

Hypoplasia of endocrine and exocrine pancreas in homozygous transgenic TGF- β 1

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Abstract

We generated the homozygous transgenic mice with expression of the active form of TGF- β 1 by the glucagon promoter (homozygous NOD-TGF- β 1). The homozygous NOD-TGF- β 1 showed severe diabetes in 84.6%, impaired glucose tolerance, and low serum insulin levels. The final size of endocrine and whole pancreas decreased, respectively, to 6 and 34%, compared to wild-type mice. The homozygous N₂ backcross to C57BL/6 (B6-TGF- β 1) showed no diabetes, but impaired glucose tolerance and low serum insulin levels. In homozygous NOD-TGF- β 1, the expression of p15^{INK4b} was induced by 3.4-fold in pancreatic islets than that in wild-type mice. Based on these, we conclude first that excessive paracrine TGF- β 1 signaling in islets results in endocrine and exocrine pancreatic hypoplasia, second that TGF- β 1 decrease the final size of endocrine and exocrine pancreas presumably through regulating cell cycle via p15^{INK4b} at least in endocrine pancreas, and third that hypoplastic action of TGF- β 1 of pancreatic islets is independent of the genetic background.

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1. Introduction

The optimal organ function requires a match between the organ size and physiologic demands of the host. Islet size, the number of islets, the relative ratio of islet β cells to other islet cells, and the balance between endocrine and exocrine cells

must be at the right balance to maintain proper glucose homeostasis. TGF- β signaling components including TGF- β 1, activin, their respective receptors, secretory factors including Notch, Hedgehog, FGF, and EGF likely regulate cell interaction necessary for proper histological development and functional maturation of the pancreas (Kim and Hebrok, 2001; Massague and Chen, 2000). TGF- β 1, a multifunctional cytokine, regulates cell growth, differentiation courses in many systems, and other functions such as immune suppression (Massague, 1990; Roberts and Sporn, 1990).

The final endocrine islet size or exocrine pancreas in vivo is decreased by the decreased TGF- β signaling through defective type II TGF- β , type IIA or IIB activin receptors (Kim et al., 2000), or decreased receptor functions by transgenic expression of a dominant negative type I activin (Yamaoka et al.,

Abbreviations: NOD-TGF- β 1, transgenic NOD mice expressing pTGF- β 1 in islet α cells; B6-TGF- β 1, backcrossed mice in N₂ generation which are homozygous for pTGF- β 1 and generated by crossing NOD-TGF- β 1 with C57BL/6 mice; ETCL, end trimming and cassette ligation; MoMuLV, Moloney murine leukemia virus; BG, blood glucose; ipGTT, intraperitoneal glucose tolerance test; HE, haematoxylin and eosin; Cdk, cyclin-dependent kinase

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1998) or type II TGF- β receptor (Bottinger et al., 1997). The final islet size or exocrine pancreas was also decreased by the increased TGF- β signaling through transgenic expression of a constitutively active type I activin receptor (Yamaoka et al., 1998). Thus, TGF- β signaling in vivo both in its excess and deficiency may determine the relative amount of endocrine and exocrine pancreas, and decrease islet size. But the mechanisms to control the relative ratios of endocrine and exocrine pancreas or the final islet size in vivo are not well understood.

Differentiation of pancreatic endocrine cells, especially islet β and PP cells, but not pancreatic exocrine cells were promoted by TGF- β 1 in vitro in embryonic mouse pancreas (Sanvito et al., 1994). Transgenic TGF- β 1 in vivo with the insulin promoter resulted in the accumulation of extracellular matrix (Lee et al., 1995), normoglycemia with unchanged β cell mass (Sanvito et al., 1995), or small islets to about 5% (Grewal et al., 2002), but the effect of excessive paracrine TGF- β 1 with the glucagon promoter on the final size of the endocrine or exocrine pancreas remains unknown.

It was recently reported that increase in the final islet size might be determined by the cell cycle factors. Mice with a loss of cyclin-dependent kinase (Cdk) 4 expression develop diabetes associated with hypoplastic islets (Rane et al., 1999; Tsutsui et al., 1999), while Cdk 4 activation is associated with islet hyperplasia (Rane et al., 1999). These observations strongly suggest that the normal islet size requires regulated entry into, passage through, and exit from cell cycles. TGF- β 1 induces the expression of p15^{INK4b}, a Cdk 4/6 inhibitor, causing G₁ arrest in many cell types including lung, thyroid, and mammary epithelial cells, astrocytes, and human keratinocytes (Massague et al., 2000; Hannon and Beach, 1994) by increasing mRNA levels or protein stability (Sandhu et al., 1997).

We generated homozygous transgenic mice with the expression of the active form of porcine TGF- β 1 (pTGF- β 1) under the glucagon promoter to determine the effects of excessive paracrine TGF- β 1 in vivo first on the final size of endocrine α or β cells, second on the size of exocrine pancreas, and third on the expression of p15^{INK4b} as the mediator of the hypoplastic action of TGF- β 1 on endocrine pancreas.

2. Material and methods

2.1. Production of homozygous NOD-TGF- β 1

Two lines of hemizygous NOD-TGF- β 1 (Moritani et al., 1998) were intercrossed to generate homozygous NOD-TGF- β 1. Hemizygous or homozygous transgene incorporation was quantitated with Southern blot analysis after PCR-based diagnosis.

2.2. Genetic backcross

To examine whether diabetes in homozygous NOD-TGF- β 1 is limited only to the NOD genetic background, male

hemizygous NOD-TGF- β 1 were crossbred twice to female C57BL/6 mice. The resulting hemizygous N₂ mice were then intercrossed to obtain the homozygous N₂ backcrosses (B6-TGF- β 1), which carry in average 75% genetic background of C57BL/6 mice.

2.3. Copy number assessment of the transgene with Southern blot analysis

Genome DNA was extracted from the tail at 4 weeks of age by phenol–chloroform method. Seven μ g of genome DNA were digested with *EcoRI* and applied to 1% agarose gel, and transferred to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, England) by capillary transfer. The membrane was hybridized with the [³²P]-labeled 1300 bp pTGF- β 1 cDNA probe obtained from a plasmid of pRB 601 (Moritani et al., 1998) with *SalI* and *NotI* digestion. The hybridization was performed according to the published method (Sambrook et al., 1989). The copy number of the integrated transgene were semi-quantitated using the computer software of NIH image with the intensity of each radioactive band compared with the indication band of endogenous mouse TGF- β 1.

2.4. Genetic diagnosis of transgene

Genotype of transgene in NOD-TGF- β 1 or B6-TGF- β 1 was determined by PCR. To discriminate the homozygous or hemizygous transgene with PCR, we determined the transgene's flanking sequence in the mouse genome with end trimming and cassette ligation (ETCL)-PCR method (Iwahana et al., 1994). Genomic DNA (0.5–1 μ g) from hemizygous NOD-TGF- β 1, NOD, or C57BL/6 mice was digested with three different groups (C1–C3) of restriction enzymes (Iwahana et al., 1994). The 3'-ends of DNA after digestion with restriction enzymes was end-trimmed by filling with one of four dNTPs using Klenow fragment. Three different cassettes were ligated with a DNA ligation kit (Takara, Kyoto, Japan). Ligated products were amplified with nested PCR. The amplified fragments were subcloned to TA vectors (Invitrogen Corp., Carlsbad, CA), and the DNA sequences were determined with the 3100 capillary sequencer (ABI, Foster City, CA). Genomic DNA from homozygous NOD-TGF- β 1 was specifically amplified with a primer set of A1 + B2 (A1: 5'-ATGATATTGAATAGAGATAGAG-3', and B2: 5'-GGTTTAGAGTTTGGCAACAT-3'), and genomic DNA from hemizygous NOD-TGF- β 1 was amplified with both primer sets of A1 + A2 (A2: 5'-GCTCTTTGACAACCTCACAC-3') and A1 + B2.

2.5. Detection of transgene and p15^{INK4b} expression in islets

Mouse islets were isolated using collagenase (Moritani et al., 1996), and the total RNA was isolated with RNeasy 96 (QIAGEN GmbH, Hilden, Germany) and

prepared with DNase treatment. The expression levels of the transgene and p15^{INK4b} were determined with quantitative real time RT-PCR using ABI 7900 sequence detection system (ABI, Foster City, CA). The sense primer was 5'-GGATCCTGAGAACTTCAGGCTC-3', the antisense primer was 5'-TTCTTTGCCAAAATGATGAGACA-3', and the minor groove binder (MGB) TaqMan probe was 5'-FAM-TGGGCAACGTGCTGGTTGTTGTG-3'-TAMURA for the transgene. For p15^{INK4b}, the sense primer was 5'-TGGATGTGTGTGACGCCTG-3', the antisense primer was 5'-AGATACCTCGCAATGTACGG-3', and the MGB probe was 5'-FAM-TGCCGGTAG-ACTTGGCTGAAGAGCAG-3'-TAMURA. The reaction mixture (TaqMan One-Step reverse transcriptase-PCR Master Mix Reagent Kit; ABI) contained the final concentration of 1 × PCR master buffer, 0.25 U/μl of Moloney murine leukemia virus (MoMuLV) reverse transcriptase, 0.4 U/μl of RNase inhibitor mix, 900 nM of each primer, and 250 nM of probe. The RT-PCR conditions were based on the manufacturer's protocols. All measurements were normalized to the transgene/GAPDH or p15^{INK4b}/GAPDH ratio, and performed in three independent experiments using five mice in each group.

2.6. Incidence of diabetes

Changes in blood glucose (BG) concentrations in time course were monitored in homozygous NOD-TGF-β1 and compared with those in hemizygous NOD-TGF-β1 and their wild-type mice. Mice were considered diabetic when non-fasting BG concentrations were greater than 14 mM.

2.7. Intraperitoneal glucose tolerance test (ipGTT)

After fasting for 16 h, 2 mg/g body weight of glucose in physiological saline were intraperitoneally injected at 8 weeks of age. BG concentrations were monitored at 0, 30, 60, and 120 min after glucose injection.

2.8. Insulin assay with ELISA

After fasting for 16 h, blood samples were collected. Serum insulin concentrations were assayed by an ELISA kit for insulin with a mouse insulin standard (Seikagaku Kogyo, Tokyo, Japan). Plasma insulin concentrations were assayed at 0 and 30 min after glucose injection.

2.9. Histological analysis

At 8 weeks of age, morphometric analysis using pancreatic tissue obtained from homozygous ($n = 11$ including 6 females and 5 males), and age-matched hemizygous NOD-TGF-β1 ($n = 7$), and their wild-type mice ($n = 7$) were performed. Histology of homozygous B6-TGF-β1 was examined at 8 weeks of age. Pancreata of mice were fixed with 4% formalin in phosphate-buffered saline, embedded in paraffin, sectioned,

and stained with haematoxylin and eosin (HE). Insulin and glucagon were stained, respectively, with a guinea-pig polyclonal antibody for porcine insulin and with a rat polyclonal antibody for human glucagon (Dako, Glostrup, Denmark).

2.10. Statistical analysis

Results were expressed as means ± S.D. *P*-value denotes the significance level in Student's unpaired *t*-test; ** $P < 0.01$, * $P < 0.05$.

3. Results

3.1. Detection of the copy numbers and transgene mRNA expression

The intensities of radioactive pTGF-β1 bands in Southern blot analysis of homozygous and hemizygous NOD-TGF-β1 were 8.3- and 3.5-fold higher, respectively, than those of endogenous mTGF-β1 bands. The intensity of pTGF-β1 assessed by the band-density in homozygous NOD-TGF-β1 was 2.3-fold higher than that in hemizygous NOD-TGF-β1 (Fig. 1A). The level of transgene in homozygous NOD-TGF-β1 was 6.1-fold higher than hemizygous NOD-TGF-β1 ($P < 0.005$), using the GAPDH as an internal standard for normalization (Fig. 1B). The level of transgene expression correlated with their copy numbers both in homozygous and hemizygous NOD-TGF-β1. No transgene was detected in wild-type mice. The similar results were observed in three independent experiments.

3.2. Incidence of diabetes in the natural course

Eleven homozygous NOD-TGF-β1 in two lines including six and five from each line developed overt diabetes with the mean BG concentrations of 16.9 mM at 15 weeks of age (Fig. 2). The cumulative incidence of diabetes was 46.2% (6/13) at 7 weeks, and 84.6% (11/13) at 9 or 15 weeks of age. In contrast, none of the hemizygous NOD-TGF-β1 ($n = 12$, 9) became diabetic even at 15 weeks of age. In wild-type mice ($n = 4$, 4), 1 out of 8 spontaneously started to develop diabetes at 12 weeks of age, and cumulative incidence of diabetes was 12.5% (1/8) at 15 weeks of age (Fig. 2). Wild-type mice spontaneously started to develop diabetes at 12 and 26 weeks of age, respectively, in female and male mice and cumulative incidence of diabetes was 62% in female and 22% in male at 35 weeks of age (data not shown). This incidence of diabetes is comparable to that in our NOD mice colony of 63% in female and 25% in male.

3.3. ipGTT in the NOD genetic background

For non-diabetic homozygous NOD-TGF-β1, ipGTT were carried out. In homozygous NOD-TGF-β1, ipGTT showed significant hyperglycemia in comparison with their

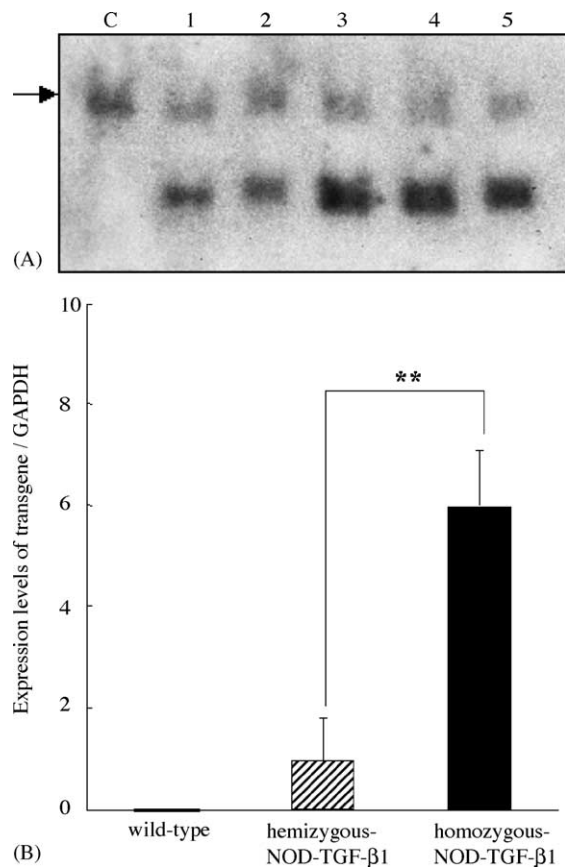


Fig. 1. Detection of the copy numbers by Southern blot analysis and transgene mRNA expression. (A) Southern blot analysis was performed with a [32 P]-labeled pTGF- β 1 probe (1.3 kbp). Lanes 1 and 2: hemizygous NOD-TGF- β 1, lanes 3–5: homozygous NOD-TGF- β 1, and lane C: wild-type mice. Arrow indicates the endogenous mouse TGF- β 1. (B) Total RNA (1 μ g) isolated from islets of homozygous or hemizygous NOD-TGF- β 1, and wild-type mice were reverse-transcribed using random hexamer primers. A black column denotes wild-type mice, a hatched column denotes hemizygous, and a solid column denotes homozygous mice.

wild-type mice. BG concentrations in male (Fig. 3A) and female (Fig. 3B) homozygous NOD-TGF- β 1 ($n = 5, 7$) were significantly higher than those in their male and female hemizygous NOD-TGF- β 1 ($n = 8, 8$) or wild-type mice ($n = 6, 7$) at 30 ($P < 0.0001$ for both), 60 ($P < 0.01$ for both), and 120 min ($P < 0.01$ and $P < 0.05$) after glucose injection, respectively. BG concentrations before glucose injection were in the normal range (2.5–4.0 mM), respectively, in homozygous, hemizygous NOD-TGF- β 1, and their wild-type mice.

3.4. ipGTT in the C57BL/6 genetic background

None of homozygous mice ($n = 3, 4$) became diabetic with the mean BG concentration in the upper normal range of 8.0 and 9.1 mM at 15 weeks of age, respectively, in male and female mice. None of homozygous B6-TGF- β 1 developed diabetes, and ipGTT were carried out. Homozygous B6-TGF-

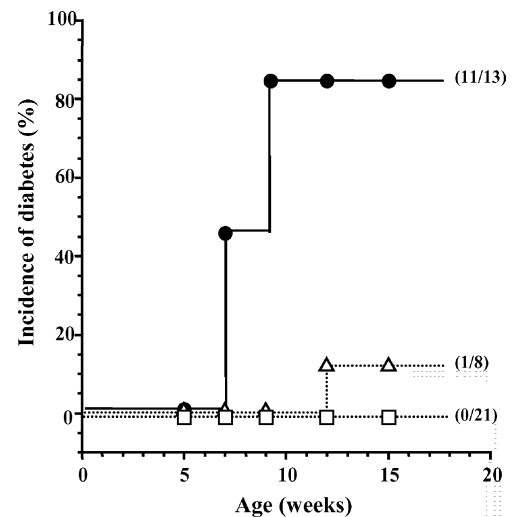


Fig. 2. Incidence of diabetes. Time courses of the incidence of diabetes are shown in homozygous NOD-TGF- β 1 (a solid line with filled circles), hemizygous NOD-TGF- β 1 (a broken line with open boxes), and wild-type mice (a broken line with open triangles). Numbers in parentheses denote the final incidence of diabetes at 15 weeks of age.

β 1 showed impaired glucose tolerance in comparison with normal glucose tolerance in hemizygous B6-TGF- β 1 ($n = 5, 7$) or their wild-type mice ($n = 5, 4$). BG concentrations in male (Fig. 4A) and female (Fig. 4B) were significantly higher

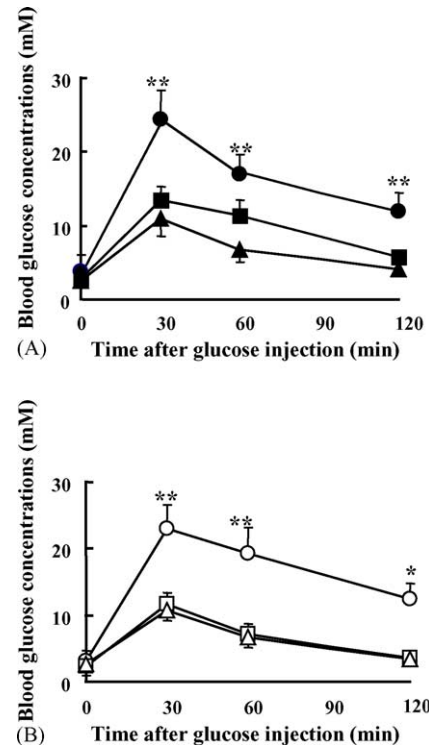


Fig. 3. ipGTT in non-diabetic NOD-TGF- β 1. BG concentrations in ipGTT after fasting for 16 h were shown before and at all time points after glucose injection in homozygous (circles), hemizygous NOD-TGF- β 1 (boxes), and wild-type mice (triangles) in non-diabetic NOD-TGF- β 1: (A) male and (B) female.

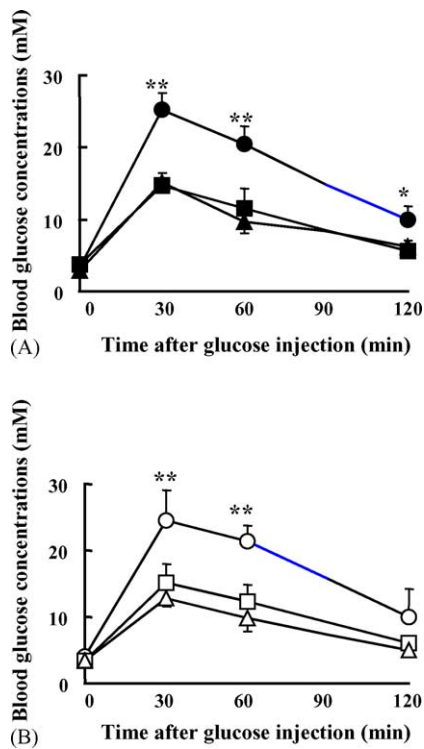


Fig. 4. ipGTT in B6-TGF- β 1. BG concentrations in ipGTT after fasting for 16 h were shown before and at all time points after glucose injection in homozygous (circles), hemizygous NOD-TGF- β 1 (boxes), and wild-type mice (triangles) in B6-TGF- β 1: (A) male and (B) female.

than those in hemizygous B6-TGF- β 1 or their wild-type mice at 30 ($P < 0.01$ for both), 60 ($P < 0.01$ for both), and 120 ($P < 0.05$ for in male) min after glucose injection, respectively. BG concentrations before glucose injection were in the normal range of 3.2–3.5 mM in homozygous, hemizygous, and their wild-type mice.

3.5. Serum insulin levels

The serum insulin concentrations (ng/ml) in response to glucose injection in male (Fig. 5A) or female (Fig. 5B) NOD-TGF- β 1 at 30 min were, respectively, 1.64 ± 0.06 or 1.42 ± 0.1 in wild-type mice ($n = 4, 4$), 1.17 ± 0.35 or 1.10 ± 0.41 in hemizygous NOD-TGF- β 1 ($n = 4, 4$), and 0.39 ± 0.12 or 0.16 ± 0.10 in homozygous NOD-TGF- β 1 ($n = 5, 5$). In homozygous NOD-TGF- β 1, the serum insulin concentrations decreased to 19% compared with their wild-type mice, which was significantly smaller than those in hemizygous NOD-TGF- β 1 ($P < 0.01$ for male, $P < 0.05$ for female) and their wild-type mice ($P < 0.005$ for both). The serum insulin concentrations in hemizygous NOD-TGF- β 1 and their wild-type mice were comparable in both sexes. In homozygous B6-TGF- β 1, the serum insulin concentrations (0.56 ± 0.19 or 0.45 ± 0.20) were lower than those in hemizygous B6-TGF- β 1 (1.36 ± 0.47 or 1.15 ± 0.41) ($P < 0.01$ for male and $P < 0.05$ for female), and their

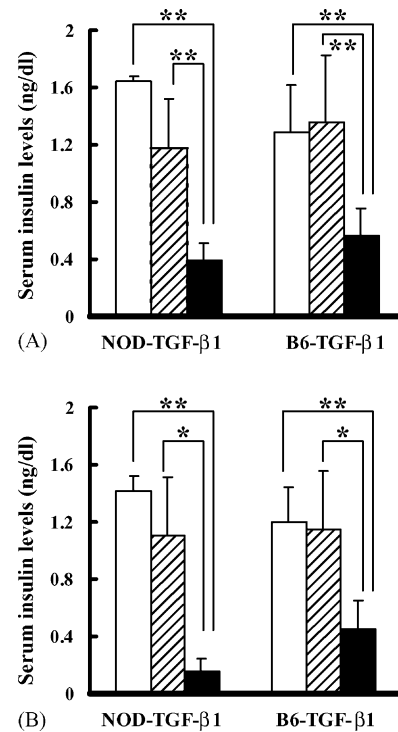


Fig. 5. Insulin levels in response to glucose in NOD-TGF- β 1 and B6-TGF- β 1. Insulin levels were determined in ipGTT. Open columns denote wild-type mice, hatched columns denote hemizygous, and solid columns denote homozygous in each generation: (A) male and (B) female.

wild-type mice (1.29 ± 0.33 or 1.20 ± 0.24) ($P < 0.01$ for both).

3.6. Area of whole pancreas or islet

The percentage islet area relative to the whole pancreas area (the mean percentage islet area relative to those in wild-type mice) were $0.19 \pm 0.10\%$ (18%) in homozygous ($P < 0.001$; compared to wild-type mice) or $0.42 \pm 0.37\%$ (40%) in hemizygous NOD-TGF- β 1 ($P < 0.001$; compared to wild-type mice). While, the percentage islet area relative to the whole pancreas area in wild-type mice was $1.06 \pm 0.32\%$ (100%) (Fig. 7A). In homozygous NOD-TGF- β 1, the whole pancreas area was decreased in the mean percentage to 34 and 37% compared with those in their wild-type mice and hemizygous NOD-TGF- β 1 ($P < 0.0001$ for both) (Figs. 6 and 7B). While, the percentage of whole pancreas area in hemizygous NOD-TGF- β 1 was 93% of those in their wild-type mice without significant difference. The absolute percentage hypoplasia of endocrine pancreas was as small as 6% (18×0.34) in homozygous and 37% (40×0.93) in hemizygous NOD-TGF- β 1 compared with those in wild-type mice (Fig. 6). The observation that most islets in homozygous NOD-TGF- β 1 exhibited almost no lymphocytic infiltration, strongly supports that the diabetes in homozygous NOD-TGF- β 1 is non-autoimmune. Immunohistochemistry of pancreas for insulin and glucagon confirmed islet hypoplasia in homozygous and

hemizygous NOD-TGF-β1, and homozygous B6-TGF-β1 (Fig. 8).

3.7. Expression levels of p15^{INK4b}

Expression levels of p15^{INK4b} in pancreatic islets of homozygous NOD-TGF-β1 were 3.4-fold higher than that of hemizygous NOD-TGF-β1 ($P < 0.01$) or their wild-type mice ($P < 0.01$) (Fig. 9). The similar results were obtained in three independent experiments.

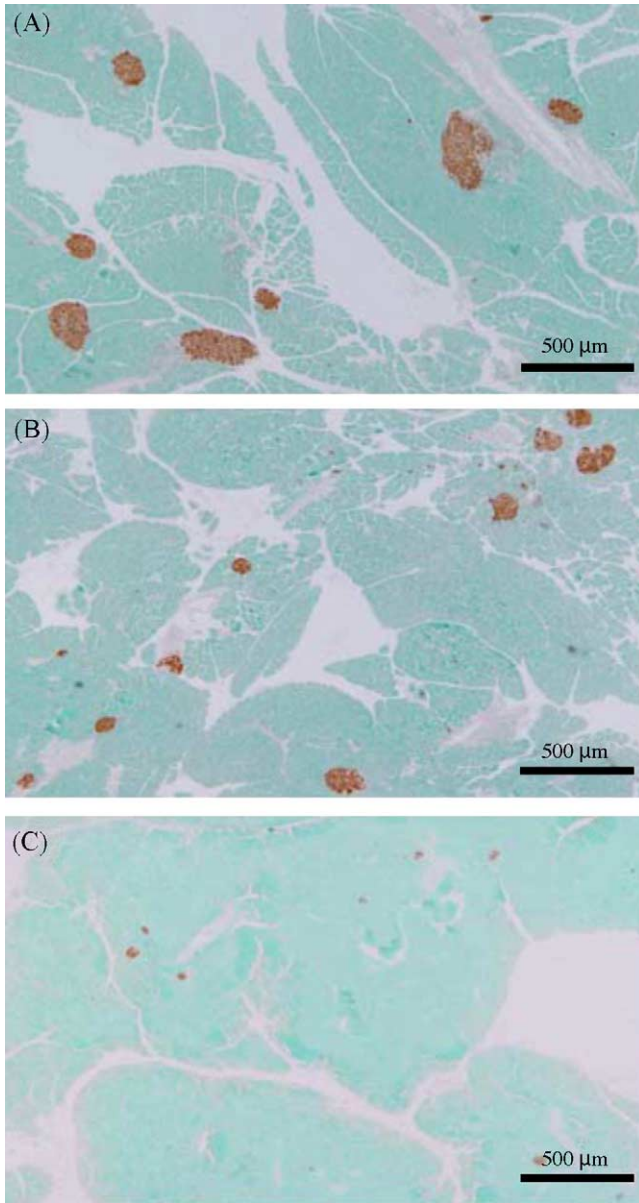


Fig. 6. TGF-β1-dependent decrease of whole pancreas area at 8 weeks of age. Representative anti-insulin immunohistochemistry of the whole pancreas at 8 weeks of age is shown. (A) Section of the pancreas from a wild-type mouse, (B) section of the pancreas from a hemizygous NOD-TGF-β1 and (C) section of the pancreas from a homozygous NOD-TGF-β1. (A)–(C) are at the same magnification.

4. Discussion

TGF-β1 is expressed in the pancreatic epithelium just after budding from the foregut endoderm and in the pancreas throughout embryonic development (Sanvito et al., 1994; Christopher et al., 2000; Millan et al., 1991). The expression and activity of TGF-β1 increase at the end of gestation (Christopher et al., 2000; Crisera et al., 1999). Various isoforms of TGF-β are expressed in the pancreatic epithelial cells, which will differentiate into both exocrine and endocrine pancreas in adult mice. TGF-β1 is strongly and specifically expressed in the human exocrine pancreas compared to other isoforms (Yamanaka et al., 1993), and highly regulated TGF-β signaling pathway exists in human pancreatic islets (Shalev et al., 2002). These expression patterns of TGF-β1 in the embryonic mouse and human pancreas suggest that TGF-β1 may regulate organogenesis of the developing pancreas. Hemizygous TGF-β1 mice by the insulin promoter did or did not decrease the organ size of exocrine or endocrine pancreas (Grewal et al., 2002; Lee et al., 1995; Sanvito et al., 1995). In this study, we generated homozygous NOD-TGF-β1 to examine the role of excessive TGF-β1 by

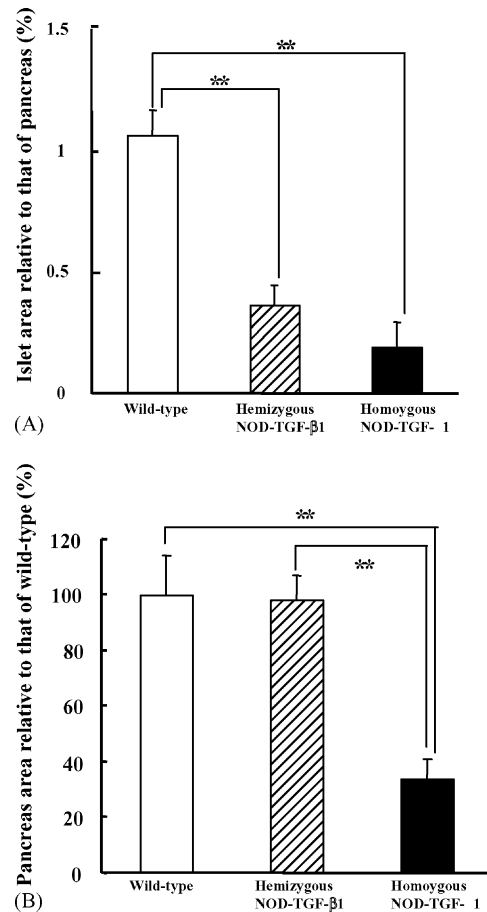


Fig. 7. Morphometric analysis of pancreas at 8 weeks of age. The percentage islet area relative to the whole pancreas area (A) and the mean percentage whole pancreas area (B) in NOD-TGF-β1 are shown.

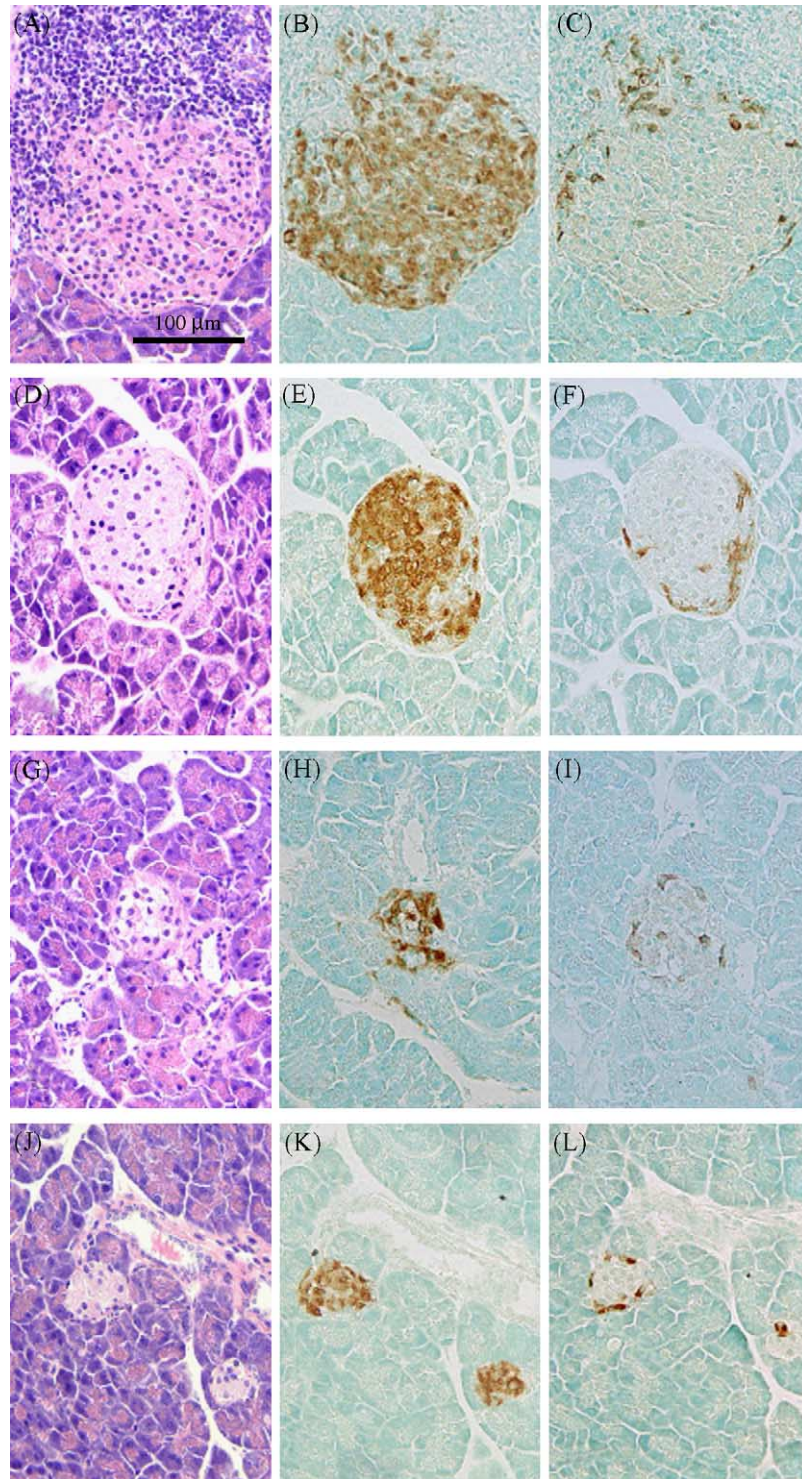


Fig. 8. Histology of pancreas in NOD-TGF- β 1: (A)–(C) sections of the pancreas from wild-type mouse at 8 weeks of age, (D)–(F) sections of the pancreas from hemizygous NOD-TGF- β 1 at 8 weeks of age, (G)–(I) sections of the pancreas from homozygous NOD-TGF- β 1 at 8 weeks of age and (J)–(L) sections of the pancreas from homozygous B6-TGF- β 1 at 8 weeks of age. (A), (D), (G), and (J) HE staining, (B), (E), (H), and (K) insulin staining and (C), (F), (I), and (L) glucagon staining. (A)–(C), (D)–(F), (G)–(I), and (J)–(L) are at the same magnification.



Fig. 9. Expression levels of p15^{INK4b} in pancreatic islets. Total RNA (1 μg) isolated from islets of homozygous or hemizygous NOD-TGF-β1, and wild-type mice were reverse-transcribed using random hexamer primers. The open column denotes wild-type mice, the hatched column denotes hemizygous, and the solid column denotes homozygous NOD-TGF-β1.

the glucagon promoter on the development or the final size of endocrine and exocrine pancreas.

The expression level of the transgene in homozygous NOD-TGF-β1 was 6.1-fold higher than that in hemizygous NOD-TGF-β1 (Fig. 1B), although the copy number of homozygous NOD-TGF-β1 was exactly twice of that in hemizygous NOD-TGF-β1 (Fig. 1A). In contrast to normoglycemia in hemizygous NOD-TGF-β1, all homozygous NOD-TGF-β1 showed overt diabetes (84.6% at 15 weeks of age) and they became diabetic even before 7 weeks of age (46%). In female wild-type mice, the onset and the incidence of overt diabetes increases as age advances, up to 65% at the age of 30 weeks. The incidence and age of onset in homozygous NOD-TGF-β1 are clearly different from these in wild-type mice. The comparable ratio of diabetes in two lines of NOD-TGF-β1 suggests that not insertional mutagenesis, but the transgene expression is the reason for diabetes. Non-diabetic homozygous NOD-TGF-β1 (both sexes) also showed impaired glucose tolerance (Fig. 3) and had small glucose-induced insulin secretion (Fig. 5) compared to their wild-type mice or hemizygous NOD-TGF-β1. These results suggest that excess of paracrine TGF-β1 signaling induces the severe morphometric change of islets.

Morphometric analysis showed the decrease to 34% in the area of whole pancreas in homozygous NOD-TGF-β1 compared to their wild-type mice (Figs. 6 and 7B). The absolute islet area in all homozygous NOD-TGF-β1 was as low as 6% of that in their wild-type mice (Figs. 6 and 7A). Many hypoplastic islets in homozygous NOD-TGF-β1 paralleled the decrease in the number of both islet β or α cells. These findings support that paracrine overexpression of TGF-β1 in its active form equally inhibits the normal fetal development of pancreatic precursor cells to both islet β and α cells leading to islet hypoplasia. The absolute islet area decreased to 37% without obvious decrease in the area of exocrine pancreas

to 93% in our hemizygous NOD-TGF-β1 by the glucagon promoter. Hypoplastic islets of 5% were observed in another transgenic mice by the insulin promoter with the small area of exocrine pancreas to 40–51% (Grewal et al., 2002). Based on these, we hypothesized that the magnitude, timing, and duration of local TGF-β1 might regulate not only the development, but also the final size of endocrine and exocrine pancreas.

In the *in vitro* study, TGF-β1 promotes the differentiation toward pancreatic endocrine cells, and inhibits the development of exocrine pancreas in the embryonic mouse pancreas (Sanvito et al., 1994), or the growth of adult mouse pancreatic acinar cells (Logsdon et al., 1992). The apparently paradoxical hypoplastic action of TGF-β1 signaling *in vivo* on pancreatic islets was, however, observed after the expression of a constitutively active form of type I activin receptor (Yamaoka et al., 1998). This is consistent with islet hypoplasia and low insulin content with impaired glucose tolerance. These results suggest that TGF-β1 *in vivo* plays an important role to decrease the final size of both endocrine and exocrine pancreas, with the former more severe than the latter.

Homozygous B6-TGF-β1 showed impaired glucose tolerance (Fig. 4) and small glucose-induced insulin secretion (Fig. 5) in contrast to normal glucose tolerance in their wild-type mice. We observed small sized islets in homozygous B6-TGF-β1. The magnitude of hypoplasia is comparable with that in homozygous NOD-TGF-β1. The NOD genetic background was shown not essential for impaired glucose tolerance, but the homozygous high-level expression of TGF-β1 was shown crucial for TGF-β1-induced diabetes or impaired glucose tolerance. Reduced incidence of diabetes in C57BL/6 genetic background is considered to be due to the presence of genetic modifier alleles in C57BL/6 to protect mice from diabetes and the disappearance of recessive diabetic genes in wild-type mice. Because genetic background is the important determinant of diabetic phenotypes under polygenic regulation, QTL analysis is under way in our laboratory to identify the responsible genetic differences between these strains.

The possible mechanisms of TGF-β1-induced growth inhibition *in vivo* include down-regulation of cyclin and Cdk expression, especially the decreased amount of Cdk4 either through the suppressed protein synthesis (Ewen and Sluss, 1993) or suppressed transcription (Geng and Weinberg, 1993; Slingerland et al., 1994), and increased inhibition of Cdk2 or Cdk4 by p15^{INK4b} (Hannon and Beach, 1994; Sandhu et al., 1997) or p21^{kip/sip} (Polyak et al., 1994; Reynisdottir et al., 1995; Reynisdottir and Massague, 1997). p15^{INK4b} specifically inhibits the Cdk4 and Cdk6 by inactivating the catalytic activity through binding to a Cdk subunit (Massague et al., 2000). Because the activation of Cdk4 *in vivo* by the constitutively active form of Cdk4 in knock-in mice (Rane et al., 1999) or in transgenic mice expressing a constitutively active form of Cdk4 in islet β cells (Hino et al., *in press*) lead to islet hyperplasia, cell cycle regulation is supposed to be a strong determinant of the final islet organ size. Homozygous NOD-TGF-β1 in our study demonstrated for the first time that

overexpressed paracrine TGF- β 1 increases the expression of p15^{INK4b} (3.4-fold) in pancreatic islets (Fig. 9) in association with the endocrine and exocrine hypoplasia (Fig. 7). We regard the absence of difference in p15^{INK4b} mRNA in islets of “hemizygous NOD-TGF- β 1” and “wild-type mice” to be possibly due to the assay limit of RT-PCR that is not sensitive enough to detect a small change in p15^{INK4b} mRNA. It is another possibility that p15^{INK4b} causes endocrine pancreatic hypoplasia at the level of precursor cells during the fetal life without obviously changing the p15^{INK4b} mRNA at adult stage. Based on our results, it is interpreted that TGF- β 1 induced growth arrest of endocrine islet cells via increased p15^{INK4b} expression in the G₁ phase. The hypoplastic role of p15^{INK4b} in islets β cells, however, has to be confirmed in the separate experiment by its transgenic expression in islet β cells.

Few apoptotic β cells were observed in homozygous NOD-TGF- β 1 (data not shown). TGF- β 1 induces apoptosis in various epithelial cell types (Massague et al., 2000; Rotello et al., 1991), but apoptosis of β cells may be playing the minimum role for the decrease in the number of hypoplastic islets and it may simply represent the result of glucose toxicity in overt diabetes in homozygous NOD-TGF- β 1.

Based on these, we conclude first that excessive paracrine TGF- β 1 signaling in islet compartment in vivo leads to endocrine and exocrine pancreatic hypoplasia presumably through its action at the level of precursor cells for both endocrine and exocrine pancreas, second that TGF- β 1 decreases the final size of endocrine and exocrine pancreas, presumably through regulating cell cycle via p15^{INK4b} at least in endocrine pancreas, and third that hypoplastic action of TGF- β 1 on pancreatic islets is independent of the genetic background.

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