

Expression and localization of *Wolfram syndrome 1* gene in the developing rat pancreas

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involved in some aspects of pancreatic development and further research on *WFS1* may provide new evidences to prove the interactions between mesenchyma and epithelia at the same time.

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Abstract

AIM: To investigate the expression and function of *Wolfram syndrome 1* gene (*WFS1*) during the development of normal pancreas.

METHODS: Pancreas from Sprague-Dawley rat fetuses, embryos, young and adult animals were used in this study. Expression levels of *WFS1* in pancreas of different development stages were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. To identify the cell location of *WFS1* in the developing rat pancreas, double-immunofluorescent staining was performed using antibodies to specific cell markers and *WFS1*, respectively.

RESULTS: Compared to E15.5, the highest level of *WFS1* mRNA was detected at E18.5, the level of *WFS1* mRNA in E15.5 and P0 was less, and at a lowest at adult ($P < 0.05$ vs P0 and adult), respectively. Compare to E15.5, the highest level of *WFS1* was at P14 and lowest at P21 ($P < 0.05$ vs P14 and P21), respectively. The *WFS1* positive staining is expressed in the normal developing rat pancreas mainly in the islet beta-cells and mesenchyme at each stage tested.

CONCLUSION: These results indicate that *WFS1* may be

INTRODUCTION

The pancreas is a complex organ composed of 2 different cell populations, exocrine and endocrine cells. The acini and ducts form the exocrine pancreas, which produces and transports digestive enzymes into the duodenum. The endocrine component contains 4 types of cells that secrete hormones to regulate glucose metabolism and other physiological processes^[1]. In rats, the pancreas develops from the foregut endoderm in 3 major steps: endoderm formation [embryonic day (E) 7.5], pancreatic morphogenesis (E10.5), and differentiation of endocrine and exocrine cells (E13.5). At approximately E16, the islet progenitor cells leave the contiguous epithelium, migrate through the adjacent extracellular matrix into the surrounding mesenchyme, and aggregate to form the islets of Langerhans. The islets are not completely formed until shortly before birth on E18-E19^[2-4] and undergo further remodeling and maturation for 2-3 wk after birth^[5]. Thus, the developing pancreas presents a challenge for developmental biologists because of the complex morphogenetic processes underlying the development of this organ. Until now, the factors that control the progressive development of pancreatic architecture and function have remained unclear. To

identify these functional factors we performed a gene array at E15.5, E18.5, birth and adult stages. The *Wolfram syndrome 1* gene (*WFS1*), was one of the factors found to be abundantly expressed in islet cells at E15.5 and E18.5 (data not shown). Wolfram syndrome (WS) is an autosomal recessive, progressive, neurodegenerative disorder characterized by diabetes mellitus and optic atrophy^[6]. This disorder is also associated with diabetes insipidus and deafness, hence the acronym DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness). Other manifestations such as atonic bladder, ataxia, nystagmus and predisposition to psychiatric illness may also be present^[7,8]. *WFS1*, discovered in 1998, was mapped to chromosome 4p16.1 by positional cloning^[9,10]. The gene is composed of 8 exons spanning 33.4 kb of genomic DNA. The 3.6 kb mRNA encodes an 890-amino acid polypeptide named wolframin^[9,11] with 9 predicted transmembrane domains, and belongs to a novel gene family. Biochemical studies in cultured cells indicate that *WFS1* protein is an integral, endoglycosidase H-sensitive membrane glycoprotein primarily localized in the endoplasmic reticulum^[12]. Postmortem studies of the pancreas from subjects with WS have shown β -cell loss^[10]. Mice with a disrupted *WFS1* were also reported to exhibit impaired glucose homeostasis accompanied by a progressive reduction of β -cell mass^[13]. Thus, *WFS1* seems to play a role in the normal function of β cells, but little is known about its function during embryogenesis. To the best of our knowledge, no study has investigated the relationship between *WFS1* expression and pancreas development, and the expression of *WFS1* during pancreatic development in rats is poorly understood. Knowledge of the regional and temporal expression of *WFS1* will be useful in understanding its potential roles in pancreatic development. Therefore, we further examined the expression patterns of *WFS1* in rat pancreas during development.

MATERIALS AND METHODS

Animals and preparation of rat pancreatic tissue

Sprague-Dawley (SD) rats were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). SD rats (2:1, male:female) were mated overnight. At noon the next day, if a vaginal plug was discovered, it was considered as Day 0.5 of gestation (E0.5). Embryos were removed at E15.5 and E18.5 from the uterus of pregnant rats, which were sacrificed by cervical dislocation. Pancreata from E15.5 and E18.5 rat embryos were isolated as previously described^[14] under a stereomicroscope. Rat pancreata at postnatal (P) days 0, 7, 14, 21 and from adults, were directly isolated by the unaided eye. All experiments were conducted in accordance with the Chinese Law for Animal Protection and were approved by the local animal care committee. Five rats were used at each age stage. Dissected tissues were immediately rinsed 3 times with phosphate-buffered saline (PBS) to remove serum proteins, and fixed with 4% paraformaldehyde in PBS overnight for histology, or frozen in liquid nitrogen for RNA and protein isolation.

RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from pancreata at each time point with TRIZOL reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis using ethidium bromide staining. For each PCR, 2 μ g of DNA-free total RNA with oligo (deoxythymidine) primers and reverse transcriptase were used. PCR was performed in 25 μ L reactions containing 25 ng of cDNA, 0.2 nmol of each primer pair, and 0.3 μ L of Taq DNA polymerase. PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for *WFS1* primers. We used the following primer pairs: *WFS1*, forward: 5'-CTGCTCTTTTGCTGGTTCT-3', reverse: 5'-GATGTCCTTGGTGATGTCG-3' (497 bp); and 18S rRNA, forward: 5'-ACGAACCAGAGCCGAAAGC-3', reverse: 5'-GGACATCTAAGGGCATCACAG-3' (514 bp). PCR conditions were as follows: 2 min at 94°C, followed by up to 35 cycles of 94°C for 30 s, 54.3°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. To estimate the linear range of the nested reactions, we analyzed the PCR products for 10, 15, 20, 25, 30, and 35 cycles. The amplified products were analyzed on 1% agarose gels and visualized by ethidium bromide staining. The data were normalized for 18S rRNA expression.

Preparation of protein samples

The pancreata was homogenized in a detergent lysis buffer containing 8 mol/L urea, 2% CHAPS, 40 mmol/L Tris, 65 mmol/L DTT and 2% IPG buffer. The lysate was then centrifuged at 15000 *g* for 1 h at 4°C. The total protein concentration of each sample was analyzed using a modified Bradford assay. All samples were stored at -80°C prior to the electrophoresis.

Western blotting analysis

An equal amount of protein samples (50 μ g protein in 20 μ L buffer) from each time point were boiled in 3 \times loading buffer (10 mmol/L Tris-HCl, pH 6.8 including 3% SDS, 5% β -mercaptoethanol, 20% glycerol and 0.6% bromophenol blue) for 3 min and separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). For blocking, membranes were incubated with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween-20 (TBST) overnight at 4°C. The membranes were then incubated with the primary antibody (NB100-1918, rabbit polyclonal antibody to *WFS1*, diluted 1:1000, Novus Biologicals Littleton, CO, USA) (sc-47778 mouse polyclonal antibody to β -actin, diluted 1:1000, Santa Cruz Biotechnology Inc., USA) for 2 h at room temperature. After washing in TBST, the membranes were incubated with the peroxidase-linked goat anti-rabbit IgG conjugates (PIERCE, Prod 1858415) or peroxidase-linked goat anti-mouse IgG conjugates (sc-2055, Santa Cruz Biotechnology Inc. USA) for 1 h at room temperature. The membranes were then washed

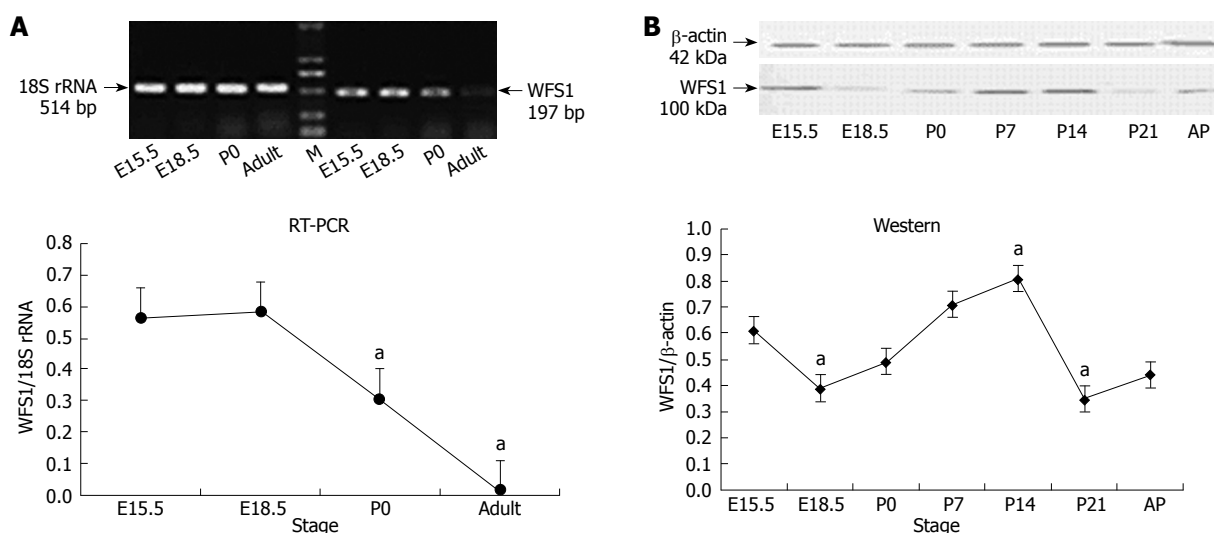


Figure 1 *Wolfram syndrome 1 (WFS1)* mRNA (A) and protein expression (B) in the developing rat pancreas. A: *WFS1* mRNA measured by reverse transcription-polymerase chain reaction (RT-PCR). upper: from embryonic day (E) 15.5 to adulthood: *WFS1* mRNA expression was highest at E15.5 and E18.5. M: Marker; lower: *WFS1* mRNA expression was analyzed and normalized to 18S rRNA. Results are indicated in percentage above the 18S rRNA value and are representative of three independent experiments. Compared to E15.5, the highest level of *WFS1* mRNA was detected at E18.5, the E15.5 and P0 was less, and at a lowest at adult ($^*P < 0.05$ vs P0 and adult), respectively; B: Western blotting of *WFS1* protein in the pancreas of E15.5, E18.5, P0, P14, P21 and adult rats. upper: expression of *WFS1* protein (100 kDa) with β -actin (42 kDa) as a loading control. The position of the molecular weight markers are indicated on the left; lower: the expression of *WFS1* protein shows dynamic changes during rat pancreas development. Results are indicated in percentage above the E15.5 value and are representative of three independent experiments. Compare to E15.5, The highest level of *WFS1* was at P14 and lowest at P21 ($^*P < 0.05$ vs E18.5, P14 and P21), respectively.

again in TBST, incubated in enhanced chemiluminescence reagents (ECL, Amersham Life Science, Cleveland, OH, USA) for 1 min, and exposed to VA 711 B Blue Sensitive X-ray films. Densitometric quantification of bands at sub-saturating levels was performed using the Syngenetool gel analysis software (Syngene, Cambridge, UK).

Double fluorescence immunohistochemistry

Tissues were fixed in 4% paraformaldehyde overnight at 4°C followed by a standard protocol of dehydration and paraffin embedding, and 5 μ m sections were cut. The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. The non-specific binding sites were blocked in 1% bovine serum albumin for 30 min. For *WFS1* protein and insulin, glucagon or vimentin double immunofluorescence, the rabbit anti-*WFS1* primary polyclonal antibody was applied and revealed using FITC-labeled Rabbit anti-goat IgG (1:400, sc-2777, Santa Cruz Biotechnology Inc., USA). Mouse anti-insulin primary polyclonal antibody (1:1000, Santa Cruz Biotechnology Inc., USA) or mouse anti-vimentin primary monoclonal antibody (1:1000, Chemicon Temecula, CA, USA) was then applied and revealed by cy3-labeled anti-mouse IgG (1:400, Chemicon Temecula, CA, USA). Sections were placed in Gel Mount Aqueous Mounting Medium (G0918, Sigma, USA) with a cover glass, and were examined under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Images were taken at a magnification $\times 400$.

Statistical analysis

Analysis of the experimental data was performed using PD Quest 7.0 software and the paired Student *t*-test. $P < 0.05$ was considered statistically significant. Data are presented as the mean \pm SD.

RESULTS

mRNA expression of WFS1 in rat pancreas at different developmental stages

The mRNA expression levels of *WFS1* in rat pancreas during its development were examined by RT-PCR. 18S RNA as used as an internal control and assessed under the same conditions. As shown in Figure 1A, RT-PCR for the *WFS1* specific region yielded a band of the expected size (197 bp) in rat pancreas tissue at different developmental stages. The mRNA expression of *WFS1* was high at E15.5 and E18.5, started to decrease at birth, and continued to decline to adulthood.

Protein expression of WFS1 in rat pancreas at different developmental stages

We performed Western blotting using total protein samples extracted from pancreata obtained at E15.5, E18.5, P0, P7, P14 and P21 and from adult rats (Figure 1B). β -actin (42 kDa) was used as an internal control under the same conditions. Analysis of the blots showed that *WFS1* (100 kDa) protein was expressed in the pancreas. Densitometric quantification of each band showed that the expression of *WFS1* protein was high at E15.5, and then decreased until birth (P0). Subsequently, the level of *WFS1* protein increased gradually and peaked at P14. The expression of *WFS1* protein was lowest at P21. The level of *WFS1* protein was relatively high in the adult rat.

Regional localization of WFS1 protein in rat pancreas at different developmental stages

To determine the localization of *WFS1* protein during pancreatic development, serial pancreas sections (obtained at E18.5, P0, P14, P21 and in adulthood) underwent

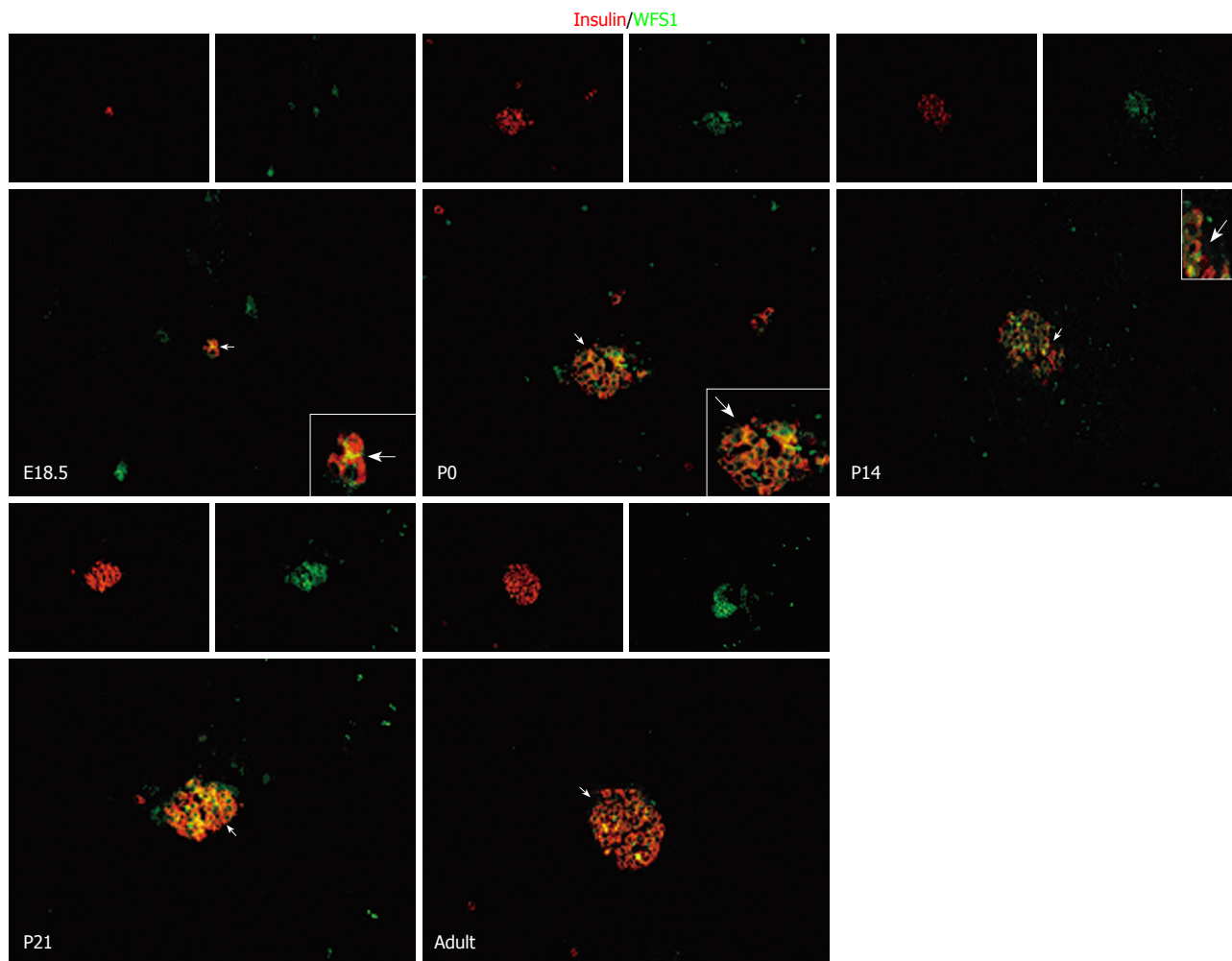


Figure 2 Immunofluorescent localization of WFS1 protein and insulin in the pancreas of E18.5, P0, P14, P21 and adult rats. The WFS1 antibody was detected with an FITC (green)-labeled secondary antibody and the insulin antibody was detected with a Cy3 (red)-labeled secondary antibody. An overlap between WFS1 (green) and insulin (red) labeling is colored orange. Modest WFS1 staining is seen in the developing islets from E18.5 to adulthood, and overlaps insulin-positive β -cells. A few WFS1-labeled cells are scattered away from the islets. Primary objective magnification, $\times 400$. Insets, higher magnifications of the areas indicated by arrows for E18.5, P0 and P14.

double immunofluorescence. We found that WFS1 protein was co-expressed with insulin and could not be detected in α -cells. As shown in Figure 2, there was a consistent overlap between the WFS1 protein-positive cells and cells labeled with insulin at each stage tested. This means that, during pancreatic development and remodeling, the WFS1 protein is continually expressed in β cells. As shown in Figure 3, there was little overlap between the WFS1 protein-positive cells and cells labeled with glucagon at any of the stages tested. This indicates that WFS1 protein is not expressed in α -cells during development. Interestingly, we also found that WFS1-positive cells were scattered throughout the pancreas. Therefore, we performed further double immunofluorescence, which confirmed that WFS1 protein was co-expressed with vimentin in mesenchymal cells (Figure 4).

DISCUSSION

Organogenesis involves 2 processes: morphogenesis, which is the shaping of an organ, and cytodifferentiation, which is the acquisition and expression of specialized

cellular functions within that organ^[15]. During pancreatic development, specialized cell types are generated from progenitor cell populations and are precisely organized into the elaborate structure of the adult organ. Previously, most researchers focused on deriving functional islets from stem cells or other cell types. Gu *et al*^[16] generated transcriptional profiles of enriched cells isolated from pancreata at 4 biologically significant stages of endocrine pancreas development (endoderm before pancreas specification, early pancreatic progenitor cells, endocrine progenitor cells and adult islets of Langerhans) to identify new endocrine regulatory genes and markers. With temporal and spatial analysis of genes expressed in embryonic endocrine cells, a database of potential progenitor cell markers was generated. In the present study, using a gene array, *WFS1* was found to be one of the genes that were specifically and highly expressed in islet cells at E15.5 and E18.5. Then, we used RT-PCR and Western blotting to analyze the expression of *WFS1* at 6 biologically significant stages of rat pancreas development: E15.5, E18.5, P0, P14, P21 and in adult rats. During the later embryonic period, the pancreatic cells

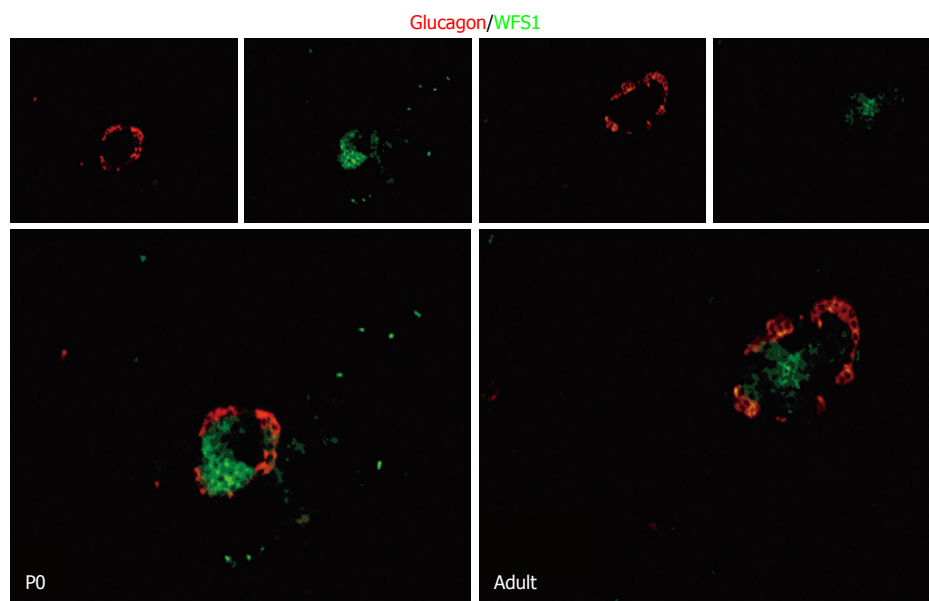


Figure 3 Immunofluorescent localization of *WFS1* protein and glucagon in the pancreas of P0 and adult rats. The *WFS1* antibody was detected with an FITC (green)-labeled secondary antibody and the glucagon antibody (mouse monoclonal antibody) was detected with a Cy3 (red)-labeled secondary antibody on the same section. The overlap between *WFS1* (green) and glucagon (red) labeling is colored orange. *WFS1*-positive cells and glucagon-positive cells can be seen in 2 pancreas sections tested. Double-labeling with antibodies to *WFS1* and glucagon reveal that the cells labeled with *WFS1* are surrounded by α -cells. Primary objective magnification, $\times 400$.

exhibit dramatic growth, differentiation and proliferation. Accordingly, it may not just be a concomitant phenomenon that the expression of *WFS1* protein is greater during the later embryonic period. The present study confirmed the changes in *WFS1* mRNA levels in the rat pancreas during development by RT-PCR.

The architecture of the islet is completely formed shortly before birth; however, the function of the islet is not fully developed at this stage. It has been reported that the pancreas of neonatal rat undergoes further remodeling and maturation for 2-3 wk after birth^[17]. The remodeling process includes a decrease in β -cell mass and enhanced islet neogenesis. The number of β -cells is mainly regulated by apoptosis and cell replication^[18]. The results of Western blotting revealed that the *WFS1* protein level was highest during the postneonatal period, particularly P14. Although the function of wolframin is still not completely known, recent studies have revealed its localization to the endoplasmic reticulum and that it may play an important role in endoplasmic reticulum (ER) homeostasis by regulating Ca^{2+} ^[11,19]. During islet development, particularly in the ER at E15.5 and P14, secretory and transmembrane proteins involved in development regulation fold into their native conformation and undergo posttranslational modifications important for their activity and structure. Consequently, ER homeostasis and the *WFS1* protein may be important in islet development. It is well known that *WFS1* protein induces cation channel activity in ER membranes^[11] and regulates calcium levels in the ER^[19]. *WFS1* protein also plays a role in stimulus-secretion coupling for insulin exocytosis in pancreatic β cells^[13,16,19,20]. Therefore, enhanced expression of *WFS1* protein in adults suggests that *WFS1* may retain its function in adult islets.

Surprisingly, the pattern of *WFS1* protein expression was not consistent with the pattern of *WFS1* mRNA

expression, as described above. This difference may result from post-transcriptional control, although the mechanisms need further investigation. RNA binding protein controls the various steps and the rate of transcription of microRNA, which regulates the destruction of RNA and changes in chromatin structure may be involved in the regulation of *WFS1* expression.

During remodeling in the perinatal period, apoptosis is an important mechanism that decreases β -cell mass. Apoptosis of β -cells is significantly increased in neonates compared with adult rats, and peaks at 2 wk of age^[18], while the highest level of *WFS1* protein was detected at the same time. It has been reported that ER stress induces *WFS1* expression in pancreatic β -cells^[21] and the expression of *WFS1* is regulated by inositol requiring 1α (*Ire1* α) and PKR-like ER kinase (PERK), central regulators of the unfolded protein response^[22]. It has also been reported that *WFS1*-deficient pancreatic β -cells exhibit increased phosphorylation of RNA-dependent protein kinase-like ER kinase, chaperone gene expression and active XBP1 protein levels, indicating an enhanced ER stress response. Furthermore, the increased ER stress response was accompanied by impaired β -cell proliferation and increased caspase-3 cleavage^[21]. Since the *WFS1* protein attenuates ER stress, maintains cell cycle progression and represses the apoptotic pathway, specifically in pancreatic β -cells, it may play an important role in the maintenance of β -cell mass by balancing β -cell growth (differentiation and proliferation) and β -cell death (apoptosis) during pancreatic remodeling.

During organogenesis, specialized cell types are generated from progenitor cell populations and are precisely organized into the elaborate structure of the adult organ. Numerous cell-cell communications and the initiation of complex inter-regulating genetic networks are involved to ensure the fidelity of organogenesis. Several

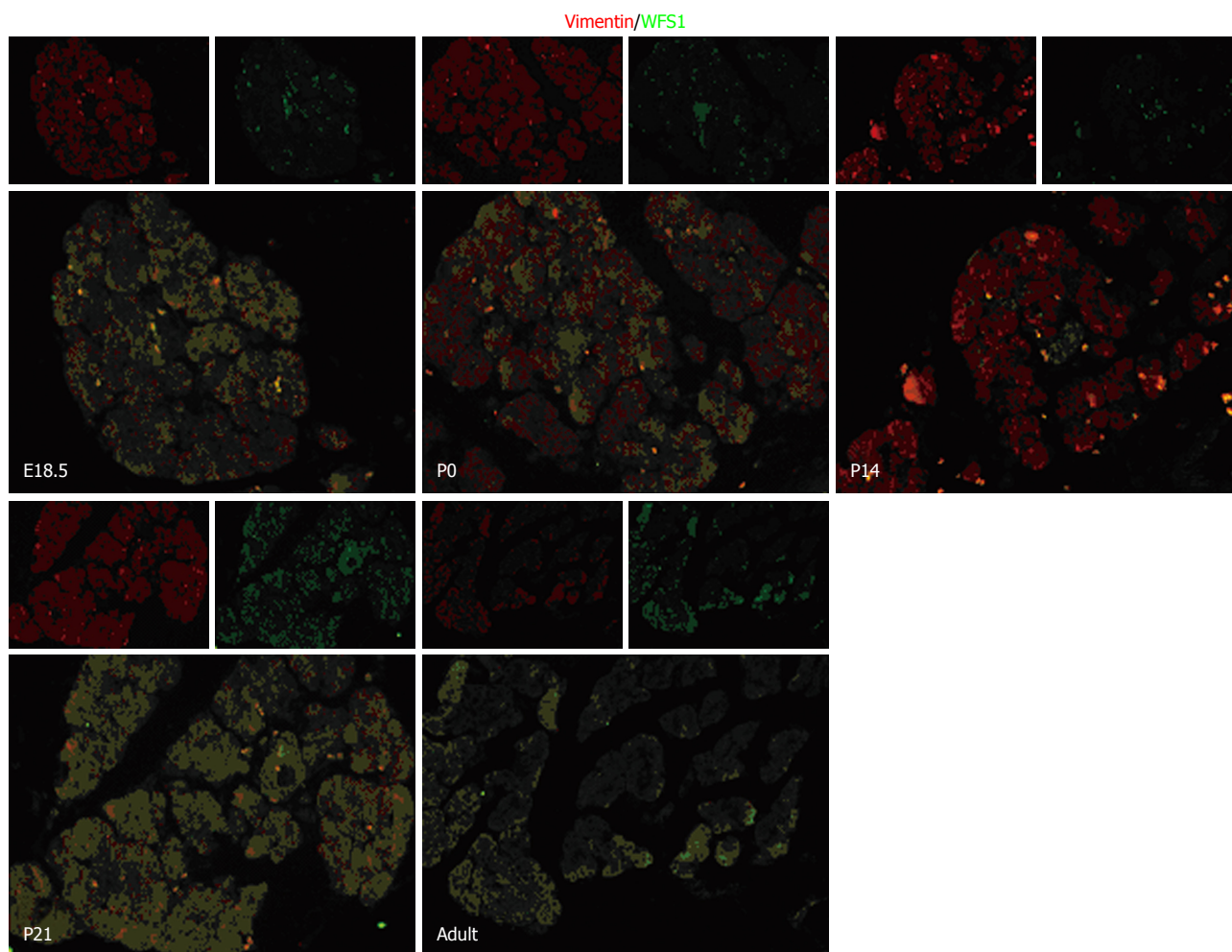


Figure 4 Immunofluorescent localization of WFS1 protein and vimentin in the pancreas of E18.5, P0, P14, P21 and adult rats. The WFS1 antibody was detected with an FITC (green)-labeled secondary antibody and the vimentin antibody was detected with a Cy3 (red)-labeled secondary antibody on the same section. The overlap between AFP (green) and vimentin (red) labeling is colored orange. WFS1-positive cells can be detected in pancreas sections from E18.5 to adult. Double-labeling with antibodies to WFS1 and to vimentin revealed colocalization of these proteins in the same cells from E18.5 to adult. Primary objective magnification, $\times 400$.

studies have reported the role of mesenchymal and/or epithelial interactions in pancreatic development and suggested that multiple diffusible mesenchyme-derived factors may act on the developing epithelia to influence cell division, cell fate, differentiation, and to determine the proportion of endocrine vs exocrine tissue^[23-26]. Examination of the ontogeny of *WFS1* in the developing rat pancreas revealed an intimate relationship between WFS1 protein and the mesenchymal and/or epithelial interactions^[27] in pancreatic development. Vimentin is a marker for mesenchymal cells^[28,29]. In the present study, the results of double immunofluorescence revealed that WFS1 protein was co-expressed with vimentin. This indicates that WFS1 protein is localized to the pancreatic mesenchyme shortly before birth on E18.5 and after birth. Previous studies revealed that the late embryonic stage is a key phase in late pancreatic organogenesis, in which a dramatic increase in the number of endocrine and exocrine cells with high levels of insulin and exocrine enzymes are observed. The islets are fully formed shortly before birth on E18.5 and undergo further remodeling and maturation after birth^[26]. Considering the previous

reports of WFS1 protein function and the results of our experiments, we suggest that WFS1 protein plays an important role in many aspects of pancreas development. However, the underlying mechanism is not fully understood and needs further research, which may also provide new evidence to confirm the interactions between mesenchymal and epithelial cells.

In summary, our present study has, for the first time, demonstrated that WFS1 protein is localized to the mesenchyme in the rat pancreas by immunofluorescence. The dynamic expression of *WFS1* in the various developmental stages of the pancreas indicates that *WFS1* may be involved in many aspects of pancreatic development.

COMMENTS

Background

Wolfram syndrome (WS), caused by the mutant of the *Wolfram syndrome 1* gene (*WFS1*), is an autosomal recessive, progressive, neurodegenerative disorder accompanied by diabetes mellitus and optic atrophy. Although it has been reported that WFS1 protein is found to be enriched in endocrine progenitors vs non-endocrine progenitor cells at embryonic day (E) 13.5, its expression and function during the development of normal pancreas remain unknown.

Research frontiers

It is thought that the pancreatic endocrine cells including beta cells arise from duct epithelial cells, however much research has been done to explore a different source. Novel strategies would obviously benefit from the use of beta stem cells/progenitor cells.

Innovations and breakthroughs

WFS1 expression has never been assessed in the developing pancreas and it is highly expressed in P14 on which the apoptosis rate of beta cells is at a high point. *WFS1* protein has been proved to be a downstream factor in the endoplasmic reticulum stress signal pathway.

Applications

This study reveals the possible mechanism of apoptosis in pancreas remodeling and may lead to new possibilities for diabetes therapy.

Peer review

This is a very interesting study that provides novel information that will be useful for the understanding of the role *WFS1* in pancreatic development as well as disease.

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