nature portfolio

Peer Review File

Low Expression of CCKBR in the Acinar Cells is Associated with Insufficient Starch Hydrolysis in Ruminants

Corresponding Author: Dr Yan Cheng

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study assessed adult and neonatal goat by proteomics and sc-RNA sequencing to further evaluate fundamental basis of pancreatic exocrine insufficiency. Authors claimed that they identified mature acinar cell phenotype and increased exocytosis response in adult goat. Unfortunately, presented data only consist of simple comparison between adult and neonatal goats, which should be interpreted as developmental differences. To evaluate underlying mechanism of PEI, authors should utilize adequate animal model. Such models have been published already, and simple assessment of pancreatic tissues yields limited information in this field. To address these points, therapeutic interventions besides examination of disease model are indispensable. In addition, detected differences need validation in pancreatic tissues, which also missing in this manuscript. Due to above mentioned defects, I regret to inform authors that this manuscript is not suitable for publication in its present form. Extensive revise and reconsideration of the study design will be necessary.

Reviewer #2

(Remarks to the Author)
Major remark: authors should consider an intermediate age stage corresponding to the weaning which must be integrated in the paper. By looking at the Materials and Methods, the assumption is that in the study pre-weaning (only fed with milk) versus adult animals (fed with a solid and well-balanced diet?) was compared. However, an important point in the pancreatic hypoplasia theory is missing: the diet effect which may modulate the pancreatic cell developmental activity. This factor can only be discovered if authors do consider a third age stage: animals at the weaning. This could corroborate also the transcriptomic data, which are quite weak.

lines 35-37 – the term hypoplasia appears for the first time as the conclusion of the 1st paragraph. However, the rest of the paragraph does not include reference to hypolastic phenomena. Please improve this part. Notably, results stem from hypoplasia concept. This makes the clarification even more relevant. And it is not clear which the main focus of all experimental approach is: are all omics addressed to investigate the adult related pancreatic hypoplasia?

The switch from acinar to B-cells is really interesting and the data would be further corroborated by correlating the plasma levels of insulin in neonatal and adult goats.

As for the immunostaining, formalin fixed tissues raise concerns about antigen masking. This may methodologically affect the outcomes. Furthermore, higher magnification of cells is deemed necessary. In Fig. 6A and C cells can be barely appreciated.
lines 330-332: receptors are generally exposed along the apical part of cytoplasmic membrane, unlikely ca be found at the basal portion. This sounds as a redundancy.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors addressed my questions and concerns adequately. This manuscript is now suitable for publication.

Reviewer #2

(Remarks to the Author)
Some improvements have been incorporated within the manuscript, such as the serum insulin correlation.

Persisting concerns:
- the comparison between neonatal and adult goat (fed wit starch and thus the comparison between the two different dietary regimen (milk vs solid feed) has not comprehesively addressed. How the rumen development affects the adult digestion is well known: here the key question is more related to the glucidic source. Has milk a comparable amount of starch/glucose to be compared with the solid diet? Can authors include, in supplementary files, the macro and micronutrients composition of milk and diet to exclude this variable? Is the diet or the developmental process responsible of shaping the pancreatinf physiology? The best would be to include a third group at weaning, to go in-depth into the transition and effectively test the hypothesis.
- the immunohistochemical staining are still not of good quality, very low magnification, and the nuclei counterstaining hampers the immunopositive signal.
- lines 311-315 and Fig. 7E: what do authors intend for intestinal type I cell? How do they demonstrate that those cells are CCK-immunoreactive? It seems there is any co-labeling over the text. Moreover, in the ileum the immunostaining appears in the connective axis which supports the intestinal villus, not along the intestinal epithelium.

Reviewer #4

(Remarks to the Author)

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I reviewed the manuscript, several critical points were improved, but others are not addressed and the answers sound weak. For instance, in the lines of rebuttal letter they cite
- "These cells, commonly known as CCK-I cells, have been well-characterized in the literature (Buffa et al., 1976) and are predominantly located in the small intestine." and they do mention a very old paper (1976).
- "The localization of immunoreactivity in the connective axis supporting the intestinal
villi is indeed unusual, as enteroendocrine cells are typically located within the epithelial layer of the intestine. We hypothesize that this may either be a species-specific feature in goats or
potentially a result of non-specific staining in the connective tissue." This hypothesis could be easily tested by using specificity controls.
However, given the huge number of experiments ad data collected, these may turn as minor concerns, and I leave you Editors the final word on the decision.
According to me, it could be accepted for publication despite these inconsistencies.

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In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.
The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
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Author Rebuttal

(Manuscript ID: COMMSBIO-24-1747)

Dear Reviewers,

Thank you for your detailed and constructive feedback on our manuscript. We appreciate the opportunity to address your comments and provide clarifications. We have carefully considered all the comments and have made the appropriate revision. All changes are highlighted by staining with yellow. Below, we have outlined our responses to the key points raised:

Comments to Author:

Reviewer #1:

This study assessed adult and neonatal goat by proteomics and sc-RNA sequencing to further evaluate fundamental basis of pancreatic exocrine insufficiency. Authors claimed that they identified mature acinar cell phenotype and increased exocytosis response in adult goat. Unfortunately, presented data only consist of simple comparison between adult and neonatal goats, which should be interpreted as developmental differences. To evaluate underlying mechanism of PEI, authors should utilize adequate animal model. Such models have been published already, and simple assessment of pancreatic tissues yields limited information in this field. To address these points, therapeutic interventions besides examination of disease model are indispensable. In addition, detected differences need validation in pancreatic tissues, which also missing in this manuscript. Due to above mentioned defects, I regret to inform authors that this manuscript is not suitable for publication in its present form. Extensive revise and reconsideration of the study design will be necessary.

Adequate Animal Models:

Response: We understand the importance of using adequate animal models to study PEI. Our study aims to investigate the changes in pancreatic exocrine function associated with the transition of digestion patterns following rumen development in ruminants. Specifically, we focus on the differences between neonatal (non-ruminating mode) and adult goats (ruminating mode). This aspect is crucial and may not be adequately addressed by existing PEI models, which are typically designed to study disease states rather than normal physiological processes (Saloman JL, Albers KM, Cruz-Monserrate Z, Davis BM, Edderkaoui M, Eibl G, Epouhe AY, Gedeon JY, Gorelick FS, Grippo PJ, Groblewski GE, Husain SZ, Lai KKY, Pandol SJ, Uc A, Wen L, Whitcomb DC. Animal

Models: Challenges and Opportunities to Determine Optimal Experimental Models of Pancreatitis and Pancreatic Cancer. Pancreas. 2019 Jul;48(6):759-779. doi: 10.1097/MPA.0000000000001335. PMID: 31206467; PMCID: PMC6581211.).

Line 23-44: Starch digestibility in the small intestine is generally lower in ruminants than in monogastric animals, and there are indications that ruminants have a limited capacity to enzymatically hydrolyze starch. Previous studies have shown that starch digestibility in the small intestine is quite low in adult ruminants, with approximately 40-60% of rumen bypass starch remaining undigested. This suggests that pancreatic function and regulation may differ between ruminants and monogastric animals, likely due to the majority of dietary nutrients being fermented in the rumen. We hypothesized that the transition in digestion patterns following rumen development may contribute to the limitation of pancreatic function. Our study aims to elucidate the underlying mechanisms by comparing neonatal and adult goats, with a focus distinct from studying pancreatic exocrine insufficiency (PEI) in a disease context.

Therapeutic Interventions and Disease Models:

Response: We recognize the need for therapeutic interventions to provide more comprehensive insights. However, it's not the scope of this study. The primary objective of our study was to investigate the changes in pancreatic exocrine function associated with the transition of digestion patterns after rumen development in ruminants. We believe that understanding these fundamental pancreatic digestive differences is a crucial first step before exploring potential therapeutic strategies.

Validation of Detected Differences:

Response: In Lines 281-286, we have already performed additional validation experiments using immunohistochemistry and Western blotting to confirm the expression levels of key proteins and genes identified in our analyses. This would strengthen the robustness of our findings and provide more conclusive evidence.

Reviewer #2:

Major remark: authors should consider an intermediate age stage corresponding to the weaning which must be integrated in the paper. By looking at the Materials and Methods, the assumption is that in the study pre-weaning (only fed with milk) versus adult animals (fed with a solid and well-balanced diet?) was compared. However, an important point in the pancreatic hypoplasia theory is missing: the diet effect which may modulate the pancreatic cell developmental activity. This factor can only be discovered if authors do consider a third age stage: animals at the weaning. This could corroborate also the transcriptomic data, which are quite weak.

lines 35-37 – the term hypoplasia appears for the first time as the conclusion of the 1st paragraph. However, the rest of the paragraph does not include reference to hypolastic phenomena. Please improve this part. Notably, results stem from hypoplasia concept. This makes the clarification even more relevant. And it is not clear which the main focus of all experimental approach is: are all omics addressed to investigate the adult related pancreatic hypoplasia?

Response: In Lines 23-44, 54-57, there are two digestion modes for the digestion of nutrients in ruminating ruminants---rumen fermentation-dependent and enzymatic hydrolysis-dependent (small intestine) --- rumen fermentation dominates, followed by enzymatic chemical digestion. The starch digestibility in the small intestinal lumen is generally lower in ruminants than in monogastric animals, and there are indications that the capacity to enzymatically hydrolyze starch in ruminants is limited. It is worth noting that newborn ruminants are known to have the same digestive pattern as monogastric animals, characterized by the dominant enzymatic hydrolysis mode, depending on the secretion of trypsin, lipase, and α-amylase from the pancreas to the small intestine. With the development of the rumen, ruminants have a unique structure of the stomach, which is different from monogastric animals, including the rumen, reticulum, omasum, and abomasum, reflecting the significant transition in digestion patterns. Thus, the pancreas function and regulation may differ between ruminants and monogastric animals because the majority of dietary nutrients may be fermented in the rumen. We hypothesized that the transition of digestion patterns after rumen development may lead to limited pancreatic function, which ultimately induce insufficient starch digestibility in the small intestine of ruminants. Thus, we use proteomic and 10x Genomics scRNA-seq approaches to elucidate the precise mechanisms of pancreatic functional transformation that underlie the two extreme digestion modes--- non-ruminating mode (neonatal goats) and ruminating mode (adult goats).

The switch from acinar to β-cells is really interesting and the data would be further corroborated by correlating the plasma levels of insulin in neonatal and adult goats.

Response: We greatly appreciate your suggestions. We found that serum insulin level in neonatal goats was higher than that in adult goats. The switch from acinar to ELC-cells (characterized by high GCG, INS, and SST expression) was further corroborated by correlating these findings with plasma levels of insulin. These results are consistent with the hypothesis that the activated NOTCH pathway may modulate the enhanced pancreatic exocrine function of AC-s of adult goats by preventing acinar cells from adopting an endocrine fate, as described in Lines 199-208.

As for the immunostaining, formalin fixed tissues raise concerns about antigen masking. This may methodologically affect the outcomes. Furthermore, higher magnification of cells is deemed necessary. In Fig. 6A and C cells can be barely appreciated.

Response: Thanks for the comments. To minimize antigen masking, we added buffer salt to the 10% formalin and adjusted it to a neutral pH. In addition, the samples were immediately fixed with formalin at room temperature after collection, and the fixation time was carefully controlled to reduce the impact on immunohistochemistry outcomes. We have updated the image quality. However, it is worth noting that immunohistochemistry doesn't typically provide detailed images of cell morphology, although the target protein was successfully marked positive in our experiment.

lines 330-332: receptors are generally exposed along the apical part of cytoplasmic membrane, unlikely ca be found at the basal portion. This sounds as a redundancy.

Response: Thanks for your suggestion. We have deleted this sentence.

We have carefully revised the manuscript, resulting in significant improvements. We believe that this updated version effectively addresses all of the reviewers' concerns and comments.

Yours sincerely

All authors

Author Rebuttal

(Manuscript ID: COMMSBIO-24-1747)

Dear Reviewers,

Thank you for your detailed and constructive feedback on our manuscript. We appreciate the opportunity to address your comments and provide clarifications. We have carefully considered all the comments and have made the appropriate revision. All changes are highlighted by staining with yellow. Below, we have outlined our responses to the key points raised:

Comments to Author:

Reviewer #2:

Some improvements have been incorporated within the manuscript, such as the serum insulin correlation.

Response: We thank the reviewer for acknowledging the improvements made in the manuscript, including the incorporation of serum insulin correlation. The correlation between serum insulin levels and pancreatic development/function, along with the distribution of CCK-I cells, has provided deeper insights into the endocrine and exocrine relationships within the goat model. We believe this addition strengthens the physiological relevance of our findings. In Lines 203-206, we have already provided detailed information about the correlation between serum insulin and the manuscript. The activated NOTCH pathway prevents adjacent cells from adopting an endocrine fate. In the manuscript, the expressions of JAG1 and NOTCH1 were elevated at the late developmental stage of AC-s, while the progressive increases of islet GCG, INS and SST expressions were only observed in AC-i during the late developmental stage. Baeyens et al. reported that inhibition of Notch signaling promotes β-cell neogenesis via inhibiting expression of Ngn3, Pdx1, and insulin. Consistent with this, serum insulin content in newborn goats was higher than that in adult goats (Fig. 4F). Thus, the above results were consistent with the possibility that the activated NOTCH pathway may modulate the enhanced pancreatic exocrine function of AC-s of AG by preventing acinar cells from adopting an endocrine fate.

Persisting concerns:

- the comparison between neonatal and adult goat (fed wit starch and thus the comparison between the two different dietary regimen (milk vs solid feed) has not comprehesively addressed. How the

rumen development affects the adult digestion is well known: here the key question is more related to the glucidic source. Has milk a comparable amount of starch/glucose to be compared with the solid diet? Can authors include, in supplementary files, the macro and micronutrients composition of milk and diet to exclude this variable? Is the diet or the developmental process responsible of shaping the pancreatinf physiology? The best would be to include a third group at weaning, to go in-depth into the transition and effectively test the hypothesis.

Response: We acknowledge that differences in diet composition between the neonatal and mature goat groups may contribute to the observed variations in pancreatic development. These differences are likely the result of both developmental age and dietary factors. While the pancreas naturally matures over time, transitioning from a milk-based diet in neonates to a starch-based diet in mature goats likely plays a significant role in shaping its structure and function.

To address the potential confounding effect of diet, we agree that it would be valuable to include the nutrient composition of both milk and solid feed as supplementary information. Providing detailed data on the carbohydrate, protein, fat, and micronutrient content of each diet will help clarify how these differences may influence pancreatic physiology and development. In lines 421-422, the nutrient contents of the two groups were provided.

Although adding a weaning group would offer valuable insights into the transition phase from milk to solid feed, it may not fully resolve the dietary confounding issue, as the weaning diet differs from both neonatal and adult diets. However, future studies will aim to incorporate this transitional phase to provide a more comprehensive analysis of how diet and development interact to shape pancreatic physiology.

- the immunohistochemical staining are still not of good quality, very low magnification, and the nuclei counterstaining hampers the immunopositive signal.

Response: We greatly appreciate your suggestions. We re-sliced and performed immunohistochemical staining. We have provided better quality images, including higher magnification images in Fig. 6 (25x; 600 dpi).

- lines 311-315 and Fig. 7E:

what do authors intend for intestinal type I cell?

Response: We appreciate the reviewer's inquiry and would like to clarify that by "intestinal type I cells", we are referring specifically to enteroendocrine cells that secrete cholecystokinin (CCK). These cells, commonly known as CCK-I cells, have been well-characterized in the literature (Buffa et al., 1976) and are predominantly located in the small intestine. These cells have an apical membrane that contacts the intestinal lumen and a basal region where secretory granules containing CCK peptides are stored (Gilliam-Vigh et al., 2021). This is consistent with the description we provided in the manuscript, and we have updated our terminology to "CCK-I cells " to ensure clarity for the readers.

How do they demonstrate that those cells are CCK-immunoreactive? It seems there is any co-labeling over the text.

Response: We demonstrated that these cells are CCK-immunoreactive via the use of immunofluorescence (IF) using an antibody specific to CCK peptides. As described in the methods section, we used a well-characterized anti-CCK antibody, which has been validated in previous study, to specifically label CCK-producing cells (Zhang et al., 2024). The CCK-immunoreactive cells were primarily found within the mucosal layer of the small intestine in this study, consistent with prior findings in humans and other mammals (Buffa et al., 1976; Gilliam-Vigh et al., 2021; Zhang et al., 2024). In this study, we did not perform co-labeling with additional markers. However, the antibody used has been validated before, and the observed staining is specific for CCK-immunoreactivity. Given the robustness of the CCK antibody, we believe that our identification of these cells is accurate. However, we acknowledge the importance of co-labeling for more comprehensive identification and will consider this in future studies to validate our findings further.

Moreover, in the ileum the immunostaining appears in the connective axis which supports the intestinal villus, not along the intestinal epithelium.

Response: The localization of immunoreactivity in the connective axis supporting the intestinal villi is indeed unusual, as enteroendocrine cells are typically located within the epithelial layer of the intestine. We hypothesize that this may either be a species-specific feature in goats or potentially a result of non-specific staining in the connective tissue.

Literature References:

Baeyens et al. Notch Signaling as Gatekeeper of Rat Acinar-to-beta-Cell Conversion in Vitro. Gastroenterology 2009, 136(5):1750-1760.

Buffa R, Solcia E, Go VL. Immunohistochemical identification of the cholecystokinin cell in the intestinal mucosa. Gastroenterology 1976, 70:528–30.

Gilliam-Vigh et al. Expression of Cholecystokinin and its Receptors in the Intestinal Tract of Type 2 Diabetes Patients and Healthy Controls. Journal of Clinical Endocrinology & Metabolism 2021, 106:2164-2170.

Zhang et al. Micro/nanoplastics impair the feeding of goldfish by disrupting the complicated peripheral and central regulation of appetite. Science of the Total Environment 2024, 946.

We have carefully revised the manuscript, resulting in significant improvements. We believe that this updated version effectively addresses all of the reviewers' concerns and comments.

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All authors

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Response: We have now included more recent studies that further characterize CCK-secreting enteroendocrine cells and their localization within the intestinal epithelium. For instance, a study by [Rashmi Chandra and Rodger A. Liddle, 2018] provides a comprehensive analysis of CCK-positive cells in the gastrointestinal tract, confirming their typical epithelial localization and functional roles in various species (https://pancreapedia.org/molecules/cholecystokinin).

We have carefully revised the manuscript, resulting in significant improvements. We believe that this updated version effectively addresses all of the reviewers' concerns and comments.

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