*This is certainly a possibility. In the same paragraph mentioned in point 1 we now note that the modest changes in these transcription factors also argue against major defects in cell differentiation and/or development. However, we also note the possibility that expression changes in key cell fate regulators have occurred, but were not detected in our analyses.*

3. Is it known whether the electrical excitability of cells is important in the development of the intestine, pancreas or the olfactory epithelium?

*This is an interesting possibility. As far as we are aware, there have been no studies examining the role of electrical excitability in the development of the intestine, pancreas or olfactory epithelium so we have not commented on this in the paper. However, a colleague recently showed me some very convincing unpublished data showing that rhythmic electrical stimulation of cardiac muscle precursor cells facilitates their differentiation into beating cardiomyocytes. Thus, it is possible that it plays a role in other these other tissues as well.*

4. The authors mentioned the potential relevance of the current findings to the observation of side effects of loop diuretics. If this connection is real, it may suggest that the cell fate defects coming from impairment of NKCC1 function occur during the continual cellular regeneration in adult intestine as opposed to some defective checkpoint early during development (point 1).

*In the final paragraph we include two references to intestinal dysfunction related to use of loop diuretics and comment on the possibility that this could involve developmental abnormalities in intestinal epithelial cells and/or a response to impaired chemosensing.*

**Reviewer 2 (03119158)** This study aimed to analyze mRNA expression changes in the intestine of Nkcc1-/- mice. In my view, methods were properly used to identify cell types, and results were also presented clearly. However, the manuscript has to be improved on the basis of the followings before it can be possibly published. Comments:

(1) The title and the aim stated in Abstract seemed not to be in consistency. Actually, the title of the manuscript should be more directly relevant to the focus of this study.

*We changed both the title and the Aim to better indicate that we were measuring differential expression of mRNAs in response to the loss of NKCC1. The new title is: "Differential Expression of Pancreatic Protein and Chemosensing Receptor mRNAs in NKCC1-null Intestine".*

(2) The Conclusions part in Abstract should be brief. And statement about the evidence to prove conclusions can be deleted.

*We shortened the conclusion and removed the speculative statements as suggested.*

(3) Results of this study were partially demonstrated in Introduction, while it's the background information and the aim of this study that should be stated. Therefore, please adjust.

*In the final paragraph of the Introduction, we removed the three sentences mentioning the primary results of the microarray analyses. We agree that this is appropriate as these results are clearly stated in the Abstract.*

(4) Main methods, such as RT-PCR, HE/AB/PAS staining and immunofluorescence mentioned in text should also be indicated in Abstract.

*We have included this in the Abstract, which now mentions Northern blot analysis, quantitative Real Time-PCR, and immunofluorescence and histological staining.*

(5) Please provide the full name of "FVB/N" when it appeared for the first time in the text.

*In the introduction (at the first mention of FVB/N) we now note that FVB/N refers to Swiss mice carrying the Fv1b sensitizing allele. It should be noted that FVB/N is not an abbreviation of the name of the strain so it requires some explanation to understand the designation. As for most mouse strains, the letter and number designations for FVB/N come from previous strains used to derive the current strain. We have added this explanation in the first section of Methods.*

(6) Information on the origin of WT mice and its total number should be given.

*We now include the origin of the WT mice (Jackson Labs) used to derive the inbred NKCC1-null strain and provide the numbers of mice used in each experiment. Also, we now explain (in Materials and Methods) that both WT and knockout mice were obtained from the same litters after mating of NKCC1 heterozygous mice that were on a highly inbred FVB/N background. Thus, the WT mice are identical to the KO mice except for loss of NKCC1.*

(7) It's preferable to list primer sequences used for northern blots and real-time PCR analysis in a table, which would be easier to read. And if possible, please add the results of negative controls by northern blots and real-time PCR analysis.

*We now present the primers for northern blots and real-time PCR in new Table 1. Analyses of the negative controls (WT in which NKCC1 has not been ablated) has been performed for all samples. These data are presented in Fig. 1 and used to derive fold-change values presented in the tables.*

(8) I suggest to present the morphology of the gastrointestinal tract in figures.

*Work from different laboratories using two different NKCC1 knockouts have reported extensively on the functional differences between NKCC1-null and WT intestine but have observed no histological or morphometric differences between the two genotypes in the adult intestine. We now cite these previous studies and briefly explain that no morphological differences were observed. These previous studies included morphometric measurements using both light and electron microscopy, so it is now well established that the morphology of the WT and KO intestine are the same.*

We greatly appreciate the efforts of you and the reviewers in handling this manuscript and look forward to submitting additional manuscripts to WJGP in the future.

Best wishes,

Gary E. Shull, Ph.D.
Professor