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β cells can be generated from cytokeratin 5-positive cells after cerulein-induced pancreatitis in adult mice

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ABSTRACT

Clinical studies have revealed that some patients will develop glucose tolerance dysfunction after recovering from acute pancreatitis (AP), which indicated the importance of investigating the potential therapies for restoration of islet β cell function. Cytokeratin 5 (Krt5)-positive cells are considered to function as stem or progenitor cells in the regeneration of lung and salivary gland following injury. In the present study, AP was induced by six hourly intraperitoneal injections of 100 μg/kg cerulein for 4 consecutive days in adult mice, in order to determine the role of Krt5-positive cells in pancreatic regeneration, especially in the restoration of β cell function and the underlying mechanisms. Results showed that glucose homeostasis were deteriorated partly during the recovery process after AP. Furthermore, clusters of Krt5-positive cells were significantly increased in the damaged pancreas marked by inflammatory cells infiltration and acinar cell eradication. In addition, cells co-labelling insulin and Krt5 were found in the injured region after cerulein administration, part of these cells were immuno-positive for GLUT2. Taken together, our data demonstrated that Krt5-expressing cells could be involved in the natural pancreas self-healing process and the renewal of β cells after AP in adult mice. It is promising that promoting conversion of Krt5-expressing cells into functional β cells may be a novel method to mitigate the development of diabetes mellitus after AP in vivo.

1. Introduction

Acute pancreatitis (AP) is a sudden inflammatory process of the pancreas, with a rising incidence worldwide [1–3], and an overall low mortality of approximately 1% [4–6]. Traditionally, transient rising in blood glucose is the common early feature that completely resolves in almost all patients [7]. Some patients will develop endocrine pancreatic dysfunction after recovering from AP, which draw more and more attention than before in recent years as more patients survive from severe AP [8,9]. Recently, a meta-analysis revealed a pooled prevalence of diabetes after acute pancreatitis of 23%, and a statistically significant 2.7-fold increased risk of developing diabetes mellitus (DM) 5 years after an AP episode [10]. Another study including 2966 first-attack AP patients and 11,864 non-AP general controls showed that the overall risk of DM more than doubled in up to 10 years after the first-attack AP [11]. These observations indicated that AP damages not only acinar cells but pancreatic islet β cells. Therefore, it is essential to investigate the potential regeneration therapies that allowing for restoration of islet β cell function after AP.

Adult stem/progenitor cells have been proposed to have significant roles in wound healing responses, tissue homeostasis, and regeneration [12–14]. Keratin filaments are important structural stabilizers of epithelial cells. In both humans and mice, Cytokeratin

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5 (Krt5)-immunopositive cells are localized in the basal layers of the stratified epithelium and other types of epithelia lining the epidermis, mammary gland, salivary glands, trachea, prostate, and urothelium, and are considered to be functioned as stem or progenitor cells [15,16]. Previous studies have revealed that Krt5-positive cell might function in the regeneration of lung and submandibular salivary gland following virus infection or radiation injury [17,18]. Up to now, little was known about whether Krt5-positive cells can contribute to pancreas healing or regeneration upon injury. Thus, the present study was designed to investigate the role and mechanism of Krt5-positive cells for β cell dysfunction in AP induced by cerulein in mice.

2. Materials and methods

2.1. Animals and induction of pancreatitis

8-week-old male C57BL/6J mice were obtained from Vital River Company (Beijing, China). All animals were housed in groups in 12 h light/dark cycles under standardized environment with free access to standard laboratory rodent chow and sterile water, and acclimated for more than 3 days prior starting the experiment. The study was performed in compliance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and approved by the Committee on Ethics of Animal Experiments of Renmin Hospital of Wuhan University. AP was induced by six hourly intraperitoneal injections of 100 μg/kg mouse weight cerulein (Sigma-Aldrich, St. Louis, USA) dissolved in 0.9% saline administered on 4 consecutive days, and the control group received saline instead. At day 1 (1d), day 3 (3d) and day 7 (7d) after the last injection of cerulein, eight mice per group were sacrificed and pancreatic tissues were taken for subsequent analyses. No lethality was observed.

2.2. Fasting blood-glucose and glucose tolerance test

For the intraperitoneal glucose tolerance test (IPGTT) at different time points, mice were fasted overnight (12 h) and a basal blood sample was harvested from the tail tip (t = 0 min). Mice received an intraperitoneal injection of 2 g/kg glucose solution, additional blood samples were taken at 15, 30, 60, 90 and 120 min [19]. A handheld OneTouch Ultra glucose meter (LifeScan, Shanghai, China) was used to determine blood glucose levels using test strips. To reflect the glucose tolerance, the areas under the glucose concentration time curve of the glucose concentration after the glucose injection during 120 min were calculated.

2.3. Pancreas histopathological examination

According to our previous methods [20], pancreatic tissue samples, which fixed in the 4% phosphatebuffered formaldehyde, were embedded in paraffin blocks, stained with hematoxylin and eosin and examined with a light microscope finally. The histopathological scoring analysis of pancreas was performed blindly according to the severity and extent of edema, inflammatory cell infiltration and acinar necrosis, as described by Schmidt et al. [21].

2.4. Immunofluorescence analysis

Sections for immunofluorescence were processed with a method described previously [22] and modified as following. The paraffin-embedded pancreas sections were deparaffinized by xylene and hydrated by sequential washing with graded ethanols. Antigen retrieval was conducted in boiling Tris-EDTA buffer with 0.05% Tween 20 (pH 9.0) in a pressure cooker. Then, the sections were permeabilized with 0.2% Triton X-100 (Vetec, Wuxi, China) in PBS and blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, USA) to avoid the unspecific staining. The sections were incubated overnight at 4 °C with the following antibodies: cytokeratin 5 (1:100, Abcam, Cambridge, UK, Cat#ab53121), insulin (1:400, Santa Cruz, USA, Cat#sc-7838), GLUT2(1:200, Absin, Shanghai, China, Cat# abs119876), CD45(1:200, Servicebio, Wuhan, China, Cat#GB11066), CD68 (1:200, Abcam, Cambridge, UK, Cat#ab125212). Alexa Fluor 488 or 594-conjugated secondary antibody (1:200, Abcam, Cambridge, UK) was added to sections and incubated in the dark for 1 h at room temperature. Fluoroshield Mounting Medium With DAPI (Abcam, Cambridge, UK) was applied to visualize the nucleus. The negative control experiments were performed in which PBS was substituted for the primary antibodies. Representative images were captured with an Olympus BX63 microscope (Olympus, Tokyo, Japan).

2.5. Statistical analysis

Data were expressed as means ± SD. The data were analyzed with GraphPad Prism software version 5 (GraphPad Software Inc, San Diego, USA). Statistical significance between two groups were determined by Student's *t*-test, and that among multiple groups were determined by One-way analysis of variance (ANOVA) and the Bonferroni post-hoc test. The correlation of cytokeratin 5-positive cell counts with pancreas histopathological score was analyzed using Spearman correlation test. Differences were considered statistically significant at a value of $P < .05$.

3. Results

3.1. Glucose tolerance and islet injury

Fasting blood-glucose and IPGTT were performed at different time points (1d, 3d and 7d) after the last injection of cerulein. The blood glucose levels during the IPGTT were showed in Fig. 1A. Both AP3d and AP7d groups showed significant high fast blood glucose (FBG) levels compared to the CON group ($P < .05$) (Fig. 1B). The areas under the curve (AUC) for the glycemia over the 120 min time period was represented in Fig. 1C; the AUC in the AP3d and AP7d group significantly increased compared to the CON group ($P < .05$). However, there were no significant differences between the AP1d group and the CON group for above parameters.

Morphological analysis and immunofluorescence staining for insulin expression were performed in pancreatic islet, and confirmed a substantial loss of islet endocrine cells in AP mice at 1d post treatment (Fig. 1D and E).

3.2. Pancreatic histological damage, Krt5 expression and β cell neogenesis

The changes in pancreatic histology were evaluated at 1, 3, and 7 days after the last cerulein injection. Normal pancreas architecture was observed in all control mice. In AP mice, extensive acinar cell injury and necrosis, abundant inflammatory cell infiltration in pancreatic tissue were found at 1d. At 3d, the pancreatic tissue morphology was in the process of recovering and at 7d, it appeared almost complete recovery (Fig. 2A).

To study the role of Krt5 in acute pancreatitis, its expression was measured in pancreas of mice. In CON group, rare Krt5-positive cells interspersed in the interlobular and intercellular spaces. Compared with CON group, the expression of Krt5 was significantly increased in pancreas of mice at 1d after the last cerulein injection, when the severity of acute pancreatitis was at its peak. Correspondingly, at 3d and 7d post treatment, Krt5 expression was

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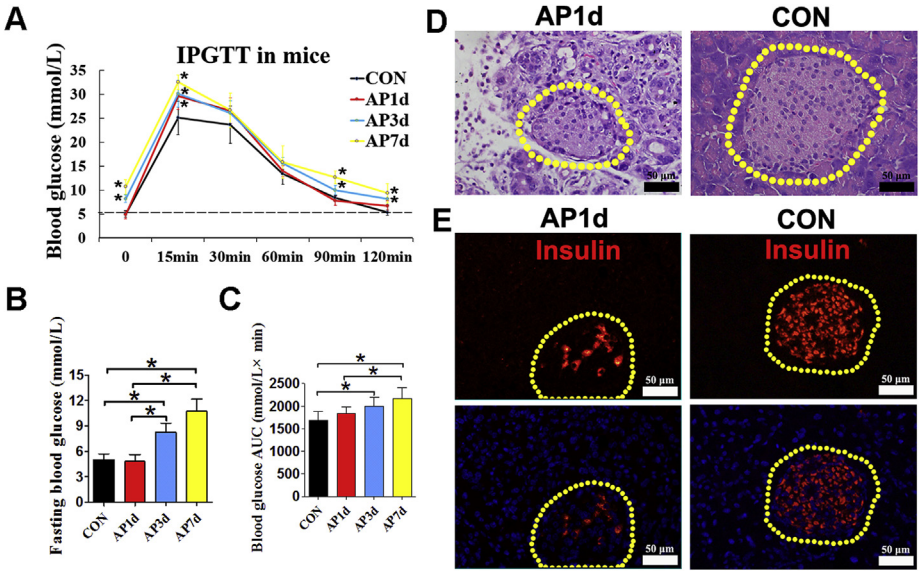

Fig. 1. Impaired glucose tolerance and islet injury in response to cerulein-induced acute pancreatitis. (A) Blood glucose levels during the intraperitoneal glucose tolerance test (IPGTT), *$P < .05$ vs. CON group. (B) Fasting glucose levels, (C) AUC of the glycemia over 120 min, *$P < .05$ indicate a significant difference between the marked groups. (D) Hematoxylin and eosin staining of the pancreatic islet in mice were analyzed at control group and 1d after cerulein treatment, (E) Immunofluorescent staining for insulin expression in islet. Note the decline in the number of β cell in AP1d mice. The dashed line marks an islet. Scale bar = 50 μm.

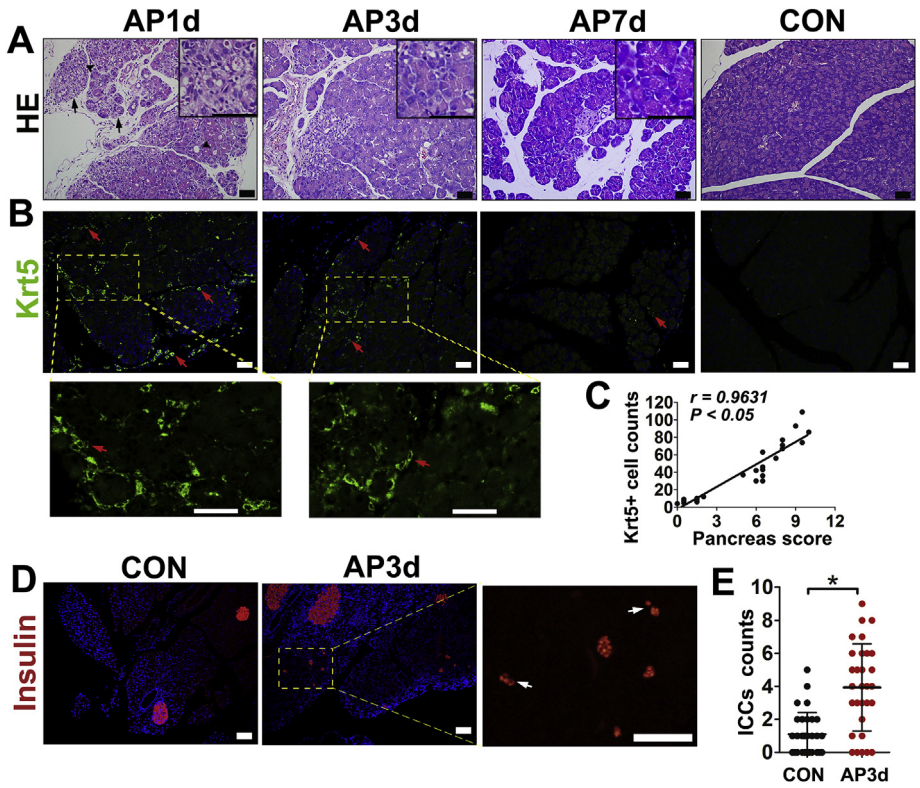

Fig. 2. Pancreatic histopathological damages, dynamics of Krt5-expressing cell population and β cell neogenesis after cerulein-induced AP. (A) Representative images of pancreas at control, 1, 3 and 7 days after the last cerulein injection were shown. Note the appearance of marked edema, strong leucocytic infiltration (black arrowheads), disruption of the acinar cell architecture (black arrows) in AP1d mice, and the injuries were decreased in mice of AP3d and AP7d. Scale bar = 50 μm. Insets, high magnification. (B) Immunofluorescent staining of Krt5 (green) in pancreas at control, 1, 3 and 7 days after the last cerulein injection were shown. Clusters of Krt5-positive cells (red arrows) spread over interlobular and intercellular spaces in pancreas of AP1d mice and less expression of Krt5 was observed in AP3d and AP7d. DAPI (blue) was used to counterstain nuclei. Scale bar = 50 μm. Insets, high magnification. (C) Correlation analysis revealed that Krt5-positive cell counts were positively correlated with pancreas histopathological score. (D) Number of small islet-like cell clusters (ICCs) was significantly increased in mice of AP3d (white arrows). DAPI (blue) was used to counterstain nuclei. Scale bar = 100 μm. (E) The number of ICCs that contain five or less insulin-positive cells was counted. Values are representative of ten low power fields per pancreas section of each mouse and from three mice per group. *P < .05 indicate a significant difference between the marked groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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reduced in the regions undergoing regenerative repair (Fig. 2B). Moreover, Spearman correlation analysis revealed that Krt5-positive cell counts were positively correlated with pancreas histopathological score ($r = 0.9631$, $P < .05$) (Fig. 2C), suggesting that Krt5 expression was consistent with the severity of pancreatic injury following AP.

In CON group, the small islet-like cell clusters (ICCs)–with five or less insulin-positive cells, were either rare or undetectable in the pancreas in our observation. Interestingly, a significant increase in the number of ICCs was seen at 3d after the last cerulein injection (Fig. 2D and E), suggesting that ICCs may be increased compensatorily for the loss of β cells in islet after AP.

3.3. Co-localization of Krt5-positive cell and inflammatory cell infiltrate

For further characterization, sequential sections were analyzed using different cell markers (Fig. 3). In AP1d mice, the regions in pancreas marked by Krt5-positive cells showed intermingled CD45-positive immune cells and CD68-positive macrophages in the immediate proximity. The presence of Krt5-expressing cells was lessened in less densely infiltrated regions where leukocytes were eliminated in the process of pancreatic recovery at 3d post cerulein treatment. These indicated that the expression of Krt5 corresponded to the infiltration of inflammatory cells in pancreas after AP.

3.4. Conversion of Krt5-positive cells into β cells

Here, it was determined whether Krt5 would be stained with β cell marker– insulin. There were no Krt5-expressing cells in pancreatic islet in CON group mice. At 1d and 3d after cerulein treatment, the number of dispersed insulin-positive ICCs was increased. Besides, scattered cells with co-expression of Krt5 and insulin can be identified in the impaired pancreas (Fig. 4A, right, dashed rectangle). While speculative, this observation reflected that a population of Krt5-expressing cells could be a source of stem cells for β cell regeneration after acute pancreatitis.

GLUT2 is responsible for glucose uptake in β cells and partially accounts for the glucose-sensing mechanism of β cells. Furthermore, by using sequential sections from mice of AP3d, double-label immunofluorescence assays demonstrated that cells expressing Krt5 and insulin were also stained with GLUT2, a functional β cell indicator (Fig. 4B, white arrows). This observation suggested that Krt5-expressing cells could be converted into functional β cells.

4. Discussion

Due to the progression of clinical treatment technology, the blood glucose will return to normal soon after AP attack [23]. However, part of the patients could not fully recover, and glycemia of some patients could arise again after a short recovery. Some patients even develop DM in the end and need further treatment with antidiabetic or insulin in the remainder of life [24,25]. Therefore, it is crucial to monitor endocrine function in the early phase of AP, thus postpone the development of impaired glucose tolerance and DM.

In the present study, obvious acinar necrosis and substantial loss of islet β cells were noticed in the histological sections of AP1d mice, while the acinar injury was almost recovered in AP7d mice. This agrees with existing knowledge that pancreatic regeneration after AP induced by cerulein is largely completed within a week, when the pancreas histology is similar to that of control mice [26]. Moreover, the fasting blood glucose levels and AUC during IPGTT procedure were gradually increased from 1d to 7d post cerulein treatment. These results demonstrated that the fasting blood glucose levels and glucose intolerance in mice were deteriorated during the restoration process in our AP model, which might progress into DM naturally.

Further study was conducted to investigate the role of Krt5-expressing cell in AP by immunofluorescent staining. The results were striking: whereas Krt5-expressing cells were rarely detected in normal pancreas, their numbers rapidly increased in AP1d mice after the last cerulein administration. The activation of these Krt5-

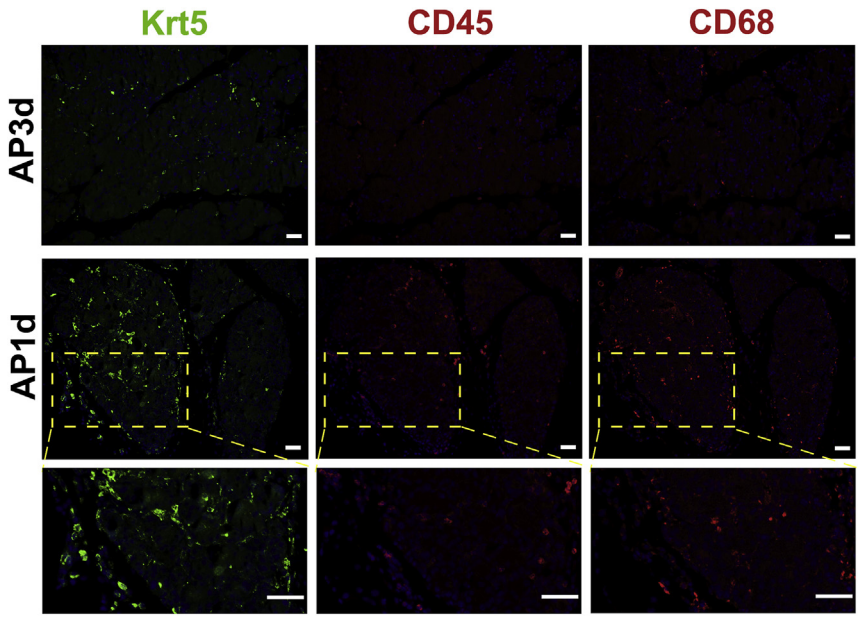

Fig. 3. Co-localization of Krt5-positive cell and inflammatory cell infiltrate. Expanded Krt5-positove cells were assembled in areas with dense infiltrates of intrapancreatic CD45-positve leukocytes and CD68-positive macrophages (dashed rectangle). DAPI (blue) was used to counterstain nuclei. Scale bar = 50 μm. Insets, high magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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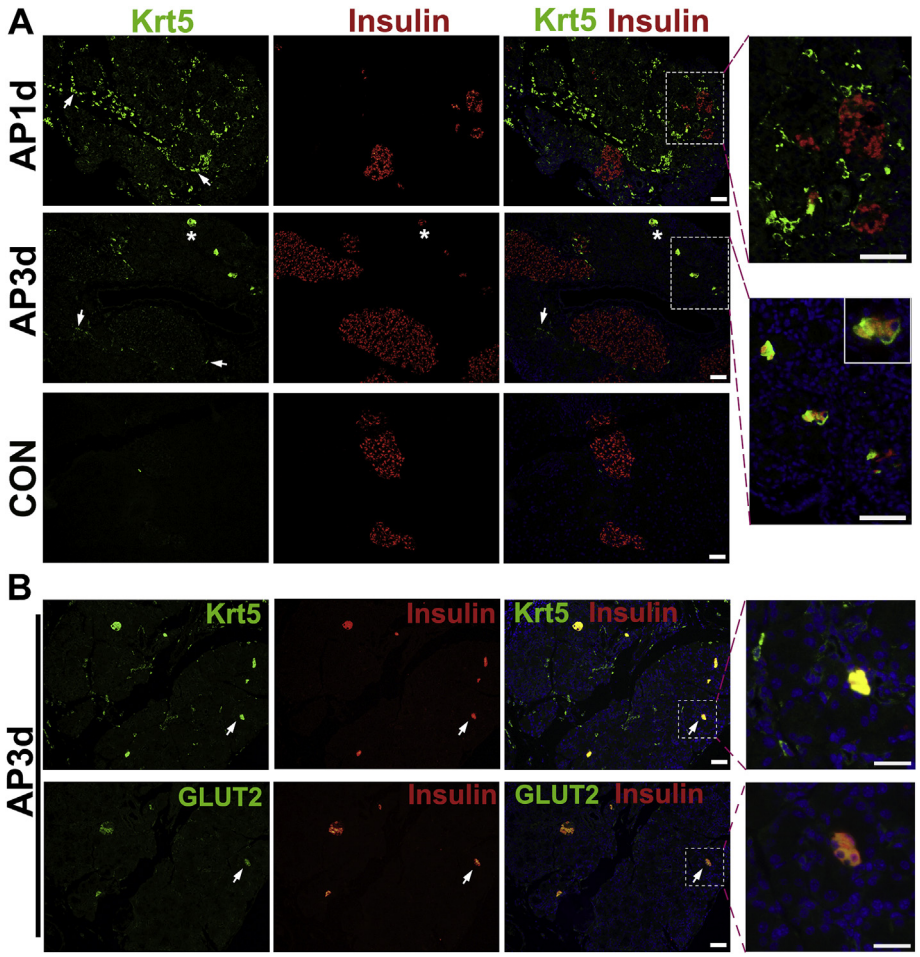

Fig. 4. Conversion of Krt5-positive cells into functional β cells in the impaired pancreas following AP. (A) Some overlap in double immunofluorescent staining of Krt5 (green) and insulin (red) in the area of injured pancreas were observed in mice of AP1d and AP3d (white asterisks). DAPI (blue) was used to counterstain nuclei. Krt5-positove cells are indicated by white arrows. Scale bar = 50 μm. Insets, high magnification. (B) Sequential sections from mice of AP3d were used to perform double-labelling immunofluorescence assays. The results demonstrated that some cells co-expressing Krt5 and insulin were also stained with GLUT2, a functional β cell indicator (white arrows). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

positive cells almost synchronously assembled in the damaged pancreas marked by a tangle of inflammatory cells and acinar cell carnage. As time went on, the number of Krt5-positive cells decreased in less densely infiltrated regions where were undergoing regenerative repair and clearing intensive immune cell infiltrates from AP3d to AP7d. In previous study, Krt5-expressing cells have been shown to possess progenitor-like properties during development [27] and to participate in gland healing after duct ligation in sialaden [28] and the lung regeneration following influenza virus infection [29,30]. Herein, it is speculated the potential that Krt5-expressing cells might be involved in the natural pancreas self-healing process after AP in adult mice. Moreover, a significant increase of ICCs in AP mice was detected, which was postulated to compensate for the loss of islet β cells. In addition, cells co-labelled with insulin and Krt5 were found in the areas of the impaired pancreas in 1d and 3d after the last cerulein injection, part of these cells were immunopositive for GLUT2, a specific glucose-sensing membrane protein in β cells. Herein, Krt5-positive cells were supposed to participate in the renewal of functional β cells and partly compensated for endocrine dysfunction when islet endocrine cells were injured after AP. Even though, the regenerative β cells derived from Krt5-positive cells could not fully reversed the exacerbation of glucose homeostasis in our AP model.

The work presented here focuses on the discovery of Krt5-expressing cells that are associated obviously with β cell regeneration after AP. Nevertheless, the present study leaves key questions unresolved. What, for instance, are the signals that trigger the activation and aggregation of Krt5-expressing cells to interlobular and intercellular sites of cerulein-damaged pancreas? What are the exact mechanisms concerning the dynamics of Krt5-expressing cells assembly? Answers to these questions, will be essential for determining the kinetics and regulation of islet cell regeneration following AP. It is possible that promoting conversion of Krt5-expressing cells into functional β cells can be a novel approach to alleviate occurrence and development of DM after AP in vivo.

Conflicts of interest

There is no conflict of interests relevant to the publication of this paper.

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