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Diabetes and pancreatic tumours in transgenic mice expressing Pa × 6

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Abstract

Aims/hypothesis. Both endocrine and exocrine cells of the pancreas differentiate from epithelial cells of primitive pancreatic ducts, and four types of pancreatic islet cells (alpha, beta, delta, and PP cells) are derived from the common pluripotent precursor cells. Although Pa × 6 is expressed in all islet cells, Pa × 4 is detected only in beta cells. In homozygous *Pa × 4*-null mice, beta cells are absent, whereas the number of alpha cells is increased. Therefore, we hypothesized that the balance of Pa × 4 and 6 is one of the determinants by which the common progenitor cells differentiate into alpha or beta cells.
Methods. To change this balance, we generated transgenic mice overexpressing Pa × 6 driven by the insulin promoter or the *PDX1* promoter.
Results. In both types of transgenic mice, normal development of beta cells was disturbed, resulting in apoptosis of beta cells and diabetes. In Insulin/Pa × 6-Tg mice, beta cells were specifically affected, whereas in PDX/Pa × 6-Tg mice, developmental abnormalities involved the whole pancreas including hypoplasia of the exocrine pancreas. Furthermore, PDX/Pa × 6-Tg mice experienced proliferation of both ductal epithelia and islet cells and subsequent cystic adenoma of the pancreas.
Conclusion/interpretation. These findings suggest that Pa × 6 promotes the growth of ductal epithelia and endocrine progenitor cells and that the suppression of Pa × 6 is necessary for the normal development of beta cells and the exocrine pancreas. [Diabetologia (2000) 43: 332–339]

Keywords Diabetes mellitus, islets of Langerhans, embryology, cell differentiation, transcription factors.

The paired box-containing (*Pax*) genes encode tissue-specific transcription factors that contain a highly conserved bipartite DNA-binding domain composed of two helix-turn-helix motifs (paired domain) [1]. In addition to the paired domain, several Pax proteins including Pa × 4 and 6 have a complete homeodomain [2].

Mouse *Pa × 6* (*Small eye*) gene [3] is homologous to *Drosophila eyeless* (*ey*) gene [4] and human *Aniridia* (*AN*) gene [5]. The Pa × 6 protein is expressed in the central nervous system, eyes, nose, and pancreatic islets [6, 7]. In the mouse pancreatic anlage, Pa × 6 expression starts from embryonic day 9.0 (E9.0), and is detected in all four types of endocrine cells, but not in exocrine cells [8]. Heterozygous *Pa × 6* mutation results in various ocular abnormalities [9, 10]. Like homozygous *Small eye* mutants [3], homozygous *Pa × 6*-null mice lack eyes and olfactory bulbs, and

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Abbreviations: BDF1, C57BL/6 × DBA2 F1; E, embryonic day; H&E, haematoxylin and eosin; Insulin/Pa × 6-Tg mice, Pa × 6 transgenic mice with the insulin promoter; *Pax* gene, paired box-containing gene; PCNA, proliferating cell nuclear antigen; PDX1, pancreatic or duodenal homeobox-1; PDX/Pa × 6-Tg mice, Pa × 6 transgenic mice with the *PDX1* promoter; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick-end labelling.

die a few minutes after birth [11]. In the pancreas of the null mice, alpha cells are absent. Although beta, delta, and PP cells are still present, they fail to form distinct islets and remain disorganized throughout the exocrine pancreas.

The Pa × 4 and 6 proteins belong to the same subgroup with the homeodomain of Pax family [12]. In contrast to a uniform expression of Pa × 6 in the pancreatic islets, the expression of Pa × 4 is restricted to beta cells and Pa × 4 mRNA is detected in the mouse pancreatic bud from E9.5. Homozygous *Pa × 4*-null mice lack beta and delta cells whereas the number of alpha cells increases [13]. Furthermore, in double mutant mice lacking both Pa × 4 and 6, no hormone-producing cells are observed [11]. These results imply that the common pluripotent precursor cells differentiate into alpha cells when only Pa × 6 is expressed and into beta and delta cells when Pa × 4 is also expressed later. We hypothesized that the balance of Pa × 4 and 6 is one of the determinants by which the common progenitor cells differentiate into alpha or beta cells. To change this balance, we generated transgenic mice overexpressing Pa × 6 under the control of the insulin promoter or the pancreatic or duodenal homeobox-1 (*PDX1*) promoter. Because the 4.5-Kb upstream region of mouse *PDX1* gene was reported to be sufficient for the specificity of the *PDX1* promoter in transgenic mice [14], we cloned a genomic fragment (6.5 Kb) including this region and used it for transgenic mice. In Pa × 6 transgenic mice with the insulin promoter (Insulin/Pa × 6-Tg mice), Pa × 6 is overexpressed in undifferentiated and mature beta cells. In Pa × 6 transgenic mice with the *PDX1* promoter (PDX/Pa × 6-Tg mice), Pa × 6 overexpression starts from the earliest stage of pancreatic development (E8.5) before the differentiation of endocrine and exocrine cells [15, 16].

Materials and methods

Preparation of transgene constructs. Mouse Pa × 6 cDNA was a kind gift from Dr. P. Gruss (Max-Planck Institute for Biophysical Chemistry, Goettingen, Germany). For Insulin/Pa × 6-Tg mice, mouse Pa × 6 cDNA was inserted into the cloning site of the vector containing the insulin promoter (1.9 Kb) for transgenic mice [17]. From the λFIX II mouse 129SvJ genome library (Stratagene, La Jolla, Calif., USA), three independent clones containing the *PDX1* promoter were obtained using the plaque screening method. The restriction maps of the overlapped region of these clones were identical. The clone containing the longest promoter region (–6.5 Kb from the start codon, ATG) was used for PDX/Pa × 6-Tg mice. The insulin promoter (1.9 Kb) in the vector for Insulin/Pa × 6-Tg mice was substituted for the *PDX1* promoter (6.5 Kb). Linearized vectors excluding plasmid-derived sequences were used as transgenes.

Generation of transgenic mice. The transgenes were microinjected into the male pronuclei of fertilized eggs obtained from superovulated BDF1 (C57BL/6 × DBA2 F1) female mice crossed with male BDF1. Injected embryos were implanted into the oviducts of pseudopregnant female mice and allowed to develop [18]. The DNA was extracted from tail snips of live offspring by the proteinase K/SDS method. The integration of the transgene into the mouse genome was detected by PCR and Southern blot analysis. The copy numbers of integrated transgenes were determined from the intensity of each radioactive band in Southern blot analysis compared with indicator bands of 1, 10, and 100 copies of the transgene. Mice of 10 to 40 weeks of age were used with sex-matched non-transgenic littermates (wild-type mice) in this study. Mice with blood glucose concentrations of more than 25 mmol/l were diagnosed as diabetics. All mice were handled according to the "Principles of Laboaratory Animal Care" (NIH publication no. 85–23, revised 1985).

Gene expression in pancreatic islets. From mouse islets collected by the collagenase method [19], total RNA was extracted with ISOGEN (Nippon Gene, Osaka, Japan). After DNase treatment, 3 μg of total RNA were reverse-transcribed to cDNA with Super Script II first-strand synthesis kit (Gibco BRL, Rockville, Md., USA). Target sequences were amplified using the first-strand cDNA as a template with 0.025 U/μl of Ampli *Taq* Gold (Perkin-Elmer, Norwalk, Conn., USA). After the initial denaturation at 94 °C for 9 min, one cycle of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 3 min was repeated 20, 25, 30, and 35 times, respectively. During the cycle number of 20 to 35, the range in which the amount of PCR products linearly increase was ascertained by polyacrylamide gel electrophoresis. On the basis of this linearity, the expressions of target genes were semiquantitatively determined with an Adobe Photoshop Version 4.0J computer program (Adobe Systems, San Jose, Calif., USA) in which the amount of each PCR product was measured as the product of the area and the mean intensity of its band after ethidium bromide staining. To distinguish the amplification of genome DNA from that of cDNA, respective primers were selected from the sequence of different exons so that one or more introns lie between a pair of primers. After amplification with several pairs of primers for each target gene in the different cycle numbers of 20 to 35, the most appropriate pair of primers for semiquantitative analysis of the target gene was chosen. Because Pa × 6 mRNA derived from transgene includes the sequences of exon 2 and 3 of the rabbit beta-globin gene, its expression was discriminated from endogenous Pa × 6 mRNA by RT-PCR with a primer set on the exon 2 and 3 of the rabbit beta-globin gene.

Histopathological examination of pancreatic islets. Mouse pancreas was stained with haematoxylin and eosin (H&E), aldehyde fuchsin for beta cells and by Grimelius' method for alpha cells. For immunohistochemistry, guinea-pig polyclonal antibodies against porcine insulin and glucagon (Dako, Kyoto, Japan) and a mouse monoclonal antibody (IgG2a) against proliferating cell nuclear antigen (PCNA; Oncogene Science, Uniondale, N.Y., USA) were used. The quantitative analysis of islet-cell area was carried out as described previously [17]. In brief, islet area, alpha-cell area and beta-cell area were firstly identified with H&E staining, glucagon immunostaining and insulin immunostaining, respectively and secondly determined by superimposing microphotographs onto graph paper ruled into 1-mm squares. Because the results obtained from staining with aldehyde fuchsin and by Grimelius' method were identical to those from the immunohistochemistry for beta and alpha cells, respectively, these data are not shown in this paper.

Determination of glucose and insulin. The glucose tolerance test and the determination of blood glucose and serum insulin were carried out as described previously [17]. For the determi-

nation of insulin content in the whole pancreas, each mouse pancreas was homogenized in 4 ml of ice-cold acid-ethanol solution (0.15 N HCl:75% ethanol) [20], and insulin was extracted at 4 °C overnight. After centrifugation at 2000 × *g* at 4 °C for 30 min, the supernatant was neutralized and diluted with PBS. Insulin concentrations were assayed in 1000- fold or 10000-fold diluted samples. Using mouse islets in primary culture, insulin secretion was measured. Five islets isolated by the collagenase method [19] were preincubated in 1 ml of modified KRB containing 115 mmol/l NaCl, 5 mmol/l KCl, 2.2 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 1 mmol/l Na_2HPO_4, 24 mmol/l $NaHCO_3$, 10 mmol/l HEPES/NaOH (pH 7.4), and 0.3% BSA with 2.8 mmol/l glucose at 37° C in an atmosphere of humidified air:CO_2 (95:5) for 60 min and stimulated by an increase in glucose concentration of 16.7 mmol/l.

Detection of apoptosis. To detect apoptotic cells in pancreatic islets, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) method was used. The paraffin sections of mouse pancreas were dewaxed by heating at 60 °C followed by washing in xylene, rehydrated through a graded series of ethanol and water and incubated with 20 μg/ml of proteinase K in 10 mmol/l TRIS-Cl (pH 7.4) at 37 °C for 30 min. After washing with PBS, the sections were incubated with TdT from calf thymus and fluorescein-labelled dUTP (Boehringer Mannheim), and the fluorescein incorporated into DNA cleavage sites was detected by Fab fragments of anti-fluorescein sheep antibodies conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) and its substrates; nitro blue tetrazolium (Wako, Osaka, Japan) and 5-bromo-4-chloro-3-indolylphosphate (Wako). For a positive control, the section was treated with 1 mg/ml of DNase I (Takara Shuzo, Tokyo, Japan) in 30 mmol/l TRIS-Cl (pH 7.5), 4 mmol/l $MgCl_2$, and 0.1 mmol/l dithiothreitol (DTT) for 10 min just before TdT reaction, to induce DNA strand breaks.

Statistical analysis. All data are presented as means ± SEM. For comparison of two means, Student's unpaired *t* test was used. For comparison of two ratios (Q_1, Q_2), a normal distribution curve with a mean of Q_1-Q_2 and a variance of $Q_1(1\text{-}Q_1)/n_1 + Q_2(1\text{-}Q_2)/n_2$ was used. Probability (p) less than 0.05 was considered statistically significant.

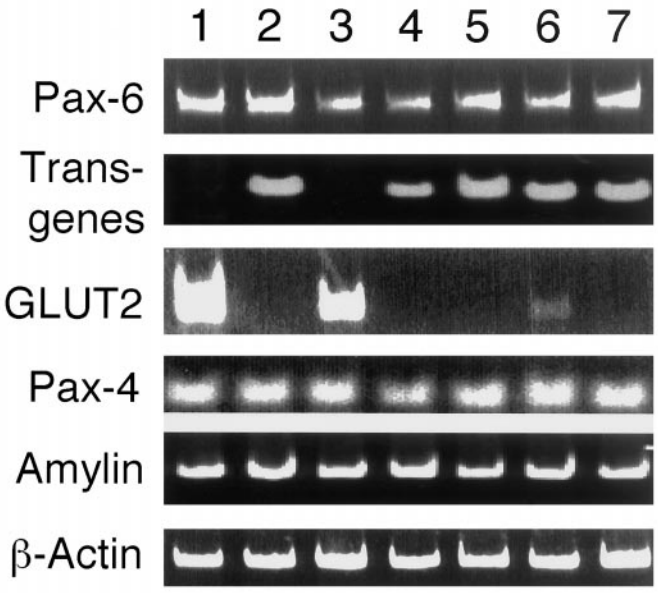

Fig. 1. Semi-quantitative analysis of gene expression in pancreatic islets by RT-PCR. Islets obtained from five mice in the same line of 16–20 weeks of age were used in one lot for RNA extraction. Lane 1, wild-type mice; lanes 2–4, Insulin/Pa × 6-Tg mice, line 1 (20 copies of transgene/mouse genome), line 2 (1 copy), and line 3 (2 copies), respectively; lanes 5–7, PDX/Pa × 6-Tg mice, line 1 (15 copies), line 2 (5 copies) and line 3 (10 copies), respectively. In transgenic lines with high copy numbers, the expressions of transgenes were also high. In line 2 of Insulin/Pa × 6-Tg mice (lane 3), the expression of transgene is not seen in this figure, but it was detected by RT-PCR with more five-thermal cycles (data not shown). The expression of transgenes correlated with the expression of total Pa × 6, which is the sum of transgene-derived Pa × 6 and endogenous Pa × 6 ($r = 0.7$). The expression of GLUT2 negatively correlated with the expressions of transgenes ($r = –0.7$) and total Pa × 6 ($r = –0.5$), whereas the expressions of Pa × 4 and amylin were similar in all lines of transgenic mice. These findings suggest that Pa × 6 overexpression inhibits the normal differentiation of beta cells

Results

Generation of transgenic mice. Both in Insulin/Pa × 6-Tg mice and PDX/Pa × 6-Tg mice, four founder mice were obtained. The copy numbers of the four Insulin/Pa × 6-Tg founders (lines 1–4) were 20, 1, 2 and 4, respectively. Those of the four PDX/Pa × 6-Tg founders (lines 1–4) were 15, 5, 10 and 8, respectively. Because both transgenic founders of line 4 could not transmit each transgene to their offspring, F1 transgenic mice of lines 1–3 with wild-type mice were used in this study. The percentage of PDX1/Pa × 6-Tg mice (66 from 199; 35%) in F1 mice at the age of 5 weeks was significantly ($p < 0.01$) lower than that of Insulin/Pa × 6-Tg mice (99 from 216; 46%), suggesting that PDX1/Pa × 6-Tg mice have more disadvantages for survival than Insulin/Pa × 6-Tg mice.

Gene expression in pancreatic islets (Fig. 1). In all transgenic mice, the expression of transgenes was confirmed by RT-PCR. The expressions (i.e. the amount of PCR products) of the transgenes correlated with their copy numbers with high correlation coefficients (r) of 0.9 for Insulin/Pa × 6-Tg mice and 0.6 for PDX/Pa × 6-Tg mice. The expressions of transgenes also correlated with the expression of total Pa × 6 ($r = 0.7$), which is the sum of transgene-derived and endogenous Pa × 6, without any change of the Pa × 4 expression. In addition to Pa × 4, further two beta-cell markers, amylin and GLUT2, were examined. Although the expression level of amylin was similar in all transgenic mice and wild-type mice, the expression of GLUT2 was negatively correlated with the expressions of transgenes ($r = –0.7$) or total Pa × 6 ($r = –0.5$). In the transgenic lines with high expression of transgenes, GLUT2 expression was not detected at all in pancreatic islets. This negative cor-

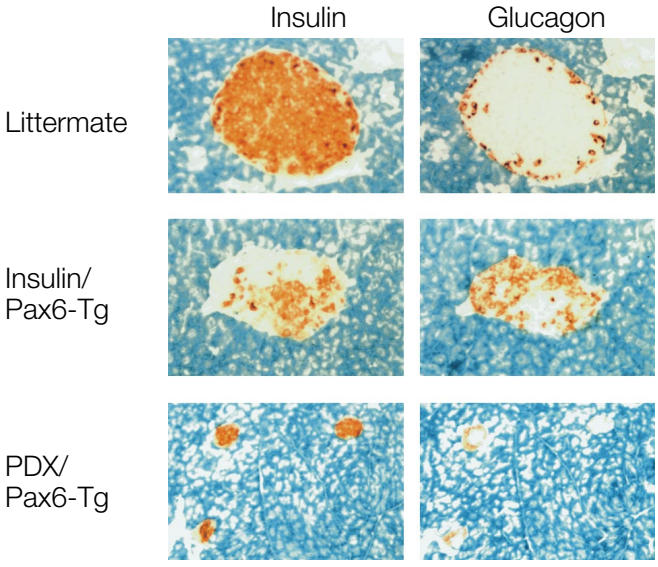

Fig. 2. Immunohistochemistry for insulin and glucagon. Each figure is a representative for seven similar observations, using pancreata obtained from seven mice of 20–28 weeks of age. In Insulin/Pa × 6-Tg mice, the number of beta cells decreased and the ratio of alpha to beta cells increased, whereas in PDX/Pa × 6-Tg mice, the numbers of alpha and beta cells decreased in parallel. All micrographs are at the same magnification (× 200)

relation indicates that Pa × 6 overexpression inhibits the normal differentiation of beta cells. Moreover, the expression of neural cell adhesion molecule (NCAM), which is regulated by Pa × 6 [21], correlated with the expressions of transgenes ($r = 0.8$) and total Pa × 6 ($r = 0.7$) (data not shown).

Phenotypes of transgenic mice. In line 1 of Insulin/Pa × 6-Tg mice with the highest copy number of 20 and in all three lines of PDX/Pa × 6-Tg mice, mice developed diabetes before the age of 10 weeks. The percentage of diabetic mice was 46 % in line 1 of Insulin/Pa × 6-Tg mice and 22 % in PDX/Pa × 6-Tg mice. In all lines of PDX1/Pa × 6-Tg mice, pancreatic cysts and the concomitant abdominal distension were frequently observed in addition to diabetes. To clarify the mechanism by which diabetes and pancreatic cysts occur, the following physiopathological examinations were carried out.

Pathogenesis of diabetes. The islet area relative to the whole area of the pancreas in Insulin/Pa × 6-Tg mice (0.71 ± 0.20 %; $n = 7$) was smaller ($p < 0.01$) than that of wild-type mice (1.72 ± 0.31 %; $n = 6$) and the islet area of PDX/Pa × 6-Tg mice showed an intermediate value (1.10 ± 0.36 %; $n = 6$). In Insulin/Pa × 6-Tg mice, the islet area negatively correlated with the copy numbers ($r = -0.7$; $n = 8$). In immunohistochemical staining, beta-cell and alpha-cell areas relative to the whole area of the pancreas were determined. In wild-type mice, beta-cell and alpha-cell areas were 1.28 ± 0.23 % and 0.44 ± 0.07 %, respectively ($n = 6$). In Insulin/Pa × 6-Tg mice, beta-cell area (0.31 ± 0.08 %; $n = 7$) decreased ($p < 0.05$), but alpha-cell area (0.40 ± 0.09 %; $n = 7$) did not change, resulting in the area of alpha cells exceeding that of beta cells (Fig. 2). In PDX/Pa × 6-Tg mice, beta-cell area (0.81 ± 0.25 %; $n = 6$) and alpha-cell area (0.28 ± 0.10 %; $n = 6$) simultaneously decreased, resulting in islet shrinkage (Fig. 2). Therefore, the ratio of alpha to beta cells in Insulin/Pa × 6-Tg mice was increased ($p < 0.01$) (1.26 ± 0.31 %; $n = 7$), although the ratios in PDX/Pa × 6-Tg and wild-type mice were similar (0.35 ± 0.12 %; $n = 6$ and 0.34 ± 0.02 %; $n = 6$, respectively). By superimposing insulin immunostaining on glucagon immunostaining between two consecutive sections, islet cells co-expressing insulin and glucagon were not found.

The insulin contents of Insulin/Pa × 6-Tg mice (13.2 ± 1.2 μg/pancreas) and PDX/Pa × 6-Tg mice (6.4 ± 1.6 μ g/pancreas) were smaller ($p < 0.01$) than those of wild-type mice (24.2 ± 1.2 μ g/pancreas). Both in Insulin/Pa × 6- and PDX/Pa × 6-Tg mice, the insulin secretion in response to high glucose concentration (16.7 mmol/l) was smaller ($p < 0.01$) than that of wild-type mice (Fig. 3).

To examine whether the number of transgenic islet cells decreased by apoptosis, the TUNEL method was used. In transgenic islets, many apoptotic cells were detected (Fig. 4). Furthermore, to examine the proliferation of islet cells, PCNA was immunohistochemically stained. We observed PCNA-positive islet cells in PDX/Pa × 6-Tg mice (Fig. 5) but detected no islets with PCNA-positive cells in Insulin/Pa × 6-Tg mice (data not shown) or in wild-type mice (Fig. 5). By superimposing consecutive sections with immunostaining, most of the PCNA-positive islet cells in PDX/Pa × 6-Tg mice were shown to be beta cells (data not shown).

Pathogenesis of pancreatic cysts. In almost all PDX/Pa × 6-Tg mice tested, a remarkable dilation of pancreatic ducts was found (Fig. 6) but no dilation in Insulin/Pa × 6-Tg mice and wild-type mice. In PDX/Pa × 6-Tg mice, PCNA-positive epithelial cells were detected in dilated pancreatic ducts but no PCNA-positive cells were detected in Insulin/Pa × 6-Tg mice and wild-type mice, in normal-sized pancreatic ducts (data not shown). Therefore, this ductal dilation in PDX/Pa × 6-Tg mice was considered to be hyperplasia of epithelial cells. Moreover, many islet cells differentiated from the ductal epithelial cells around the dilated pancreatic ducts (Fig. 6). We also detected PCNA-positive cells in these islet cells (data not shown). Such islet neogenesis was not observed in Insulin/Pa × 6-Tg mice and wild-type mice. In association with the islet neogenesis, the number of islets in PDX/Pa × 6-Tg mice (110 ± 9.0 / cm^2 of

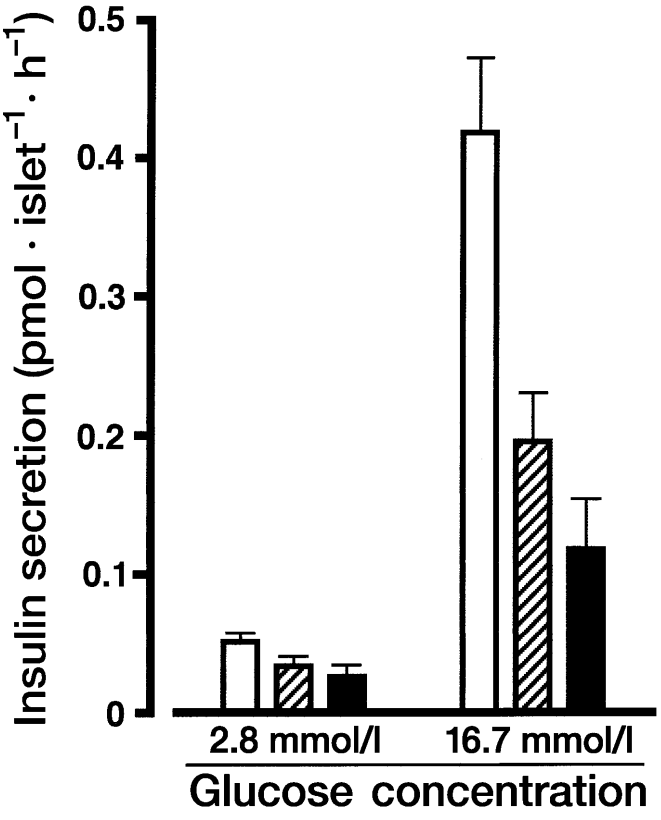

Fig. 3. Insulin secretion from isolated pancreatic islets. Open columns, wild-type mice ($n = 7$); hatched columns, Insulin/Pa × 6-Tg mice ($n = 7$); and solid columns, PDX/Pa × 6-Tg mice ($n = 8$). Both in Insulin/Pa × 6-Tg mice and PDX/Pa × 6-Tg mice, the insulin secretion in response to high glucose concentration (16.7 mmol/l) was smaller ($p < 0.01$) than that of wild-type mice

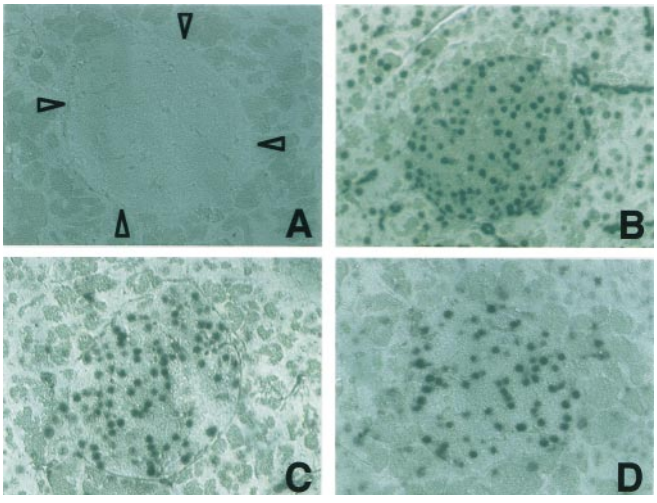

Fig. 4 A–D. Detection of apoptotic cells in pancreatic islets by the TUNEL method. Each figure is a representative for four similar observations, using pancreata obtained from four mice of 20–28 weeks of age. **A** Wild-type mouse. Arrowheads indicate an islet. **B** Wild-type mouse with DNase treatment as a positive control. **C** Insulin/Pa × 6-Tg mouse. **D** PDX/Pa × 6-Tg mouse. **A–D** are at the same magnification (× 200)

section; $n = 6$) was larger ($p < 0.01$) than that in wild-type mice (75.7 ± 7.1/cm^2 of section; $n = 6$), whereas that in Insulin/Pa × 6-Tg mice (45.7 ± 11.7/cm^2 of section; $n = 7$) was smaller ($p < 0.05$) than that in wild-type mice. The means of area of individual islets were 0.023 ± 0.002 mm^2 in wild-type mice ($n = 6$), 0.016 ± 0.001 mm^2 in Insulin/Pa × 6-Tg mice ($n = 7$) and 0.010 ± 0.001 mm^2 in PDX/Pa × 6-Tg mice ($n = 6$).

Hyperplastic ductal epithelia frequently progressed to cystic adenomas in PDX/Pa × 6-Tg mice. Many adenoma cells were PCNA-positive (Fig. 5). In cystic adenoma, islet neogenesis did not occur and islets adjacent to cystic adenomas were occasionally disorganized and shrunken in size (Fig. 5).

Development of pancreatic exocrine cells. As an indicator of the amount of exocrine pancreas, the weight of the whole pancreas was determined. In PDX/Pa × 6-Tg mice, the weight of the pancreas (5.2 ± 0.3 mg/g of body weight; $n = 10$) was lower ($p < 0.01$) than that of Insulin/Pa × 6-Tg mice (7.7 ± 0.3 mg/g of body weight; $n = 13$) or wild-type mice (7.4 ± 0.4 mg/g of body weight; $n = 20$), indicating the hypoplasia of the exocrine pancreas in PDX/Pa × 6-Tg mice but not in Insulin/Pa × 6-Tg mice.

Discussion

Diabetes developed in 46% of line 1 of Insulin/Pa × 6-Tg mice and in 22% of PDX/Pa × 6-Tg mice. The reason for only some of the transgenic mice being affected with diabetes is not clear. It could be because of the differences in genetic background in individual transgenic mice. Because BDF1, a hybrid strain of C57BL6 and DBA2, was used for generation of transgenic mice and for crossing with transgenic mice, the genetic background of transgenic offspring varied between C57BL/6 and DBA2.

Both in Insulin/Pa × 6-Tg mice and in PDX/Pa × 6-Tg mice, islet area, the number of beta cells, insulin content of the pancreas, glucose-induced insulin secretion, and GLUT2 expression decreased, resulting in diabetes. The decrease in glucose-induced insulin secretion can be explained, at least in part, by the reduction of GLUT2 expression. The sufficient expressions of amylin, another beta-cell marker and transgenes which are expressed in beta cells by the insulin promoter or the *PDX1* promoter indicate that the decreased GLUT2 expression is due to the abnormal differentiation of beta cells, rather than the decrease in the number of beta cells. Furthermore, the greater the expression level of the transgene, the lower was the expression of GLUT2. Therefore, overexpression of Pa × 6 was shown to disturb the normal

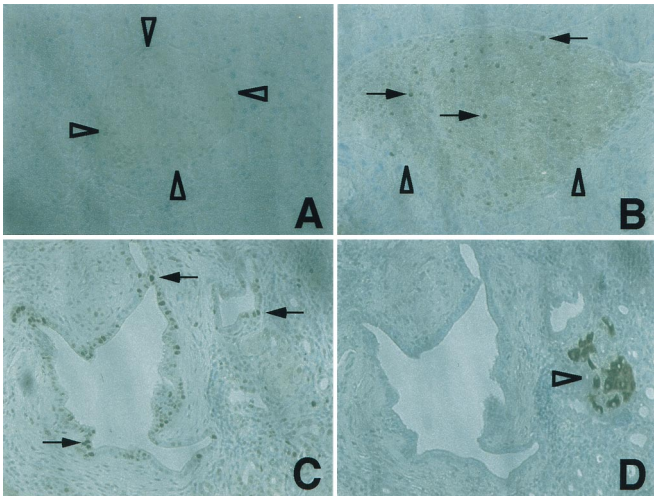

Fig. 5 A–D. Detection of proliferating cells by immunostaining for PCNA. Each figure is a representative for four similar observations, using pancreata obtained from four mice of 28–36 weeks of age. **A** An islet of a wild-type mouse. **B** An islet in a PDX/Pa × 6-Tg mouse. **C** Pancreatic cystic adenoma in a PDX/Pa × 6-Tg mouse. **D** Insulin immunohistochemistry of a cystic adenoma in a PDX/Pa × 6-Tg mouse. Islets adjacent to the cystic adenoma were occasionally disorganized and shrunken in size. Arrows and arrowheads indicate PCNA-positive proliferating cells and islets, respectively. **A** vs **B** (× 200) and **C** vs **D** (× 100) are at the same magnifications, respectively

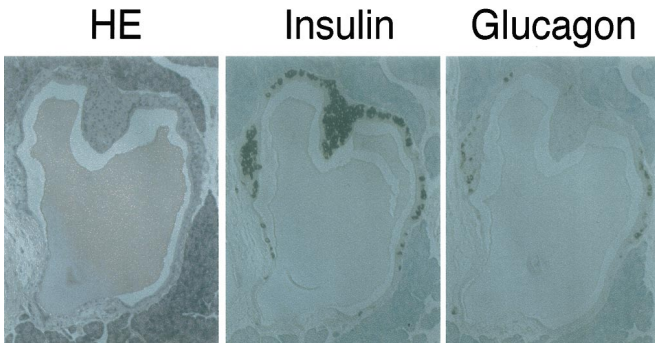

Fig. 6. Islet neogenesis around dilated pancreatic ducts in a PDX/Pa × 6-Tg mouse. This figure is a representative for seven similar observations, using pancreata obtained from seven mice of 20–28 weeks of age. Haematoxylin and eosin staining and immunostaining for insulin and glucagon are shown

development of beta cells. In the development of eyes, overexpression of Pa × 6 in transgenic mice was also reported to cause severe microphthalmia although appropriate expression of Pa × 6 rescued eye abnormalities observed in *Small eye* mice which have mutations in the *Pa × 6* gene [22].

In Insulin/Pa × 6-Tg mice, beta cells were specifically affected, whereas in PDX/Pa × 6-Tg mice, developmental abnormalities extended over the whole pancreas including endocrine and exocrine tissues. The *PDX1* promoter functions in the primitive pancreatic epithelium which differentiates into the endocrine and exocrine tissues. In PDX/Pa × 6-Tg mice, overexpression of Pa × 6 is expected to occur throughout the epithelium but in wild-type and Insulin/Pa × 6-Tg mice, Pa × 6 expression occurs only in a subset of cells in the epithelium which differentiate into endocrine cells. Furthermore, in Insulin/Pa × 6-Tg mice, the exocrine tissue developed normally. These findings suggest that overexpression of Pa × 6 in the whole of the primitive pancreatic epithelium resulted in hypoplasia of the exocrine tissue. The pancreatic weight of PDX/Pa × 6-Tg mice was 70% that of wild-type mice. Because the pancreatic weight of PDX/Pa × 6-Tg mice includes the weight of cystic adenoma, the real weight of transgenic exocrine pancreas should be even lower than 70%.

In PDX/Pa × 6-Tg mice, dilation of pancreatic ducts due to hyperplasia of ductal epithelial cells was observed, and this frequently progressed to cystic adenoma. Indeed, Pax-1, 2, 3, 6, and 8 have been reported to have an oncogenic potential, based on the transformation of cultured fibroblasts overexpressing Pax proteins and the tumour formations in nude mice bearing these cells [23]. Unlike Insulin/SV40 T-antigen-Tg mice [24], Insulin/Pa × 6-Tg mice did not develop insulinoma. Therefore, it is considered that the transforming activity of Pa × 6 is weaker than that of SV40 T-antigen, and that Pa × 6 cannot transform islet cells differentiated to the level of insulin secretion.

Although the mean of area individual islets in PDX/Pa × 6-Tg mice (0.010 mm^2) was smaller than that in Insulin/Pa × 6-Tg mice (0.016 mm^2), the number of islets in PDX/Pa × 6-Tg (110/cm^2) was larger than that in Insulin/Pa × 6-Tg mice (45.7/cm^2) because of islet neogenesis from hyperplastic epithelial cells of pancreatic ducts, and the total area of islets relative to the whole area of the pancreas was larger in PDX/Pa × 6-Tg mice (1.10%) than in Insulin/Pa × 6-Tg mice (0.71%). In contrast to the total area of islets, pancreatic insulin content of PDX/Pa × 6-Tg mice (6.4 μg) was smaller than that in Insulin/Pa × 6-Tg mice (13.2 μg) and glucose-induced insulin secretion in PDX/Pa × 6-Tg mice was also smaller than that in Insulin/Pa × 6-Tg mice. These results suggest that many poorly differentiated beta cells, which have a small amount of insulin and a low response of insulin secretion to glucose stimulation, inhabit islets of PDX/Pa × 6-Tg mice. We speculate that islet-cell remodelling occurs in the pancreas of PDX/Pa × 6-Tg mice, i.e. poorly differentiated islet cells generated one after another from the hyperplastic epithelial cells of pancreatic ducts proliferate and eventually die apoptotically by the developmental abnormality during their differentiation.

The absence of alpha cells in homozygous *Pa × 6*-null mice shows that Pa × 6 is essential for the differentiation from the common islet precursor cells to al-

pha cells [11]. In addition to Pa × 6, Pa × 4 is expressed in beta cells [13]. Although Pa × 6 is expressed in all four types of islet cells and in other organs including eyes and the central nervous system [6, 7], Pa × 4 is specifically expressed in beta cells [13]. The Pa × 6 protein binds to a common element in the glucagon, insulin, and somatostatin promoters, and transactivates the glucagon and insulin promoters [8]. In contrast to many target genes of Pa × 6, the target genes of Pa × 4 have not yet been found in spite of an extensive search. Recently, it was reported that DNA binding specificities of Pa × 4 and Pa × 6 are similar and Pa × 4 can act as a Pa × 6 repressor by the competition for binding sites [25, 26]. Possibly Pa × 4 inhibits the induction of the alpha-cell phenotype by Pa × 6 in normal beta cells. We speculate that excessive Pa × 6 overcame the Pa × 4 inhibition of Pa × 6 and that the differentiation and maturation of beta cells were disturbed in our transgenic mice.

In PDX/Pa × 6-Tg mice, many proliferating endocrine cells were detected around dilated and growing pancreatic ducts and in regenerating islets. In contrast to PDX/Pa × 6-Tg mice, obvious islet neogenesis was not found in Insulin/Pa × 6-Tg mice. These findings suggest that Pa × 6 promotes the growth of the epithelial cells of pancreatic ducts and islet progenitor cells before they mature into insulin-producing cells because Pa × 6 showed no proliferative effect under the control of the insulin promoter. Possibly Pa × 6 exhibits a dual action during pancreas development; one action is the proliferation of primitive ductal epithelia and islet progenitor cells and the other is the induction of alpha-cell phenotypes. Among homeoproteins regulating pancreatic development, the dual action of PDX1 is well known, i.e. the loss of its early function leads to agenesis of the pancreas [15] whereas its later expression in beta cells is required to maintain the beta-cell pattern of hormone production, GLUT2 expression, and normoglycaemia [27].

Overexpressed Pa × 6 induced not only the proliferation of islet progenitor cells but also apoptosis of differentiating islet cells. Similar phenomena were observed in the transgenic overexpression of c-myc [28–30] and E2F-1 [31–33]. Although these transcriptional factors lead to S-phase entry of mammalian cells in G1 phase, their overexpression without other signals for cell growth induces apoptosis. The single activation of c-myc or E2F-1 is probably recognized as an abnormal growth signal by tumour suppressor proteins such as p53 and turns on the programme for apoptosis [31–35]. Overexpression of Pa × 6 could induce apoptosis by a similar mechanism.

We suggest that Pa × 6 stimulates the proliferation of the epithelial cells of pancreatic ducts and islet precursor cells and that the suppression of Pa × 6 is required for the normal development of beta cells and the exocrine pancreas.

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