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Expression of Wnt, Frizzled, sFRP, and DKK Genes in Adult Human Pancreas

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Wnts are important signaling molecules involved in many normal developmental processes in the human body as well as some forms of cancer. Nineteen Wnt genes are found in the human genome, as well as 10 Wnt receptor genes called Frizzled. Two coreceptors called LRP 5 and 6 are critical for Wnt signal transduction. The interaction of the Wnts with the receptors is regulated by two classes of extracellular Wnt or LRP binding proteins called sFRP and Dickkopf (DKK), which modulate Wnt signaling. We have examined the expression of all Wnt family members both in the exocrine portion and in isolated islets of adult human pancreas. RT-PCR analysis of the 1-day cultured exocrine pellet fraction from the islet isolation procedure showed that Wnt 2, 2b, 3, 4, 5a, 5b, 7a, 7b, 14, and 15 were detectable. All 10 Frizzled (Frz) receptors were expressed but only Frizzled 1, 2, 4, 5, and 6 strongly. RT-PCR performed on purified human islets revealed that Wnt 2b, 3, 4, 5a, 7b, 10a, and 14 and Frz 4, 5, and 6 were the most highly expressed. DKK 1, 3, and 4 as well as sFRP 1, 4, and 5 were expressed in the exocrine fraction. sFRP 2 and 3 were detectable but only at low levels. In situ hybridization for Frz 1–7 showed that expression colocalized with the islets of Langerhans. Together the data suggest that active Wnt signaling occurs in adult pancreas and is probably important for physiological functions.

Human Wnt Frizzled sFRP In situ hybridization Gene expression PCR Pancreas

WNTs are a large family of secreted proteins which act via seven transmembrane receptors called Frizzled (13). Wnts are involved in multiple developmental processes in the embryo (12,20). In addition, Wnts play key roles in carcinogenesis in the adult human (14). We have recently described that several Wnts, Frizzled, and secreted modifiers are expressed in the mouse pancreas during development (4). To begin to investigate the role of Wnts in the adult human pancreas, we have examined the expression of all 19 human Wnts and 10 human Frizzled receptors as well as the secreted modifiers of Wnt signaling, DKK and sFRP.

MATERIALS AND METHODS

Human Materials

Human adult pancreases from heart-beating cadaveric nondiabetic donors were procured at European hospitals associated with the Eurotransplant Foundation (Leiden, the Netherlands) and with the beta cell bank of the JDRF Center, a European multicenter program involving beta cell transplantation in type I diabetic patients. The data reported in this study are based on RNA prepared from the exocrine fraction from four different pancreata from three male and one female donor with an average age of 58 years

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and a range from 51 to 64 years. The age range for the islet RNA material was 15 to 29 with an average of 20 years of age. The four different human islet cDNA samples were kindly provided by Dr. Jens H. Nielsen (Panum Institute, Univ. of Copenhagen, Denmark). Human fetal brain RNA was obtained from Stratagene and used as a positive control for the PCR reactions.

RT-PCR

Exocrine pellet fraction samples were placed in RNA later (Ambion) at 4°C. RNA was prepared using the Trizol reagent (Cinna Bio Tecx). cDNA was generated from RNA samples as previously described (6). The PCR Reddy mix from AbGene containing 1.5 mM $MgCl_2$ was used for all PCR reactions. PCR primer sequences are available upon request. Thirty to 36 cycles of PCR were run to detect low- and high-expressing genes. PCR reactions were separated on 2.5% agarose gels with Sybr Gold (Molecular Probes) incorporated and scanned on a Moleular Dynamics Typhoon Scanner.

In Situ Hybridization

RNA probes specific for the 3′ untranslated region of the different human Frizzled genes were prepared by PCR. Antisense riboprobes were synthesized using the digoxigenin RNA labeling kit from Boehringer Mannheim following the manufacturer's instructions.

In situ hybridization was performed on 6–8-μm frozen sections of adult human pancreas fixed in formalin overnight and embedded in OCT compound after overnight in a 30% sucrose/PBS solution. Slides were removed from −80°C and allowed to come to room temperature for 30 min. Probes were denatured at 75°C in hybridization buffer for 10 min and subsequently cooled on ice. The probes were then hybridized to the tissue slides overnight at 65°C. The unhybridized probe was removed by three consecutive rinses for 30 min at 65°C, with a solution containing 1× sodium chloride-sodium citrate (SSC), 50% formamide, and 0.1% Tween-20. They were then subjected to two 20-min rinses in maleic acid buffer (MABT; 100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20, final pH 7.5).

Slides were blocked with 2% Boehringer Mannheim blocking reagent, 20% sheep serum in MABT for 1 h at room temperature. Alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim) diluted 1:2500 were added to the slides and incubated overnight at room temperature. The slides were rinsed five times with MABT for 20 min, followed by two rinses for 10 min with NTMT [0.1 mol/L NaCl, 1% Tween-20, 0.01 mol/L $MgCl_2$, and 0.02 mol/L Tris (pH 9.5)]. Reaction mixture [nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boerhinger Mannheim)] was prepared in NTMT, added to the slides, and allowed incubate from 2 h to overnight at room temperature. The reaction was subsequently terminated by several rinses with PBS and was then processed for immunocytochemistry.

Immunocytochemistry

Sections were rinsed in PBS and nonspecific binding was blocked with 10% donkey nonimmune serum in PBS. For double immunofluorescence, sections were incubated with primary antibodies for 1 h at room temperature. Secondary antibodies (Cy-2 and Cy-3) were obtained from Jackson ImmunoResearch (West Grove, PA). Mouse anti-glucagon and guinea pig anti-insulin antiserum was obtained from Nordisk Gentofte A/S (Gentofte, Denmark). Images were collected with an Olympus BX51 microscope and captured using a Hamamatsu digital camera and Image Pro Plus 4.5 software interfaced with a PC computer. In situ hybridization and immunofluorescent images were overlaid in Image Pro Plus.

RESULTS

RT-PCR Analysis of the Exocrine Fraction and Islets Derived From Adult Human Pancreas

The pellet fraction of the human islet isolation after 1 day in suspension culture was used to analyze the expression of the 19 human Wnts by RT-PCR. This pellet after 1 day in culture contained 56% acinar cells, 41% duct cells, and 3% other cell types including endocrine cells determined by immunostaining for cytokeratin 19, amylase, alpha-1-antichymotrypsin, chromogranin A, and vimentin (Klein and Heller, unpublished observations). The RT-PCR screening of three representative pancreata showed that Wnt 2, 2b, 3, 4, 5a, 5b, 7a, 7b, 14, and 15 were detectable. Wnt 6, 8a, and 10b were found in only one of three pancreata analyzed (Fig. 1). RT-PCR performed on purified human islets revealed that Wnt 2b, 3, 4, 5a, 7b, 10a, and 14 were detectable. Wnt 5b, 7a, 8b, and 15 were only found in one of three islet preparations. Interestingly, Wnt 16 and the splice variant 16a, which has previously been reported in the pancreas (2), were undetectable using the published primers in any of the human pancreata screened.

There are 10 Frizzled (Frz) genes in the human genome (13) and their expression was analyzed by RT-PCR. All 10 Frizzled genes were detectable in the pellet fraction but expression of Frz 7 and 10 was quite low (Fig. 2). Frz 7, 8, and 9 were found in

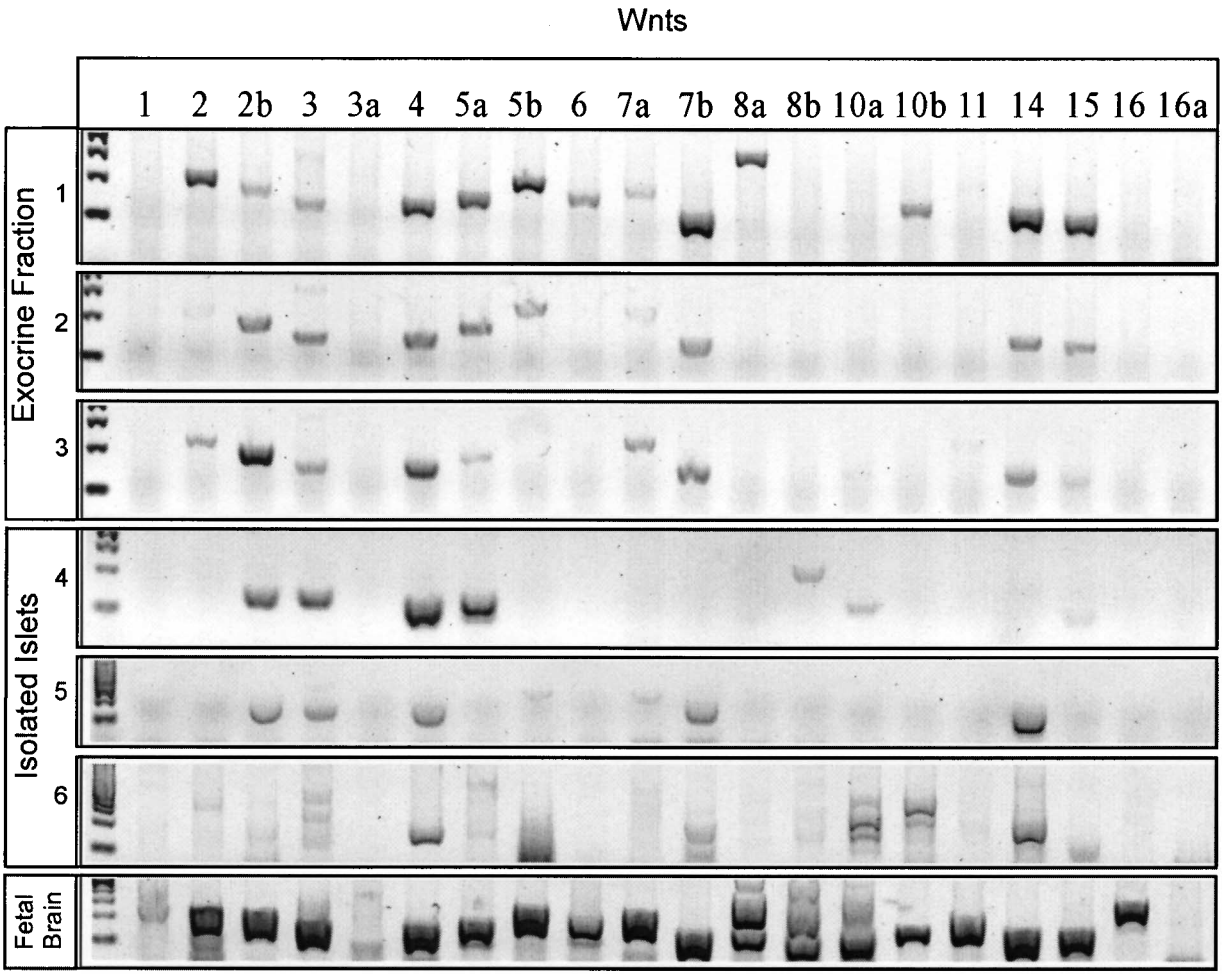

Figure 1. RT-PCR analysis of the expression of all 19 human Wnt genes. The exocrine pellet fraction analysis showed that Wnt 2, 2b, 3, 4, 5a, 5b, 7a, 7b, 14, and 15 were expressed in multiple different pancreata (#1–3). Wnt 6, 8a, and 10b were observed only in pancreata #1. Analysis of three different human islet preparations (#4–6) revealed the expression of Wnt 2b, 3, 4, 5a, 7b, 10a, and 14. Wnt 5b, 7a, 8b, and 15 were only found in one of three islet preparations. Human fetal brain cDNA was used as a control for the PCR primers. Wnt 3a and 16a primers failed to amplify any products.

two of the four preparations, while Frz 10 was only observed in pancreas 1. RT-PCR on cDNA from purified human islets revealed that all of the Frz genes were detectable with Frz 4, 5, and 6 showing the highest expression (Fig. 2).

Examination of the gene expression of the two families of Wnt signaling modulators (7,21), DKK and sFRP, showed that sFRP 1, 4, and 5 and DKK 1, 3, and 4 were detectable in the human pellet fraction (Fig. 2). Analysis of the purified human islets showed expression of sFRP 3, 4, and 5 and DKK 3 and 4 (Fig. 2).

Localization of Frizzled Genes in Adult Human Pancreas

The expression of the human Frz genes 1–10 was examined by in situ hybridization on sections of adult human pancreas. Probes specific to the 3′-UTR of the genes were used. The probes for Frz 1–7 all showed expression in the islets of Langerhans and coexpression with insulin- and glucagon-immunoreactive cell types (Figs. 3 and 4). Interestingly, Frz 3 and 5 colocalized with insulin but only weakly with glucagon-immunoreactive cells. Frz 5 and 6 also stained areas of the exocrine pancreas (data not shown), but due to the high RNAse expression in acinar cells, it is difficult to rule out the expression of any of the genes in these cells. Frz 8, 9, and 10 were not reliably detected (data not shown). These data are supported by the RT-PCR data comparing the pellet fraction (endocrine depleted) and the purified islet fraction.

DISCUSSION

Wnts are a large family of secreted proteins that regulate many developmental processes. Their func-

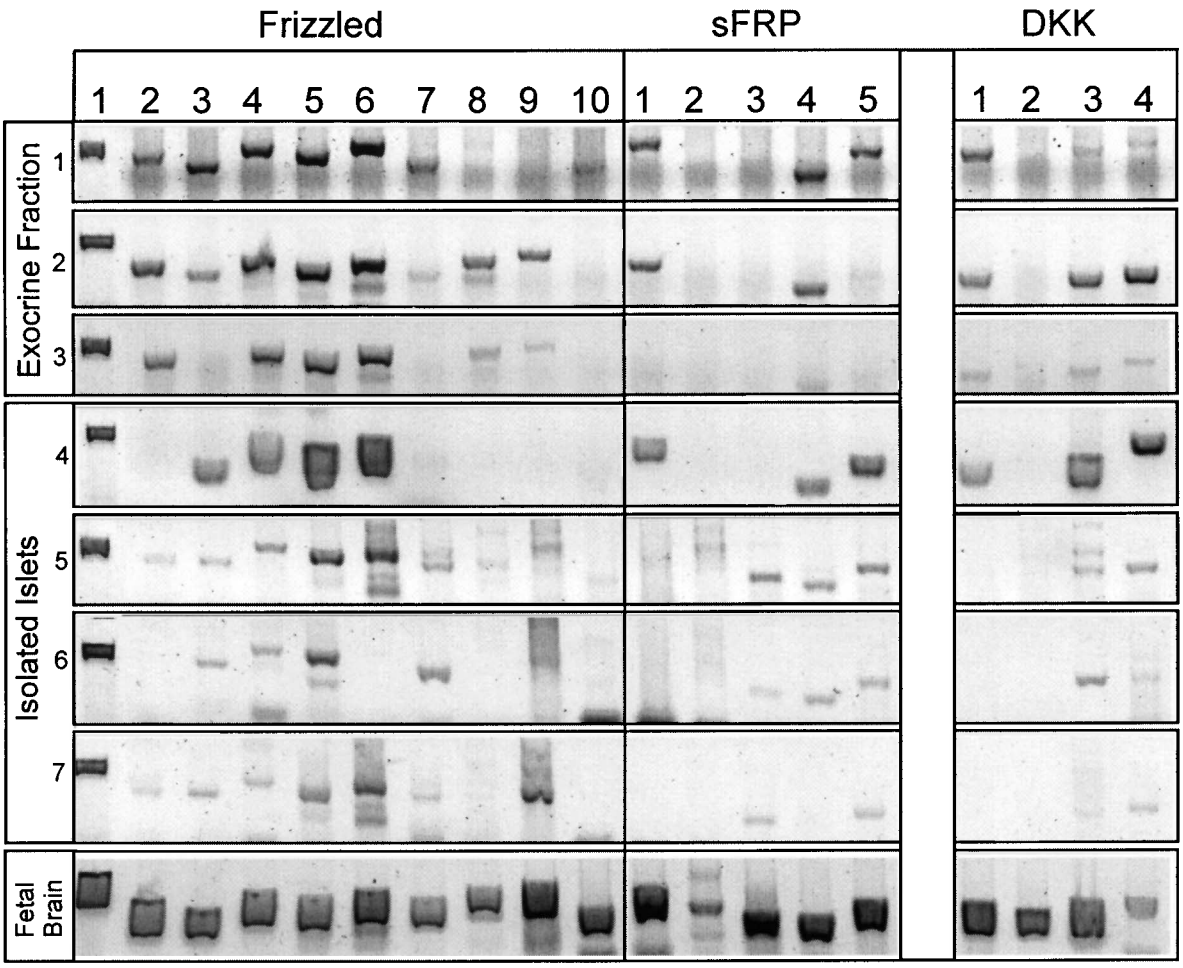

Figure 2. RT-PCR analysis of the expression of all 10 human Frizzled genes. Analysis of three different pancreatic exocrine fractions (#1–3) showed that all 10 Frizzled receptors were expressed but only Frizzled 1, 2, 3, 4, 5, and 6 were consistently observed. Frz 7, 8, and 9 were found in two of the four preparations, while Frz 10 was only seen in pancreas #1. Analysis of four different human islet preparations (#4–7) revealed that highest expression was observed for FRZ 3, 4, 5, and 6, while the others showed variable expression in the different pancreata. Expression of the Wnt signaling modifiers, sFRP and DKK, showed that in the human pellet fraction sFRP 1, 4, and 5 and DKK 1, 3, and 4 were detectable. Analysis of the purified human islets showed expression of sFRP 3, 4, and 5 and DKK 3 and 4. Human fetal brain cDNA was used as a control for the PCR primers.

tions in adult organs are much less understood, but Wnt signaling via the β-catenin pathway is a critical event in hematopoietic stem cell self-renewal (15). To investigate the possible role of Wnts in human pancreas, we undertook a PCR screen for all known members of this family. In agreement with our previous data, the expression profiles of Wnts detected using RT-PCR were similar to those of the mouse pancreas (4). Taken together, these data and a recent report showing that Wnt 3a and 5a stimulate insulin secretion in mouse islets (3) suggest that active Wnt signaling occurs in islets.

All 10 human Frizzled genes could be detected in adult human pancreas but some at higher levels than others. These data are consistent with those reported previously by Northern blot analysis of human pancreas RNA (8–10,16,17,19) and similar to data in mouse pancreas (5). In situ hybridization showed that expression of the Frizzleds localized mainly to the pancreatic islets. Interestingly, Frz 3 appeared to be more insulin cell specific and did not or only weakly colocalized with glucagon. The mouse Frz3 gene does not activate the β-catenin pathway but a protein kinase C-dependent calcium pathway in *Xenopus* embryos (11). There is some evidence to suggest that Wnt 5a may interact with mFrz3 and thus mediate some of its actions (18). This may explain the ability of Wnt5a to stimulate insulin secretion (4).

Dickkopf (DKK) and sFRP are families of secreted molecules that modulate Wnt signaling (7,21). We found multiple members of each family expressed in the pancreas. Interestingly, sFRP2, which we found

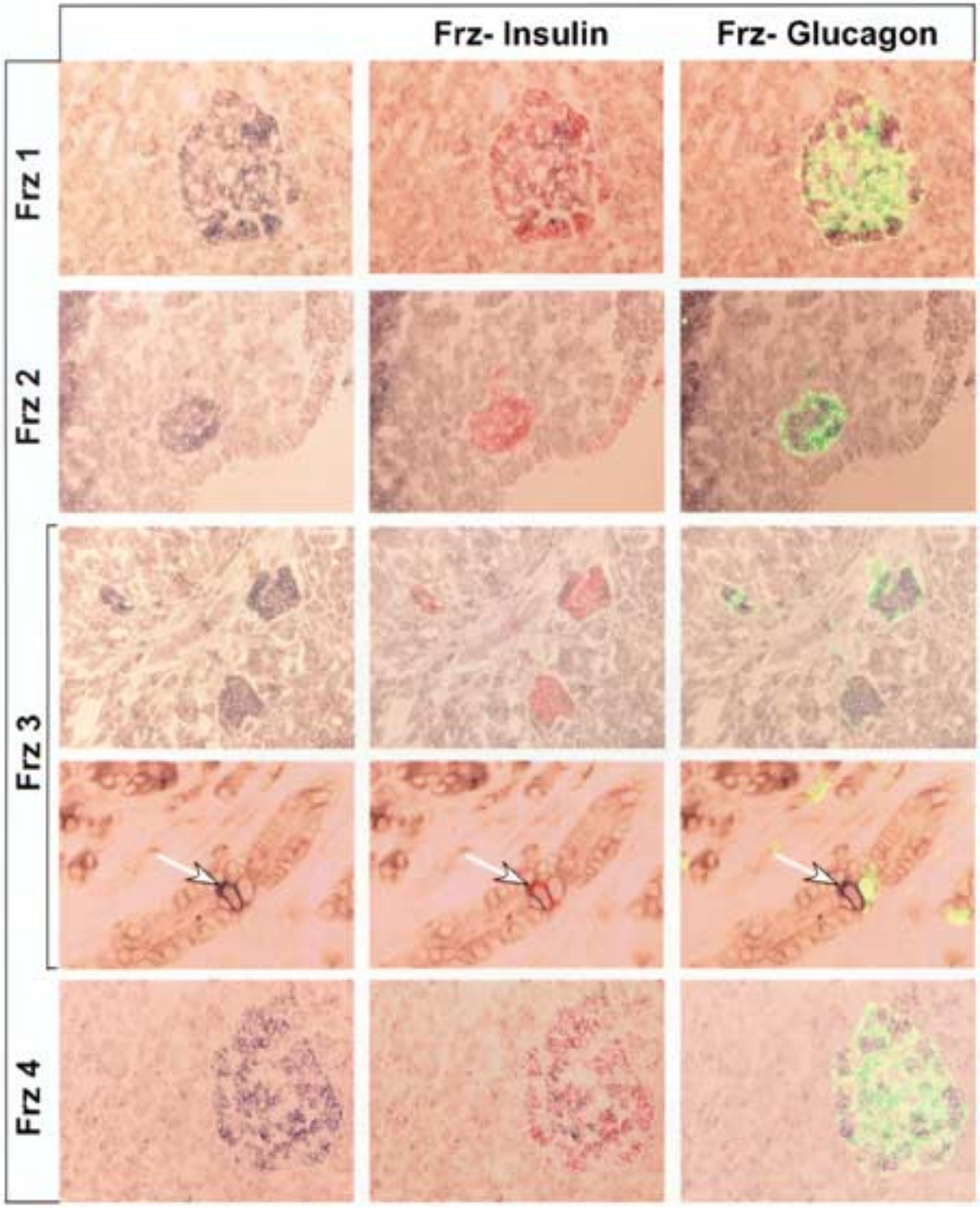

Figure 3. Expression of Frizzled 1–4 in sections of adult human pancreas. In situ hybridization was performed followed by immunocytochemistry for insulin and glucagon to denote the islet structures. Frz 1, 2, 3, and 4 were all expressed in human islets. Frz 3 was expressed mainly in insulin-immunoreactive cells. Arrows denote a Frz 3-positive cell in a pancreatic duct. Original magnification for all images was 200× except the second panel of images from Frz 3, which was 400×.

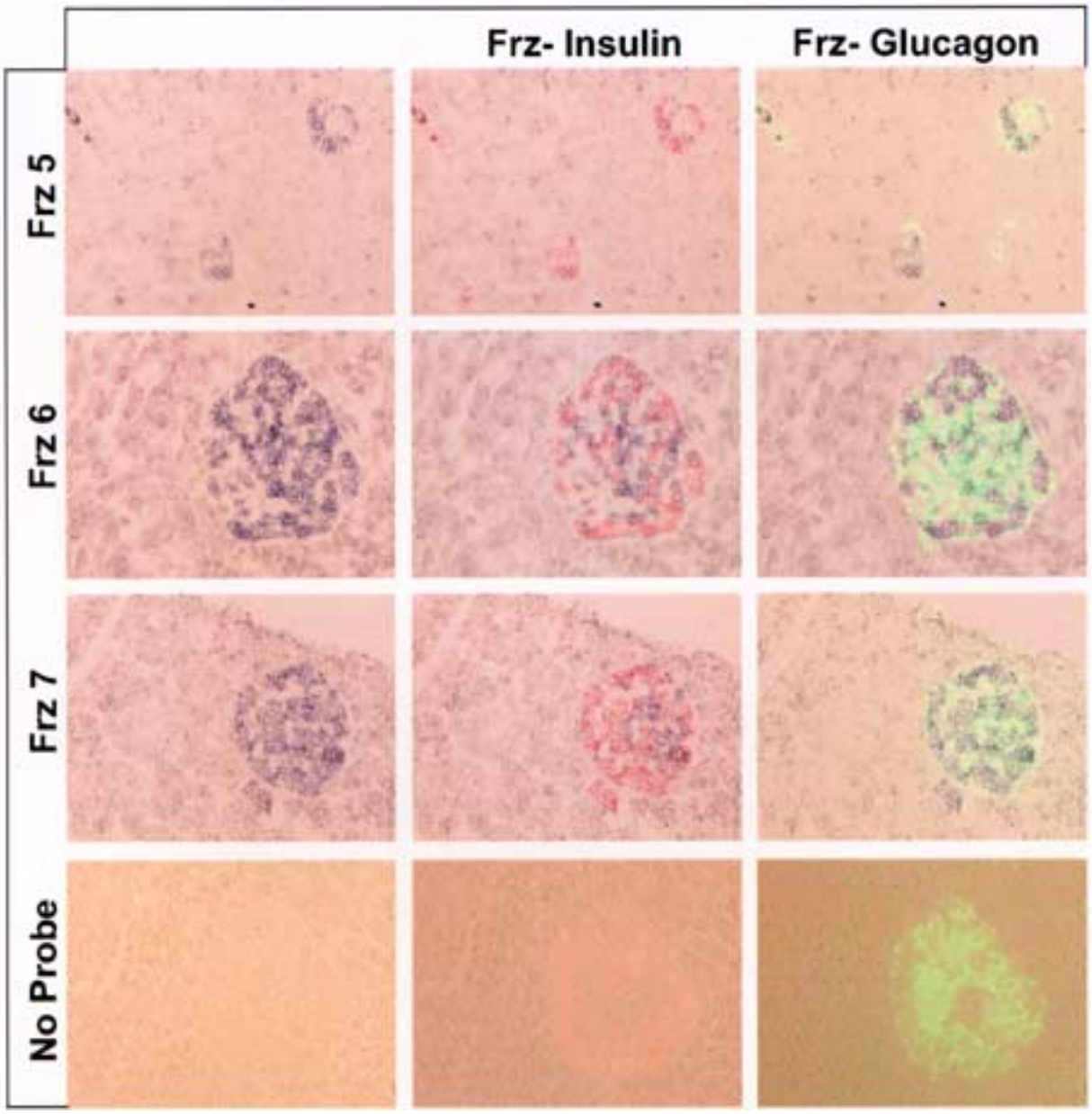

Figure 4. Expression of Frizzled 5–7 in sections of adult human pancreas. In situ hybridization was performed followed by immunocytochemistry for insulin and glucagon to denote the islet structures. Frz 5, 6, and 7 were all expressed in human islets. Frz 5 showed a stronger expression in insulin cells. Frz 6 and 7 were expressed throughout the whole islet. Sections in which no riboprobe was added showed no islet signal. Original magnification for all images was 200×.

to be expressed in mouse pancreas (4), was not expressed in human pancreas. Expression of sFRP-1 in the human pancreas has been previously reported (5). Our PCR data would suggest that sFRP-1 is not found in the endocrine portion of the pancreas.

In conclusion, multiple members of the Wnt family of genes are expressed in the human pancreas, as well as detectable levels of all Frizzled receptors. Together, this suggests a rather complex network of Wnt signaling both via canonical β-catenin pathways as well as other less well characterized pathways like calcium signaling (11) and cGMP signaling (1) in the normal adult human pancreas. Clearly, further analysis of the role of Wnt signaling in the pancreas is indicated, to understand the role of Wnts in development and pancreatic physiology.

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