

F. Ye · B. Duvillié · R. Scharfmann

Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells

Received: 19 May 2004 / Accepted: 4 September 2004 / Published online: 3 February 2005
© Springer-Verlag 2005

Abstract *Aims/hypothesis:* The fibroblast growth factor (FGF) family consists of 22 members. In rodents, several FGFs are expressed in the pancreas, where they participate in epithelial–mesenchymal interactions. Our objective was to describe the pattern of expression of FGFs in the human embryonic pancreas and to analyse their effect on pancreas development. *Methods:* The expression of FGFs was analysed by RT-PCR. To investigate the cell types expressing FGF7 and FGF10, we separated epithelial from mesenchymal cells using immunomagnetic beads linked to E-cadherin antibodies and performed real-time PCR. The effect of FGF7 and FGF10 on proliferation of human embryonic pancreatic epithelial cells was evaluated in vitro by measuring BrdU incorporation. *Results:* We found that different FGFs are expressed in the human embryonic pancreas, and we focused on FGF7 and FGF10. We defined a new approach to separating epithelial cells (containing the pancreatic progenitor cells) from mesenchymal cells. This allowed us to demonstrate that human embryonic pancreatic mesenchymal cells express both FGF7 and FGF10. We next demonstrated that FGF7 and FGF10 were able to induce the proliferation of the epithelial cells in vitro. *Conclusion/interpretation:* These findings indicate that it is now possible to efficiently separate human embryonic pancreatic epithelial from mesenchymal cells, an important step to characterize and expand progenitor cells. This method allowed us to demonstrate that human embryonic pancreatic mesenchyme expresses FGF7 and FGF10 that act on epithelial cells to activate their proliferation. Such growth factors could thus be used to expand human embryonic pancreatic epithelial cells.

Keywords Development · Embryonic · FGF · Human · Pancreas

Abbreviations BrdU: bromodeoxyuridine · EGR1: early growth response 1 · FGF: fibroblast growth factor · FGFR2b: fibroblast growth factor receptor 2b · Pdx1: pancreatic and duodenal homeobox factor 1 · RT-PCR: reverse transcriptase polymerase chain reaction

Introduction

Type 1 diabetes is caused by an autoimmune destruction of pancreatic beta cells resulting in insulin deficiency and consequent hyperglycaemia. Recent data have shown that the replacement of damaged beta cells by the transplantation of islets from donors could be used to provide a continued insulin reserve and a long-term glycaemic control [1]. The major obstacle remains the insufficient supply of cells. Thus, alternative sources of beta cells have to be found.

Recently, the potential of different human cell types and tissues to differentiate into beta cells has been evaluated. For example, human embryonic pancreata at an early stage of development were used [2]. In this model, it was shown that when human embryonic pancreata at 6–9 weeks of development that contain very rare insulin-containing cells were grafted under the kidney capsule of NOD/scid mice, the tissue grew, increasing in weight 200 times within 6 months, and the endocrine cells differentiated.

While data related to the control of pancreas development in rodents accumulate, little is known about the factors that control the proliferation and the differentiation of the precursors in the human embryonic pancreas. In rodents, it is now established that pancreas development is controlled by signals derived from different tissues that contact the endodermal regions. In the case of signals derived from the pancreatic mesenchyme, it has been shown that ligands of receptor tyrosine kinases are implicated in the control of pancreatic epithelial cell proliferation and differentiation. This is for example the case for members of the fibroblast growth factor (FGF) family. Specific ligands for fibroblast growth factor receptor 2b (FGFR2b), 47 and FGF10, are expressed in the pancreatic

F. Ye · B. Duvillié (✉) · R. Scharfmann
Faculty Necker, INSERM E363,
156 Rue de Vaugirard,
75015 Paris, France
e-mail: duvillie@necker.fr
Tel.: +33-1-40615571
Fax: +33-1-43060443

mesenchyme [3], and data based on loss or gain of function experiments indicate that both FGF7 and FGF10 are implicated in the control of pancreas development [3–6].

In the present study, we have examined the expression of FGF7 and FGF10 in human embryonic pancreata at 6–9 weeks of gestation. We next designed a new experimental approach based on the separation of epithelial from mesenchymal cells and showed that in human embryonic pancreas, FGF7 and FGF10 are expressed in mesenchymal cells. Finally, we examined the role of FGF7 and FGF10 and demonstrated that both factors activate the proliferation of embryonic pancreatic epithelial cells.

Materials and methods

Human tissues Human pancreata were extracted from embryonic tissue fragments obtained immediately after voluntary abortions performed between 6 and 9 weeks of development, in compliance with the current French legislation.

RT-PCR Total RNA was isolated by the guanidinium isothiocyanate method [7]. The oligonucleotides used for amplification were (Table 1):

Selection of pancreatic epithelial cells from mesenchymal cells and SYBR Green real-time PCR An immunomagnetic separation system (Dynabeads, Dynal) was used to separate epithelial from mesenchymal cells. Human embryonic pancreatic tissue was digested with 250 U/ml of Collagenase IV (Worthington), and the cells were mechanically dispersed through 18-, 23-, and 25-gauge needles. Cells were next incubated with anti-E-cadherin antibody raised in rats (a gift from Dr Kemler) and incubated with Dynabeads M-450 sheep anti-rat IgG (Dynal). Dynabead-bound cells were collected by Dynal magnetic particle concentrator (Dynal). Total RNAs from cells bound or unbound to beads were extracted for real-time PCR analysis and processed using the SYBR Green PCR Core Reagent Kit (Applied Biosystems) according to the manufacturer's instructions. Each sample was amplified in duplicate.

Table 1

Cyclophilin	forward: 5'-CAGGTCCTGGCATCTTGTC-3' reverse: 5'-TTGCTGGTCTTGCCATTCCT-3'
E-cadherin	forward: 5'-AGTGGGCACAGATGGTGTGA-3' reverse: 5'-TAGGTGGAGTCCCAGGCGTA-3'
Vimentin	forward: 5'-CCTCACCTGTGAAGTGGATGC-3' reverse: 5'-CAACGGCAAAGTTCTCTTCCA-3'
FGF7	forward: 5'-ATCAGGACAGTGGCAGTTGGA-3' reverse: 5'-AACATTTCCCCTCCGTTGTGT-3'
FGF10	forward: 5'-CACATTGTGCCTCAGCCTTTC-3' reverse: 5'-AGGTGATTGTAGCTCCGCACA-3'
EGR1	forward: 5'-ATCATATGCCTGACCCCTTGC-3' reverse: 5'-TTTCCCCCAAGTGAGGACCTA-3'

FGF treatment Human pancreatic rudiments were cultured on Millipore filter inserts in 12-mm tissue culture plates in RPMI 1640 medium, and treated with 50 ng/ml of human recombinant FGF7 or FGF10 (both from R&D Systems), or PBS/BSA as control, for 1 h without serum (for EGR1 expression experiment) or for 24 h with 1% serum (for cell proliferation experiment). During the last hour, 10 μ mol/l bromodeoxyuridine (BrdU, Sigma) was added.

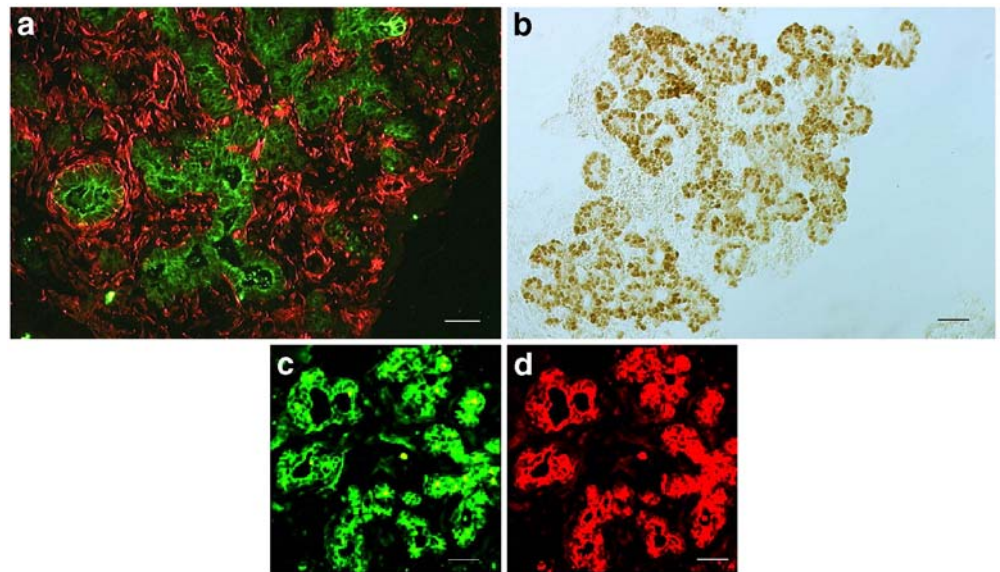
Immunohistochemistry Paraffin-embedded, 4- μ m-thick sections were processed for immunohistochemistry with antibodies directed against BrdU, pan-cytokeratin, vimentin, E-cadherin, and Pdx1 as described in [8]. The fluorescent secondary antibodies were Texas red anti-mouse, FITC anti-rat, and FITC anti-rabbit antibodies.

Results

Expression of FGF7 and FGF10 in pancreatic mesenchyme Among the 22 FGF family members analysed by RT-PCR, we found that FGF1, 2, 5, 7, 9, 10, 11, 12, 17, and 18 were present in human embryonic pancreas at 6–9 weeks of development (data not shown). We focused on FGF7 and FGF10 and first asked whether they were expressed by pancreatic epithelial or mesenchymal cells. As shown in Fig. 1, the human embryonic pancreas is composed of epithelial cells expressing cytokeratin and Pdx1 and mesenchymal cells expressing vimentin. At this stage, the epithelium cannot be mechanically separated from the mesenchyme. We thus adapted a new method to separate human embryonic pancreatic epithelial cells from mesenchymal cells. We first showed that epithelial cells express the transmembrane protein E-cadherin (Fig. 1). We next used anti-E-cadherin antibodies and the Dynabead system to separate epithelial from mesenchymal cells. After cell separation, RNA was prepared and real-time PCR was used to compare cells in the two fractions. As shown in Fig. 2, cells that attached to the beads (E-cadherin-sorted cells) expressed ten times more E-cadherin mRNA than cells that did not attach to the beads. On the other hand, cells that did not attach to the beads expressed six times more vimentin mRNA, ten times more FGF7 mRNA, and eight times more FGF10 mRNA, than cells that attached to the beads (Fig. 2). This indicates that both FGF7 and FGF10 are expressed in the mesenchyme during the early stages of human pancreatic development.

Effect of FGF7 and FGF10 on the proliferation of embryonic pancreatic epithelial cells EGR1 is an early growth response gene that is rapidly induced by growth factors. As shown in Fig. 3, both FGF7 and FGF10 treatment of the pancreatic rudiments induced the expression of EGR1 mRNA, indicating that functional receptors for such factors were expressed by human embryonic pancreatic cells. The expression of FGFR2b, the receptor for FGF7 and FGF10, by human embryonic epithelial cells was confirmed by RT-PCR (data not shown). Embryonic pancreata were next cultured with or without FGF7 or

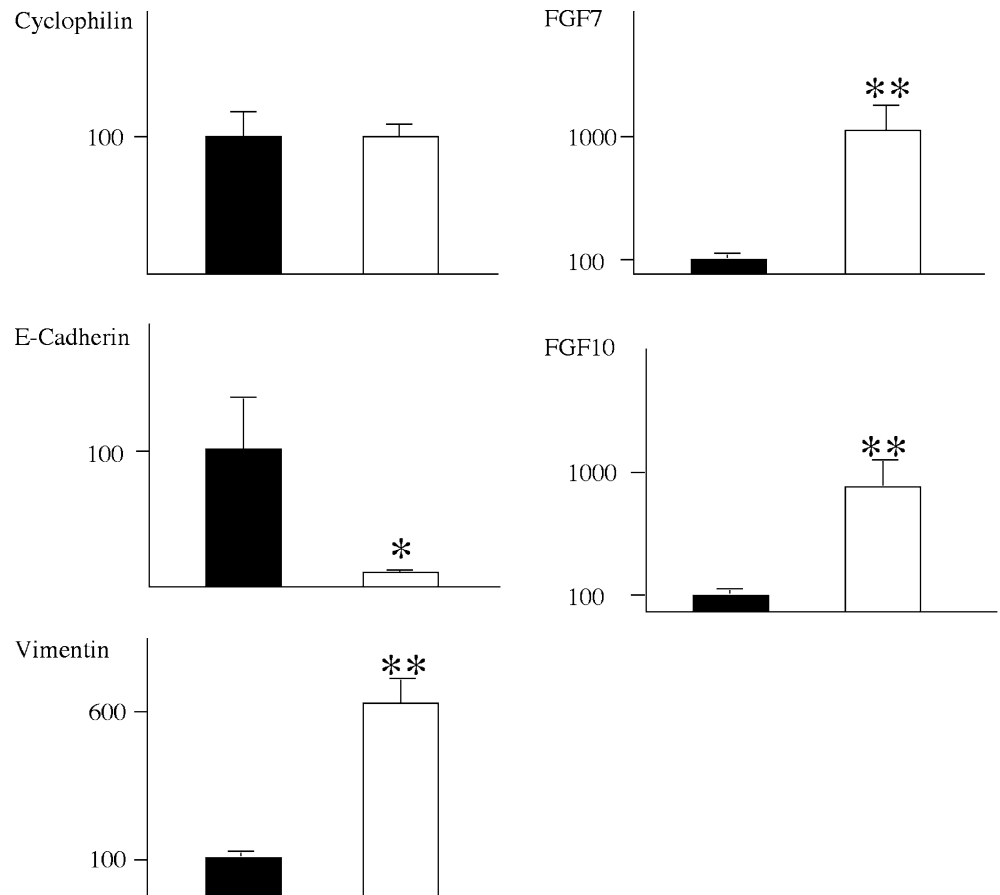
Fig. 1 Characterisation of the pancreatic epithelium and mesenchyme from 9-week-old human embryos. Pancreatic sections were stained for **a** cytokeratin (in green) and vimentin (in red), **b** Pdx1 (revealed in brown), **c** E-cadherin (in green), and **d** cytokeratin (in red). **c, d** The same section stained with anti-E-cadherin and anti-cytokeratin antibodies. Bar 50 μ m.



FGF10 for 24 h, and during the last hour, 10 μ M BrdU was added. As shown in Fig. 4, the percentage of BrdU-labelled epithelial cells considerably increased in FGF7- or FGF10-treated pancreatic rudiments as compared with untreated rudiments. In the presence of FGF7, $30.3 \pm 2.3\%$ of epithelial cells were labelled with BrdU compared to $16.8 \pm 1.7\%$ in control rudiments (1.8-fold increase, $p < 0.01$). Similarly, a 2.2-fold increase was observed in FGF10-treated

rudiments as compared to untreated rudiments (34.9 ± 4.6 vs $16.2 \pm 1.1\%$; 2.2-fold increase; $p < 0.01$). On the other hand, BrdU incorporation in mesenchymal cells was not different in FGF7/10 treated or untreated pancreata. These data indicate that both FGF7 and FGF10 stimulate human embryonic pancreatic epithelial cell proliferation in a specific fashion.

Fig. 2 Expression of FGF7 and FGF10 in the human embryonic pancreatic mesenchyme. Epithelium-rich and mesenchyme-rich fractions were separated using the Dynabead system and anti-E-cadherin antibodies as described in Materials and methods. The relative levels of cyclophilin, E-cadherin, vimentin, FGF7, and FGF10 were compared between the fraction of cells bound to the beads and the cells present in supernatant. An arbitrary value 100 was systematically given to the signal obtained with the cells attached to the beads. *Solid bars* fraction of cells bound to beads with anti-E-cadherin antibody, *open bars* fraction of cells from supernatant. Data represent mean \pm SE ($n=4$); * $p < 0.05$, ** $p < 0.01$.



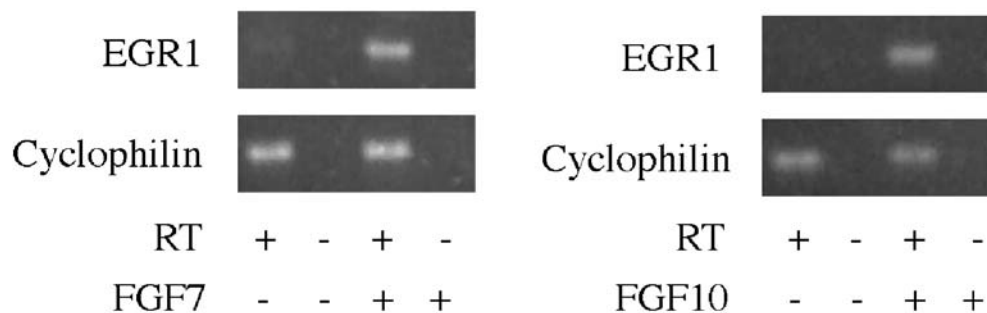


Fig. 3 Induction of EGR1 expression by FGF7 and FGF10. Human embryonic pancreatic rudiments were incubated with 50 ng/ml of FGF7 or FGF10, or vehicle for 1 h. Total RNA was extracted and submitted to RT-PCR analysis using primers specific for cyclophilin

and EGR1. *RT+* and *RT-* indicate that reactions were performed in the presence or absence of reverse transcriptase, respectively. Each reaction was repeated in three independent experiments.

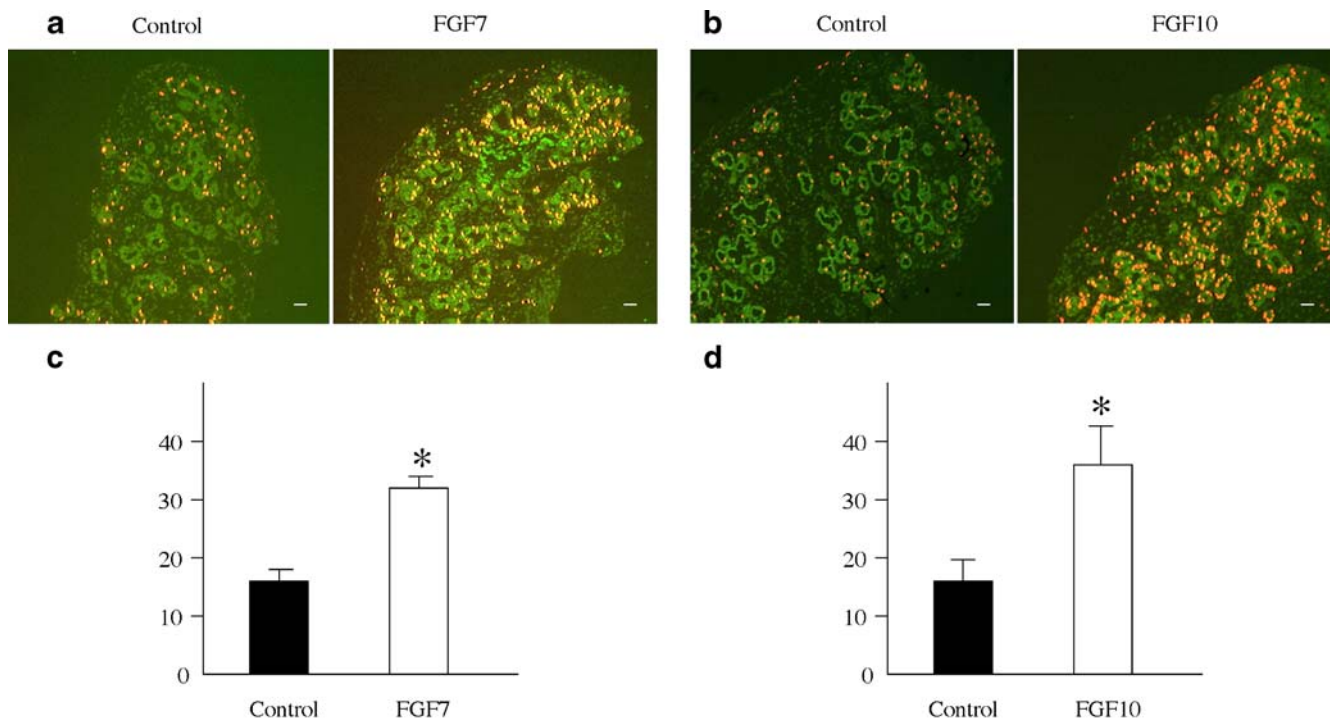


Fig. 4 Effect of FGF7 and FGF10 on the proliferation of embryonic pancreatic epithelial cells. Human pancreatic rudiments were cultured in the presence of 50 ng/ml of FGF7 (a) or FGF10 (b), or vehicle for 24 h. During the last hour, 10 μ M BrdU was added. The pancreatic rudiments were next embedded in paraffin for immunohistochemistry. Epithelial cells were labelled with anti-pancytokeratin antibody and revealed in green. Proliferating cells were

labelled using anti-BrdU antibody and revealed in red. Bar 50 μ m. **c, d** Percentage of epithelial cells that incorporated BrdU, among the total number of epithelial cells, in rudiments treated with or without FGF7 (c) or FGF10 (d). Each data point represents the mean \pm SE of four independent experiments; * p <0.01 as compared to the vehicle controls.

Discussion

In human embryonic pancreas, the inability to separate the pancreatic epithelium from the mesenchyme has represented a technical limitation to studying epithelial-mesenchymal interactions. Kobayashi et al. [9] recently defined an approach to solving this problem in the rodent pancreas at late stages of development, by using Dolichos biflorus agglutinin (DBA) as a specific marker of epithelial cells, and the Dynabead system. Here we used another epithelial marker, E-cadherin, and magnetic beads, to separate human epithelial from mesenchymal cells. Our data indicate that human embryonic pancreatic fractions en-

riched or poor in E-cadherin-expressing cells can be obtained. Such cell populations will be useful in the future to compare the transcriptome of these two fractions. Here, we used this approach combined with real time PCR as an alternative to in situ hybridization to define whether FGF7/10 was expressed by epithelial or mesenchymal cells. Using the approach described above, we demonstrated that FGF7 and FGF10 are expressed by mesenchymal cells, as in rodents. The next step was to define whether human embryonic pancreatic cells were sensitive to FGF7/10. Another group has recently shown that FGF7 treatment activated beta cell development from human fetal pancreatic cell preparations grafted to athymic rats. No ef-

fect of FGF7 was seen *in vitro*, suggesting that the growth effect of FGF7 was indirect [10]. Here, we show that FGF7 and FGF10 also have a direct effect on embryonic pancreatic cells. This was demonstrated by their capacity to rapidly induce the expression of EGR1. Moreover, we were able to show that FGF7 and FGF10 specifically activated epithelial cell proliferation. FGF7 and FGF10 could thus be used to expand human embryonic pancreatic epithelial cells that could potentially be useful for therapeutic approaches.

In conclusion, we have used a new method of separation of epithelium and mesenchyme from human embryonic pancreas, which allowed us to demonstrate that FGF7 and FGF10 were expressed by mesenchymal cells. Next, we provide evidence that FGF7 and FGF10 are capable of inducing the proliferation of the pancreatic epithelium in a direct fashion, thereby amplifying the pool of precursor cells. Our discovery of this direct mitogenic effect of FGF7 and FGF10 provides a novel approach to enhancing the quantity of pancreatic progenitor cells that represent a potential source of beta cells.

Acknowledgements We would like to thank Dr Kemler for antibodies. We are indebted to the medical staff in the department of gynaecological surgery at the hospital R. Debré (Pr Blot and Pr Oury). This work was supported by the Juvenile Diabetes Research Foundation (JDRF Center for beta cell therapy in Europe), the Association française des Diabétiques (AFD), and the Fondation pour la Recherche Médicale.

References

1. Ryan EA, Lakey JR, Paty BW et al (2002) Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes* 51:2148–2157
2. Castaing M, Peault B, Basmaciogullari A, Casal I, Czernichow P, Scharfmann R (2001) Blood glucose normalization upon transplantation of human embryonic pancreas into beta-cell-deficient SCID mice. *Diabetologia* 44:2066–2076
3. Bhushan A, Itoh N, Kato S et al (2001) Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development* 128:5109–5117
4. Elghazi L, Cras-Meneur C, Czernichow P, Scharfmann R (2002) Role for FGFR2IIIb-mediated signals in controlling pancreatic endocrine progenitor cell proliferation. *Proc Natl Acad Sci U S A* 99:3884–3889
5. Norgaard GA, Jensen JN, Jensen J (2003) FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. *Dev Biol* 264:323–338
6. Hart A, Papadopoulou S, Edlund H (2003) Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. *Dev Dyn* 228:185–193
7. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162:156–159
8. Duvillie B, Attali M, Aiello V, Quemeneur E, Scharfmann R (2003) Label-retaining cells in the rat pancreas: location and differentiation potential *in vitro*. *Diabetes* 52:2035–2042
9. Kobayashi H, Spilde TL, Li Z et al (2002) Lectin as a marker for staining and purification of embryonic pancreatic epithelium. *Biochem Biophys Res Commun* 293:691–697
10. Movassat J, Beattie GM, Lopez AD, Portha B, Hayek A (2003) Keratinocyte growth factor and beta-cell differentiation in human fetal pancreatic endocrine precursor cells. *Diabetologia* 46:822–829