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Title page

Selective Expression of CYP2A13 in Human Pancreatic α -Islet Cells*

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Running title: CYP2A13 expression in human pancreatic islets

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The abbreviations used are: CYP, cytochrome P450; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

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Abstract

Exposure to cigarette smoke is an etiological factor of human pancreatic cancer and has been associated with an increased risk of pancreatic diseases, including pancreatitis, and diabetes. The toxicants in cigarette smoke can reach pancreatic tissue and most of them require cytochrome P450 (CYP)-mediated metabolic activation to exert their toxicity. Among all the human CYP enzymes, CYP2A13 is the most efficient enzyme in the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a major tobacco-specific toxicant and a suspected human carcinogen. It also metabolically activates 4-aminobiphenyl, another toxicant in cigarette smoke.

Immunohistochemical analysis in this study demonstrated that CYP2A13 was selectively expressed in the islets but not exocrine portion of adult human pancreas. Further study using dual immunofluorescence labeling technique showed that CYP2A13 protein was mainly expressed in the α -islet but not in β -islet cells. The selective expression of CYP2A13 in human pancreatic α -islet cells suggests that these islet cells could be damaged by the toxicants existing in cigarette smoke through CYP2A13-mediated *in situ* metabolic activation. It provides a mechanistic insight for human pancreatic diseases that have been associated with cigarette smoke exposure.

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Introduction

Both epidemiological and experimental data have demonstrated that cigarette smoking is an etiological factor of pancreatic cancer and is associated with an increased risk for the development of chronic pancreatitis (Mulder et al., 2002; Wittel et al., 2006; Maisonneuve et al., 2006; Tranah et al., 2011). Recent studies indicated that cigarette smoking may also play an important role in the pathogenesis of diabetes. Passive smoking was found to be more frequent in children with type 1 diabetes or pre-diabetes (Hathout et al., 2006; Skrodeniene et al., 2008). In both healthy persons and the patients with chronic pancreatitis, the serum glucose levels in smokers were substantially higher than their non-smoker controls, whereas the insulin levels were lower (Milnerowicz et al., 2007). Mechanistic studies are in need to support these observations in humans and to establish etiological and/or pathological role of tobacco smoke in pancreatic diseases.

There are many toxic and carcinogenic chemicals in cigarette smoke, including nicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosobis(2-oxopropyl)amine and 4-aminobiphenyl (Hoffmann et al., 1993; Chowdhury et al., 1995; Standop et al., 2001). These toxic chemicals can reach the pancreas as demonstrated by their detection in the pancreatic juice from smokers and the non-smokers who were exposed to second-hand tobacco smoke (Prokopczyk et al., 2002). Most of these toxicants and carcinogens require metabolic activation, usually catalyzed by cytochrome P450 (CYP) enzymes, to generate the electrophilic metabolites that are responsible for their toxicity and carcinogenicity. The intermediate metabolites generated during the metabolic activation are very short-lived ($t_{1/2}$ often within a second or less). Therefore, it has been well accepted that CYP-mediated metabolic activation *in situ* (i.e., in the target sites) is critical for cell- or tissue-specific toxicity/carcinogenicity induced by environmental chemicals (Ding and Kaminsky, 2003).

CYP2A13, CYP2A6 and CYP2A7 are the known three members in human CYP2A subfamily

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(Fernandez-Salquero et al., 1995). CYP2A7 has no catalytic activity and CYP2A6 is a major human enzyme catalyzing the metabolism of coumarin and nicotine (Ding et al., 1995). Our previous studies demonstrated that CYP2A13 is the most efficient human CYP enzyme for the metabolic activation of NNK, a tobacco-specific toxicant and suspected human carcinogen (Su et al., 2000). CYP2A13 can also metabolically activate 4-aminobiphenyl, another carcinogen in cigarette smoke (Nakajima et al., 2006). In addition, CYP2A13 is highly efficient in metabolizing nicotine (Bao et al., 2005) as well as the metabolic activation of aflatoxin B₁ (He et al., 2006), naphthalene and styrene (Fukami et al., 2008).

Using a highly specific antibody for immunohistochemistry, we previously demonstrated a high level of CYP2A13 protein expression in the epithelial cells of human bronchus and trachea but not in human liver, heart, testis and ovary (Zhu et al., 2006). The selective expression of CYP2A13 protein in human respiratory tissue is consistent with the fact that most smoking-related human lung cancers are broncogenic. It also supports the role of CYP2A13-mediated *in situ* activation of NNK and other toxicants in smoking-related human lung cancer. It is of interest to notice a recent report in which an inactive CYP2A13 variant was found to be associated with decreased pancreatic cancer susceptibility (Mohelnikova-Duchonova et al., 2010).

We speculated that CYP2A13, if it is expressed in pancreas, may play the same important role in smoking-related pancreatic diseases. This study aimed to determine the expression of CYP2A13 in human pancreas and to identify its cellular localization.

Materials and Methods

Antibodies and reagents

Normal goat serum, biotinylated goat anti-rabbit IgG, antigen unmasking solution, Vectastain ABC reagent and peroxidase substrate kit (DAB SK-4100) were purchased from Vector Laboratories Inc. (Burlingame, CA). Mouse

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anti-proinsulin C-peptide IgG, FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG were from Millipore (Billerica, MA). Mouse anti-glucagon IgG and Mayer's hematoxylin solution were purchased from Sigma-Aldrich (Saint Louis, WA). Rabbit anti-CYP2A13 polyclonal antibody was generated in our laboratory (Zhu et al., 2006). This antibody was raised against a C-terminal CYP2A13-specific peptide sequence covering the amino acid residues 369 to 377. The selection of this antigenic peptide was based on the hydrophilicity and the side chain properties of the amino acid residues that differ most from either CYP2A6 or CYP2A7. The specificity of this antibody was validated by immunoblot and immunohistochemical analyses. It does not cross-react with any of the CYP proteins present in human liver microsomes, including CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2E1, and CYP3A4. Nor does the antibody cross-react with CYP2A6 and mouse CYP2A5, both sharing a significant protein sequence similarity with CYP2A13 (Zhu et al., 2006).

Tissue samples

Human pancreas samples were a kind gift from Department of Pathology, Tongji Medical College, Huazhong Science & Technology University, Wuhan, China. They were collected from individual patients during pancreatectomy, with the consent of patients and the approval of the local ethics committee. The sample data (including the age, gender, and pathological diagnosis of the patients) are available from Department of Pathology, Tongji Medical College. A total of twelve samples (4 pancreatic cancer tissues and 8 normal pancreatic tissues adjacent to cancer) were collected and embedded in paraffin blocks.

Immunohistochemistry

Paraffin sections (5 μ m thickness) were prepared from the paraffin blocks. The immunohistochemical analysis protocol was described in detail in our previous report (Zhu et al., 2006). The dilution of rabbit anti-CYP2A13 antiserum was 1:600. Staining specificity was appraised by substitution of the primary antibody with the pre-immune

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serum from the same rabbits. The immunohistochemical staining was performed at least three times with several adjacent sections and the results were confirmed independently by four of the authors (Y Guo, L-R Zhu, G Lu and J-Y Hong).

Dual immunofluorescence labeling

Tissue sections were first processed through the same steps as for immunohistochemistry analysis (de-paraffinization, hydration, wash, antigen retrieval, and blocking of nonspecific binding). Then for dual labeling, sections were incubated with anti-CYP2A13 antiserum (diluted 1:600) mixed with either mouse anti-proinsulin C-peptide IgG (diluted 1:200) or mouse anti-glucagon IgG (diluted 1:2000) in a humidified chamber overnight at 4°C. After washed with PBS, the sections were incubated with FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG (diluted 1:200) for 1 hr. Sections were washed in PBS again, and then mounted in glycerol.

Results and Discussion

We have used a highly specific anti-CYP2A13 antibody in immunohistochemical analysis to localize the expression of CYP2A13 protein in human pancreatic tissues. In all the 8 normal samples, strong immunostaining was observed almost exclusively in the pancreatic islet cells, but was very weak in acinar cells and was even rare in ductal cells (Fig. 1A). CYP2A13-specific staining was undetectable in the negative controls (Fig. 1B), in which the anti-CYP2A13 antibody was replaced by the pre-immune serum from the same rabbits. The specificity of CYP2A13 immunoreactivity was further evidenced by including additional controls (human bronchus and liver tissue sections) in the same immunohistochemistry experiment. CYP2A13-specific immunostaining was positive in human bronchial epithelial cells (Fig. 1C) but was absent in human liver (Fig. 1E). This is consistent with our previous reports that the expression level of mRNA and protein of CYP2A13 is high in human bronchial cells but has little or no expression in the human liver (Su et al., 2000; Zhu et al., 2006). There was no detectable CYP2A13 protein in 4 pancreatic

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cancer samples (data not shown), probably due to the absence of normal islet morphology in the cancer tissues.

The pancreatic islets consists of mainly α -cells (producing glucagon, 15-20% of total islet cells), β -cells (producing insulin, 65-80%), δ -cells (producing somatostatin, 3-10%), and PP cells (producing pancreatic polypeptide, 3-5%) (Cabrera et al., 2006). To identify which type(s) of the islet cells express CYP2A13 protein, we applied immunofluorescent double-staining assay in which anti-CYP2A13 antibody was combined with the antibody against proinsulin C-peptide, a distinct hormone secreted only by β -islet cells, or with the antibody against glucagon, a specific hormone synthesized in α -islet cells. Double staining result showed that, in all the 8 normal human pancreatic tissues, CYP2A13-expressing cells (labeled by FITC in green fluorescence) were in small quantities and mostly scattered in peripheral islet region with some single cells in the central part of the islet (Fig 2, A-1 and B-1). In contrast, insulin-expressing β -cells (labeled by rhodamine in red fluorescence) accounting for approximately 70% of total islet cells, were mainly localized in the center of the islet (Fig. 2, A-2). Consistent with the known histological distribution of α -cells in pancreatic islets and the distribution of CYP2A13-expressing cells, glucagon-producing α -cells (also labeled by rhodamine in red fluorescence) were mainly localized at peripheral or perisinusoidal space (Fig. 2, B-2). Superimposition results of the labeling patterns clearly showed that CYP2A13 protein was mainly expressed in α -cells (Fig. 2, A-3 and B-3).

Expression of several other CYP enzymes, including CYP1A2, CYP2B6, CYP2C8/9/19, CYP2E1, CYP2J2 and CYP3A, and NADPH-cytochrome P450 oxidoreductase (POR), an essential supporter of CYP-catalized reaction, in human pancreatic islets has been reported (Zeldin et al., 1997; Standop et al., 2002, 2003; Ulrich et al., 2002). Similar to CYP2A13, CYP2B6, CYP2C8/9/19, CYP2E1, CYP2J2 and CYP3A proteins are also mainly expressed in α -cells. The co-existence of these CYP and POR proteins in the islet cells indicates that the CYP enzymes in human pancreatic islets are functional in metabolizing various endogenous and exogenous substrates. This was supported by

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the presence of epoxyeicosatrienoic acids, the metabolites produced in CYP2J2-catalyzed reaction, in human pancreatic tissues (Zeldin et al., 1997).

While CYP2J2 is known as an epoxygenase for catalyzing the metabolism of arachidonic acid (Zeldin et al., 1997), the endogenous substrates of most CYP enzymes are largely unknown. Therefore, it is difficult to understand the physiological role of CYP enzymes expressed in pancreatic islets. In contrast, the toxicological significance of CYP enzymes in the islets appears to be clear based on our current understanding of the critical role of CYP-mediated metabolic activation in toxicity induced by environmental toxicants, such as CYP2A13-catalyzed activation of NNK and other toxicants in cigarette smoke (Wang et al., 2006). It is also worth mentioning that the islet cells are the first to expose to blood-borne toxicants as a large portion of arterial blood passes through the islets first in pancreatic blood supply (Standop et al., 2002). All these would make pancreatic islet a vulnerable target of toxicants that reach pancreas and undergo the metabolic activation *in situ*.

Metabolic activation of tobacco-specific toxicant NNK by CYP2A13 in α -cells is anticipated to cause cell damage. This notion is supported by a recent study which demonstrated the adverse effect of cigarette smoking on α -cells. It has been found that, the number of pancreatic α -cells in chronic pancreatitis patients was significantly decreased in smokers when compared with nonsmoking patients and healthy subjects (Milnerowicz et al., 2007). The association of smoking and α -cell damage was further confirmed by the finding that fibrosis and progressive atrophy of α -cells were observed in smoking chronic pancreatitis patients, but the pancreatic islets were normal in nonsmoking patients (Milnerowicz et al., 2007).

The damage induced by toxicants in tobacco smoke on α -cells may also lead to damage in β -cells. In human pancreatic islets, α -cells and β -cells are in close contact (Cabrerria et al., 2006). An early study found morphologically typical tight and gap junctions occurring between α - and β -cells (Orci et al., 1975). It has also been

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demonstrated that carboxyfluorescein (an impermeant fluorescent probe), after microinjection into the β -cells, can be directly transferred to neighboring α -cells and δ -cells (Meda et al., 1982). Therefore, it is reasonable to expect that the toxic metabolites generated inside α -cells could be transferred directly by gap junctions from α -cells to neighboring β -cells, causing the β -cell damage and death. In addition to this “bystander effect”, it has been well known that different types of pancreatic islets cells can interact with each other through a paracrine feedback system. Most β -cells express glucagon receptors (Kieffer et al., 1996). Glucagon modulates α - and β -cell proliferation in the establishment of islet phenotype during pancreas development (Vuguin et al., 2006). While insulin produced in β -cells can inhibit α -cells, glucagon produced in α -cells activates β -cells (Wang MB et al., 2001). Therefore, reduction in the number of α -islet cells and impaired excretion of glucagon may induce loss or dysfunction of β -cells. Indeed, loss of β -cells has been observed in chronic pancreatitis patients who were smokers (Milnerowicz et al., 2007) and deficiency of glucagon was found to be associated with pancreatogenic diabetes mellitus (Goldstein et al., 1989).

In conclusion, we demonstrated that CYP2A13 protein is almost exclusively expressed in the α -islet cells in human pancreas. The expression of CYP2A13 protein in pancreatic islet cells was also observed in human fetuses (Guo Y et al., unpublished). The presence of a highly efficient enzyme for the metabolic activation of some toxicants in cigarette smoke in the pancreas suggests that CYP2A13-mediated activation plays an important role in pancreatic diseases induced by or associated with smoking.

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Authorship Contributions

Participated in research design: Guo, and Hong.

Conducted experiments: Guo.

Performed morphological analysis: Guo, Zhu, Lu, and Hong.

Wrote or contributed to the writing of the manuscript: Guo, Wang, and Hong.

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Footnotes:

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Figure legends:

Fig 1. Immunohistochemical localization of CYP2A13 protein in adult human pancreas, bronchus and liver. A

and B: pancreas; C and D: bronchus; E and F: liver. Anti-CYP2A13 antibody was used in A, C and E. Pre-immune rabbit serum, as a negative control, was used in B, D and F. The magnification for A and B is $\times 200$, for C, D, E and F is $\times 100$.

Fig 2. Immunofluorescent double staining images of CYP2A13, pro-insulin C-peptide and glucagon in adult

human pancreatic islets (magnification $\times 400$). A-1 and B-1: Rabbit anti-CYP2A13 antibody and FITC-conjugated goat anti-rabbit IgG were used as the primary and secondary antibody, respectively; A-2: Mouse anti-proinsulin C-peptide IgG and rhodamine-conjugated goat anti-mouse IgG were used as the primary and secondary antibody, respectively; A-3: superimposition of A-1 and A-2; B-2: Mouse anti-glucagon IgG and rhodamine -conjugated goat anti-mouse IgG were used as the primary and secondary antibody, respectively; B-3: superimposition of B-1 and B-2.

Figure 1

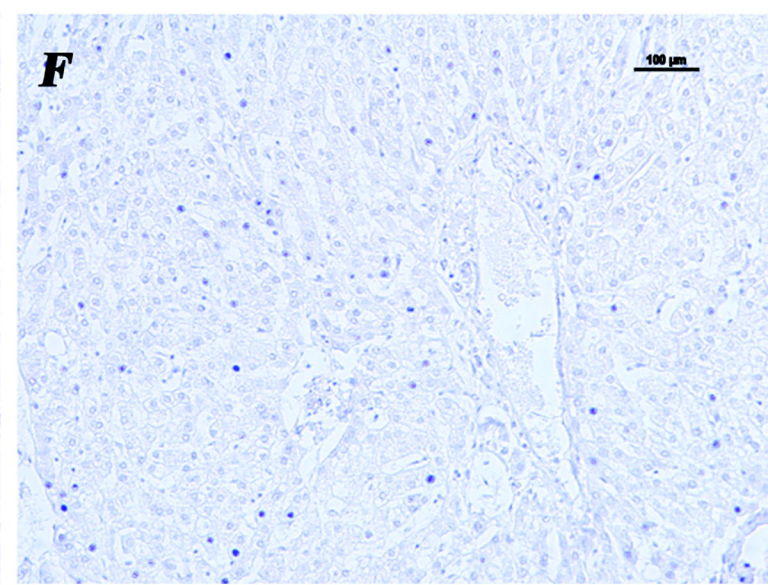
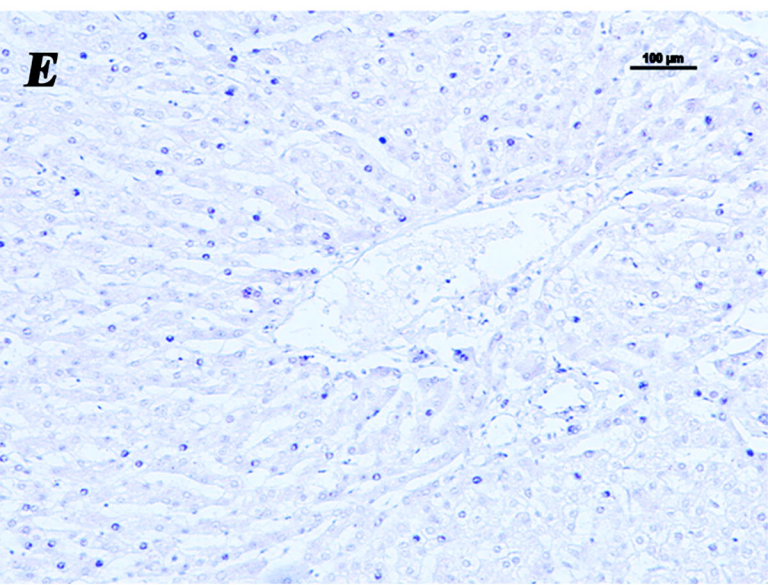
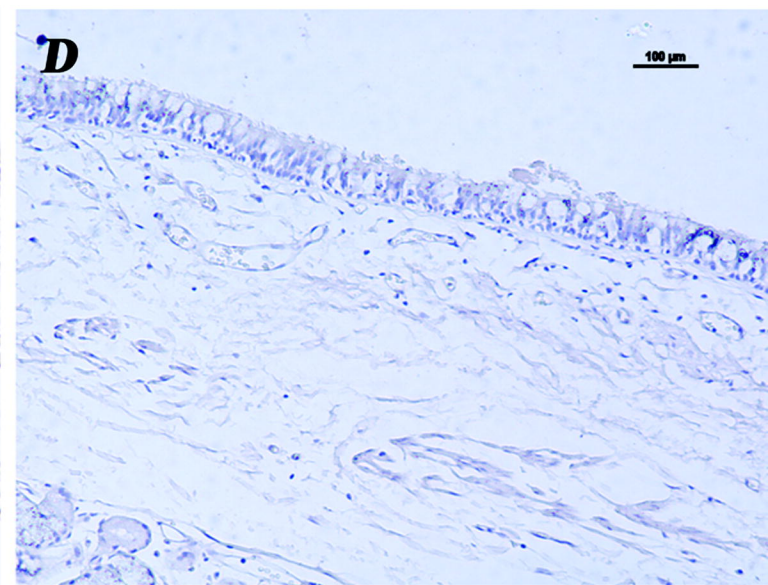
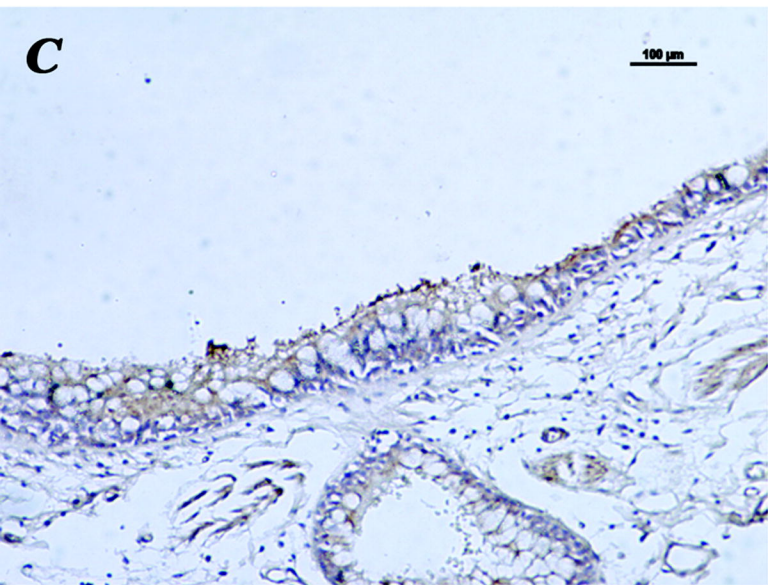
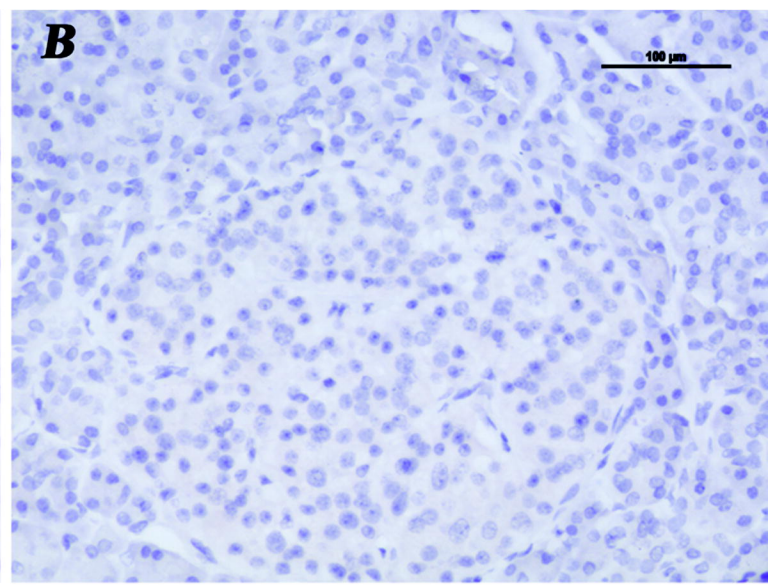
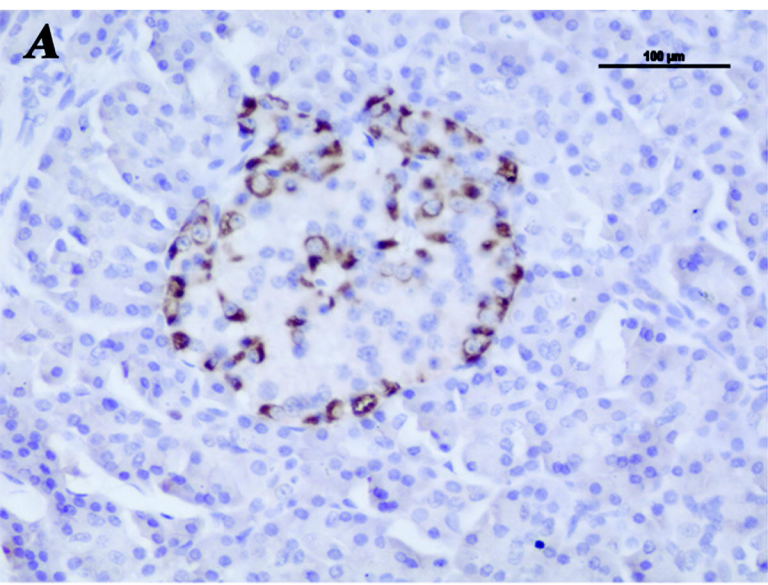


Figure 2

