Early Expression of Adenosine 5′–Triphosphate–Gated $P2X_7$ Receptors in the Developing Rat Pancreas

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Objectives: Extracellular adenosine 5′–triphosphate modulates the functions of the adult pancreas via 2 nucleotide receptor families, the P2X and P2Y receptors. Expression of the $P2X_7$ receptor has been demonstrated in islet cells of the pancreas, particularly the mature α cells that secrete glucagon. In the streptozotocin-induced diabetic model, a loss of insulin-secreting cells was accompanied by an increase in α cells that expressed the $P2X_7$ receptor.

Methods: In the present study, we have examined the expression of $P2X_7$ receptors in the developing pancreas from embryonic days 10 (E10) to E18.

Results: We detected $P2X_7$ receptor–immunoreactive cells in pancreatic islet cells as early as E11′ before glucagon expression. Subsequently, $P2X_7$ receptors were expressed in glucagon-secreting cells at E12, and complete colocalization was observed at E14. Occasional colocalization of $P2X_7$ receptors and insulin was observed in scattered cells at E12 and E14, but not at E18, when the glucagon- and insulin-secreting cells were almost completely segregated.

Conclusions: It was found that $P2X_7$ receptors were expressed early in a subpopulation of glucagon- and insulin-immunopositive cells in developing islets and subsequently became restricted to glucagon-expressing cells as development proceeded. The possible functional significance of these changes is discussed.

Key Words: ATP, $P2X_7$ receptor, rat embryo, pancreas, islets of Langerhans, glucagon, insulin

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The functional units of the endocrine pancreas are the islets of Langerhans, composed of 4 cell types: α, β, δ, and PP cells. The insulin-secreting β cells constitute most of the endocrine cell population and form the core of the islet, whereas α, δ, and PP cells, secreting glucagon, somatostatin, and a pancreatic polypeptide, respectively, make up the rest of the islet cell population. In mammals, the embryonic pancreas develops from the primitive foregut as 2 outpocketings (the dorsal and ventral buds), each of which consists of an inner endodermal epithelium surrounded by the mesenchyme.[1] Both buds subsequently proliferate to form multiple branches and fuse to make a functional organ. Among the hormones secreted from the endocrine portion of the pancreas, glucagon is the first peptide expressed in the developing pancreatic epithelium at embryonic day 9.5 (E9.5) in the mouse.[2,3] The functional significance of this early appearance of glucagon in the pancreas has not been resolved, although it has been suggested that glucagon regulates the differentiation of insulin-secreting cells.[4] However, the relationship of these endocrine cells in embryos and how the differentiation of these various types of pancreatic cells is regulated is still far from clear. It is known that the histogenesis of pancreatic endocrine cells requires complex and dynamic gene expression.[5,6]

Receptors responsible for ATP-mediated activities are subdivided into ionotropic P2X and metabotropic P2Y receptor families.[7,8] The P2X receptors mediate rapid, nonselective passage of small cations across the cell membrane, resulting in an increase in intracellular Ca^{2+} and depolarization.[7,8] Among the cloned P2X receptors to date, the $P2X_7$ receptor is structurally and functionally unique in that the cation channel of the $P2X_7$ receptor, under the continuous presence or repetitive stimulation with low doses of ATP and a low level of divalent cations, converts to a pore permeable to small molecules and ions.[9,10] The significantly longer than other P2X receptors intracellular C-terminus is associated with the induction of the nonselective pore.[10] In addition, the $P2X_7$ receptor is the only P2X receptor subunit that is unable to form heteromeric assemblies with other P2X receptor subunits,[11] and thus the $P2X_7$ receptor only exists as a homomer. The $P2X_7$ receptor has also been implicated in mediating apoptosis and/or necrosis in some tissues.[7,9,10,12–14]

Exogenous nucleotides modulate the functions of the adult pancreas.[15–17] It has been demonstrated that ATP stimulates insulin secretion, whereas adenosine inhibits insulin secretion from β cells and stimulates glucagon secretion from α cells.

The $P2X_7$ receptor has been detected in α cells but not the other pancreatic endocrine cell types.[18,19] In the present study, we have investigated the developmental expression pattern of the $P2X_7$ receptor. We have made use of specific antibodies and an immunofluorescent double-labeling method to study the spatial-temporal expression of $P2X_7$

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receptors in the early developing pancreas to find out its relationship with the glucagon and insulin-secreting cells.

MATERIALS AND METHODS

Tissue Preparation

The expression of P2X$_7$ receptor protein was studied in Sprague-Dawley rat embryonic pancreas of E10 to E18 using fluorescence immunohistochemical techniques. The day of identification of the presence of a vaginal plug was designated as E0. Pregnant Sprague-Dawley rats were killed by asphyxiation with a rising concentration of carbon dioxide (between 0% and 100%), and death was confirmed by cervical dislocation according to Home Office (UK) regulations covering schedule 1 procedures. Embryos collected were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C. Three litters of embryos were used for each embryonic stage, and 3 embryos were used from each litter. Fixed embryos were cryoprotected, embedded in Tissue-Tek, and cryosectioned at 12 μm.

Immunofluorescence Double Labeling

Air-dried sections of the entire embryonic pancreas were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 2 minutes at room temperature. Nonspecific binding sites were blocked by incubating sections in 10% normal serum (normal horse serum [NHS] for P2X$_7$ and glucagon; normal goat serum [NGS] for insulin) in phosphate buffered saline (PBS) for 1 hour. The sections were then incubated with primary antibodies, diluted in 10% NHS (or NGS) in PBS, overnight at room temperature. The primary antibodies used were rabbit anti-P2X$_7$ (1:200; Roche Palo Alto, Calif), goat antiglucagon (1:50; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) and guinea pig anti-insulin (1:500; Incstar, Stillwater, Minn). The sections were washed in PBS and incubated for 1 hour at room temperature in either fluorescein isothiocyanate– or Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, Pa) diluted in 1% NHS (or NGS) in PBS. For control experiments, the sections were incubated with the primary antibodies preabsorbed with the control peptide antigens or with NHS only. All images of immunohistochemical staining were taken with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). The images were imported into a graphic package (Adobe Photoshop, San Jose, Calif).

RESULTS

The P2X$_7$ receptor immunoreactivity was detected in pancreatic islets at E11 to E18. The P2X$_7$ receptor was not detected at E10 (data not shown) but was first expressed at E11 (Fig. 1A), the stage at which glucagon and insulin immunoreactivities were not yet detectable.[20] The P2X$_7$ receptor–immunoreactive cells were found in the islets, which

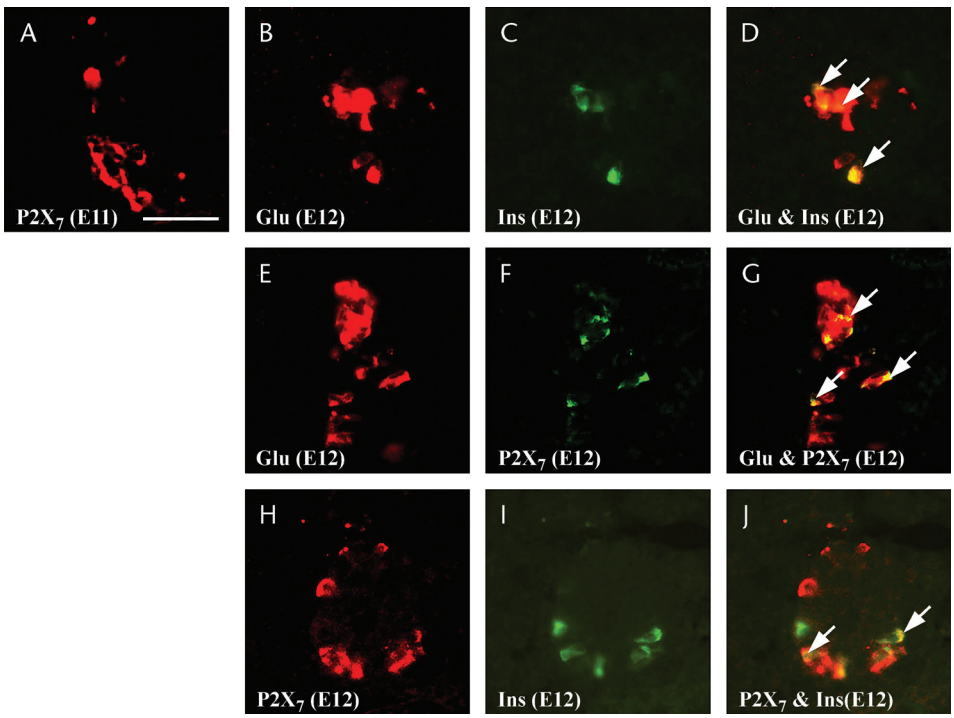

FIGURE 1. Immunohistochemical staining of P2X$_7$ receptors, glucagon and insulin in islets of E11 to E12 pancreas. The P2X$_7$ receptor–immunoreactive cells (red) are expressed in clusters in E11 pancreas (A), glucagon-expressing cells (red) at E12 (B), insulin-expressing cells (green) at E12 (C), and colocalization of glucagon and insulin (D) showing glucagon and insulin coexpressed cells (arrows). E–G, Double labeling (yellow; arrows) of glucagon (red) and P2X$_7$ receptors (green). The P2X$_7$ receptor expression (red) (H), insulin expression (green) (I), and colocalization (yellow, arrows) of P2X$_7$ receptors (red) and insulin (green) (J). Scale bar, 100 μm (A, E–J) and 114 μm (B–D).

appear as ovoid cell clusters in the pancreas. At E12, both glucagon and insulin were detected in the islets (Figs. 1B, C). Double immunofluorescent labeling showed that all the insulin-expressing cells were glucagon positive, whereas some pancreatic cells expressed glucagon only (Fig. 1D). Insulin-expressing cells constituted only a small fraction of cells within the glucagon-expressing population at E12 (Fig. 1D). Double labeling (Fig. 1G) also showed that all the $P2X_7$ receptor–expressing cells (Fig. 1F) were glucagon positive (Fig. 1E). Some of the glucagon-expressing cells, however, were $P2X_7$ receptor–negative (Fig. 1G). At this stage, a small number of islet cells were observed coexpressing $P2X_7$ receptors and insulin, whereas some populations expressing either $P2X_7$ receptors or insulin only were also identified (Fig. 1J).

At E14, expression of glucagon and insulin was detected. Unlike E12, where all the insulin-expressing cells were glucagon immunopositive, cells that express insulin but not glucagon first appeared at this stage (Figs. 2A–C). All the glucagon-immunoreactive cells showed complete colocalization with the $P2X_7$ receptor (Figs. 2D–F). Similar to that observed at E12, some but not all of the $P2X_7$ receptor–expressing cells were colocalized with insulin-immunopositive cells. Cells expressing either $P2X_7$ receptors or insulin were also observed.

A sharp increase in the population of insulin-expressing cells takes place after E14.[5] At E18, when insulin-secreting β cells became the dominant endocrine cell type and outnumbered the glucagon-immunoreactive cells (Figs. 3A–B), the glucagon- and insulin-positive cells were completely segregated (Fig. 3C). The $P2X_7$ receptor was expressed in the glucagon-expressing islet cells, indicated by colocalization of $P2X_7$ receptors and glucagon (Figs. 3D–F). Glucagon-positive, $P2X_7$ receptor–negative cells, although present, were very rare (Fig. 3F). At this stage, none of the insulin-expressing cells were $P2X_7$ receptor immunopositive (Figs. 3G–I).

DISCUSSION

The current study used a double immunohistochemical method to examine the expression of ATP-gated $P2X_7$ receptors in glucagon- and insulin-expressing cells during early development in the rat pancreas. Glucagon and insulin are secreted hormonal peptides and are common markers for α cells and β cells, respectively, in both adult and embryonic islets in pancreas. Surprisingly, we found that the $P2X_7$ receptor, which has previously been shown to be expressed specifically in glucagon-expressing cells in postnatal and in adult pancreas,[18] was detected as early as at E11, the stage at which pancreatic cells are still glucagon and insulin negative. The early appearance of $P2X_7$ receptor immunoreactivity before glucagon expression indicates that expression of the $P2X_7$ receptor does not require the presence of glucagon. Whether or not the $P2X_7$ receptor participates in regulation of glucagon expression, however, is not known. Subsequent expression of the $P2X_7$ receptor was shown to be largely associated with glucagon-positive cells, but some insulin-staining cells also expressed $P2X_7$ receptors. Eventually, $P2X_7$ receptor expression was only detected in mature α cells containing glucagon.[18] Whether $P2X_7$ receptors are involved in apoptotic death of

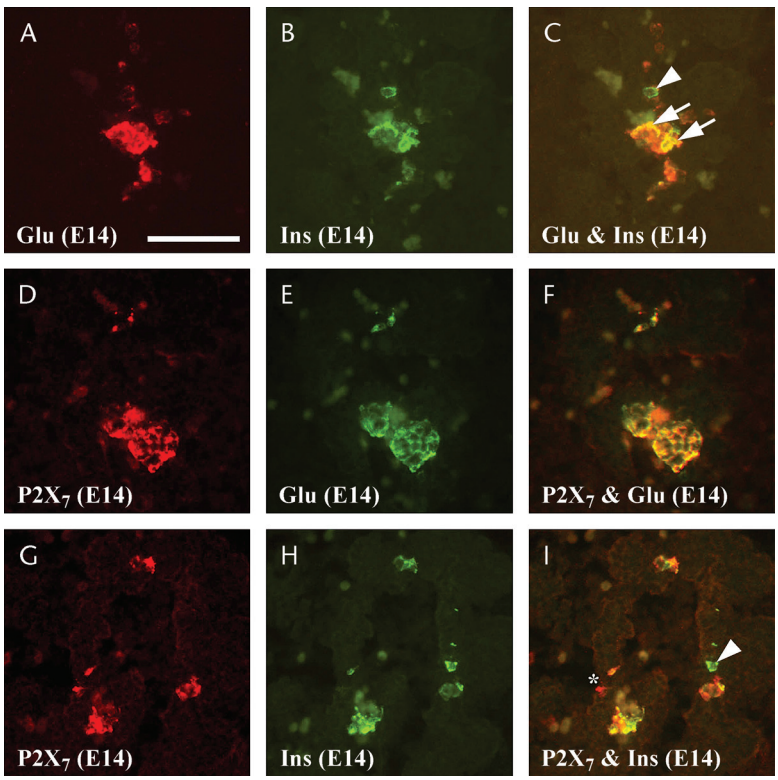

FIGURE 2. Immunohistochemical staining of $P2X_7$ receptors, glucagon, and insulin in islets of the E14 pancreas. Glucagon-expressing cells (red) (A), insulin-expressing cells (green) (B), and colocalization (yellow) of glucagon- and insulin-expressing cells (C). Note the cells expressing insulin only (arrowhead) and glucagon and insulin coexpressed cells (arrows). The $P2X_7$ receptor–immunoreactive cells (red) (D), glucagon-expressing cells (green) (E), and colocalization of $P2X_7$ receptors and glucagon (yellow) (F). The $P2X_7$ receptor–immunoreactive cells (red) (G), insulin immunostaining (green) (H), and colocalization of $P2X_7$ receptors (I) with insulin (yellow). Note cells expressing insulin only (arrowhead) and $P2X_7$ receptors only (asterisk). Scale bar, 100 μm.

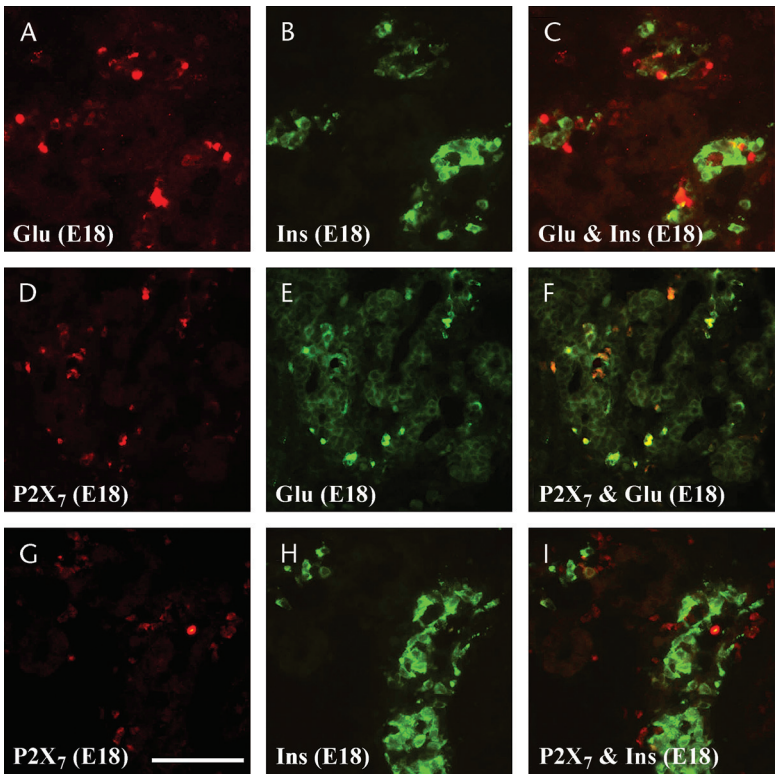

FIGURE 3. Immunohistochemical staining of P2X$_7$ receptors, glucagon, and insulin in islets of the E18 pancreas. Glucagon-expressing cells (red) (A), insulin-expressing cells (green) (B), and colocalization of glucagon and insulin (yellow) (C). The P2X$_7$ receptor–immunoreactive cells (red) (D), glucagon-expressing cells (green) (E), and colocalization of P2X$_7$ receptors and glucagon (yellow) (F). Note the sparse colocalization of P2X$_7$ receptors and glucagon. The P2X$_7$ receptor–immunoreactive cells (red) (G), insulin-expressing cells (green) (H), and colocalization of P2X$_7$ receptors and insulin (I). Note that none of the P2X$_7$ receptor–expressing cells are colocalized with insulin-expressing cells. Scale bar, 100 μm.

pancreatic cells would need further experiments, investigating the staining of apoptotic markers.

It has been shown that the early islet cell population (before E14-E15) consists of glucagon-positive cells and glucagon/insulin-coexpressing cells.[5] Our present study and also other reports[5,21,22] show that all insulin-expressing cells before E14 belong to the glucagon/insulin-coexpressing cell subpopulation. Our results also show that the P2X$_7$ receptor was expressed in both glucagon-positive and glucagon/insulin-coexpressing cells. In view of the finding in the adult pancreas that the P2X$_7$ receptor was restricted to mature α cells that secrete glucagon only,[18,19] the expression of the P2X$_7$ receptor in glucagon/insulin-expressing cells seems to be novel. It is, however, known that although the glucagon-positive cells will give rise to the mature α cells, the glucagon/insulin-expressing cells will not give rise to any of the mature α or β cells,[23] which are thought to be eliminated by apoptosis. Whether the P2X$_7$ receptor is involved in the elimination of the glucagon/insulin-expressing cells through apoptosis needs further investigation, but the finding that P2X$_7$ receptors are not found in all of the glucagon/insulin-expressing cells and are observed in glucagon-positive cells indicates that the involvement of the P2X$_7$ receptor in the pancreatic cell death is highly unlikely.

Previous studies demonstrated the first appearance of ultrastructurally recognizable β cells with a characteristic insulin-positive and glucagon-negative immunoreactivity at E14 to E15.[5] Our present study also found that the insulin-positive and glucagon-negative cells first appeared at E14.

In summary, we have shown that the expression of the P2X$_7$ receptor early in the embryonic pancreas, even before expression of hormone peptides, suggesting that expression of P2X$_7$ receptors did not depend on the presence of glucagon. Subsequent expression was detected in islet cells expressing different endocrine markers. Thus, the P2X$_7$ receptor seems to be one of the earliest markers during islet cell development. The transient expression of P2X$_7$ receptors in the glucagon and insulin coexpressing cells offers additional insight into whether ATP is involved during pancreatic development, in addition to its role in modulating insulin secretion.

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REFERENCES

1. Pictet R, Rutter WJ. Development of the embryonic endocrine pancreas. In: Steiner DF, Frenkel N, eds. *Handbook of Physiology/ Endocrinology*, vol 7. Washington, DC: American Physiological Society; 1972:25–66.
2. Rall LB, Pictet RL, Williams RH, et al. Early differentiation of glucagon-producing cells in embryonic pancreas: a possible developmental role for glucagon. *Proc Natl Acad Sci U S A*. 1973;70: 3478–3482.
3. Guz Y, Montminy MR, Stein R, et al. Expression of murine STF-1, a putative insulin gene transcription factor, in cells of pancreas, duodenal epithelium, and pancreatic exocrine and endocrine progenitors during ontogeny. *Development*. 1995;121:11–18.
4. Prasadan K, Daume E, Preuett B, et al. Glucagon is required for early insulin-positive differentiation in the developing mouse pancreas. *Diabetes*. 2002;51:3229–3236.

5. Larsson LI. On the development of the islets of Langerhans. *Microsc Res Tech*. 1998;43:284–291.
6. St-Onge L, Wehr R, Gruss P. Pancreas development and diabetes. *Curr Opin Genet Dev*. 1999;9:295–300.
7. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev*. 1998;50:413–491.
8. Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev*. 2006;58:58–86.
9. North RA. Molecular physiology of P2X receptors. *Physiol Rev*. 2002;82:1013–1067.
10. Surprenant A, Rassendren FA, Kawashima E, et al. The cytolytic P2z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science*. 1996;272:735–738.
11. Torres GE, Egan TM, Voigt MM. Hetero-oligomeric assembly of P2X receptor subunits. *J Biol Chem*. 1999;274:6653–6659.
12. Di Virgilio F. Dr. Jekyll/Mr. Hyde: the dual role of extracellular ATP. *J Auton Nerv Syst*. 2000;81:59–63.
13. Auger R, Motta I, Benihoud K, et al. A role for mitogen-activated protein kinase (Erk1/2) activation and non-selective pore formation in $P2X_7$ receptor-mediated thymocyte death. *J Biol Chem*. 2005;280: 28142–28151.
14. Bulanova E, Budagian V, Orinska Z, et al. Extracellular ATP induces cytokine expression and apoptosis through $P2X_7$ receptor in murine mast cells. *J Immunol*. 2005;174:3880–3890.
15. Loubatières-Mariani MM, Chapal J. Purinergic receptors involved in the stimulation of insulin and glucagon secretion. *Diabete Metab*. 1988;14:119–126.
16. Petit P, Hillaire-Buys D, Loubatieres-Mariani MM, et al. Purinergic receptors and the pharmacology of type 2 diabetes. In: Abbracchio MP, Williams M, eds. *Handbook of Experimental Pharmacology. II. Purinergic and Pyrimidinergic Signalling II-Cardiovascular, Respiratory, Immune, Metabolic and Gastrointestinal Tract Function*, vol 151. Berlin, Germany: Springer-Verlag; 2001:377–391.
17. Novak I, Nitschke R, Amstrup J. Purinergic receptors have different effects in rat exocrine pancreas. Calcium signals monitored by Fura-2 using confocal microscopy. *Cell Physiol Biochem*. 2002;12:83–92.
18. Coutinho-Silva R, Parsons M, Robson T, et al. Changes in expression of P2 receptors in rat and mouse pancreas during development and ageing. *Cell Tissue Res*. 2001;306:373–383.
19. Coutinho-Silva R, Parsons M, Robson T, et al. P2X and P2Y purinoceptor expression in pancreas from streptozotocin-diabetic rats. *Mol Cell Endocrinol*. 2003;204:141–154.
20. Roberts C, Roberts GA, Lobner K, et al. Expression of the protein tyrosine phosphatase-like protein IA-2 during pancreatic islet development. *J Histochem Cytochem*. 2001;49:767–775.
21. Herrera PL, Huarte J, Sanvito F, et al. Embryogenesis of the murine endocrine pancreas, early expression of pancreatic polypeptide gene. *Development*. 1991;113:1257–1265.
22. Teitelman G, Alpert S, Polak JM, et al. Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. *Development*. 1993;118:1031–1039.
23. Herrera PL. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development*. 2000;127:2317–2322.

