厚生労働科学研究費補助金

ヒトゲノム・再生医療等研究事業

膵上皮幹細胞による糖尿病細胞治療に関する研究

平成１８年度　総括研究報告書

主任研究者　宮脇　一真

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研究要旨
糖尿病は、わが国において増加の一途を辿っている生活習慣病であり、再生医療への期待も大きい。主任研究者は、マウス初期膵原基に存在する*in vivo*の膵前駆細胞と同じ遺伝子プロファイルを有する細胞を*in vitro*で単離培養することに成功した。この細胞は、未分化状態を維持したまま長期間増殖する細胞であった。本研究の遂行により、この未分化細胞から膵β細胞への分化誘導法が確立されれば、糖尿病細胞治療の実現へ向け大きく前進することが期待される。
本年度はこの未分化細胞から膵内分泌細胞への分化誘導法確立を目指して研究を行った。

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A．研究目的

2002年に実施された糖尿病実態調査によると、我が国における糖尿病患者数は約740万人、その予備軍を合わせると約1620万人にも達するとされている。さらに、糖尿病に伴う重篤な合併症（網膜症、腎症、神経障害など）を発症する患者も増加の一途を辿っており、糖尿病関連医療費の増大は医学的、社会的に深刻な問題となっている。しかし従来のインスリン治療や食事・運動療法では、1型糖尿病患者や重度の２型糖尿病患者の血糖値を生理的範囲内にコントロールし、合併症を防止することは困難であることから、糖尿病移植医療の確立に期待が寄せられている。2000年にカナダのアルバータ大学において実施されたエドモントンプロトコールによって膵島移植が好成績を修めて以来、膵島移植が糖尿病に対する根本的な治療法として注目されるようになった。しかしながら深刻なドナー不足が解消される見通しが立たないことから、多くの糖尿病患者に幅広く適用することは現実的に困難である。

本研究の目的は、このようなドナー不足の問題を解決するために、*in vitro*で長期間自己複製可能な体性幹細胞を同定し、その細胞から膵β細胞へと分化誘導する方法を開発することである。この方法が確立されれば、*in vitro*で作製したインスリン分泌細胞を移植ソースとして供給することが可能となり、より多くの糖尿病患者が移植医療の恩恵を享受できる。最近、主任研究者らはマウス新生児の膵組織から、無血清培養条件下で1年近くにわたり増殖力を保持する細胞を単離した。この細胞は非常に小型で均一な形態を示し、コロニーを形成する。この細胞の転写因子発現プロファイ

ルを数十個のコロニーに関して検証した結果、膵発生初期に存在する膵前駆細胞のプロファイルと一致していた。この様な特徴から、この細胞は未分化な膵上皮細胞であると考えられ、膵β細胞への分化能を有する前駆細胞の可能性が高いと考えられた。主任研究者らは、この細胞を膵上皮幹（Pancreatic epithelial stem: PES）細胞と名付けた。本研究では、３年の期間終了までに、マウスおよびヒトPES細胞から膵β細胞への分化誘導法を開発することを目指している。

B．研究方法

マウスを用いた段階では、その動物モデルとしての有用性を最大限に活用し、分子生物学的手法と細胞生物学的手法を組み合わせ、より厳密かつクリアーにPES細胞そのものの評価と分化能の評価を行う。主任研究者らは、マウスインスリンプロモーターGFP（MIP-GFP）マウスの新生児膵から Fluorescence Activated Cell Sorter（FACS）により成熟膵β細胞のコンタミネーションを完全に除去した試料を調整し、そこからPES細胞を樹立した。このPES細胞の膵発生関連転写因子の発現を膵β細胞と比較したところ、Neurogenin3、NeuroD、Pax4、Hlxb9、Isl1等の発現が認められなかった。

１）そこで主任研究者らは、まずPES細胞から膵β細胞への分化能を調べる第一歩として、これらの転写因子をアデノウイルスベクターによりPES細胞に発現させ、インスリン分泌関連遺伝子の発現に対する効果を調べた。加えて、単独のみならず複数の転写因子を組み合わせて導入することにより、PES細胞から膵β細胞への分化能を評価した。

２）生理的な条件によりPES細胞を分化誘導する方法を確立するために、サイトカインやNotchシグナル阻害剤である γ-secretase inhibitor等さまざまな外的因子を組み合わせることにより、遺伝子導入を必要としないPES細胞の分化誘導法を探索した。

３）分化誘導したPES細胞の機能評価として、グルコース応答性インスリン分泌試験、電気生理学的解析（Patch Clamp）など生理学的機能評価を行った。

C．研究結果

１）転写因子導入による膵β細胞への分化能を評価

PES細胞に、膵発生学上重要な転写因子11種類をアデノウイルスベクターに乗せ、PES細胞に順次導入し、膵内分泌細胞関連遺伝子90種類の発現変化を検討した。NeuroDの導入により、グルコース応答性インスリン分泌を担うATP感受性カリウムチャネル(Kir6.2、SUR1)、電位依存性カルシウムチャネル(Cav1.2、Cav1.3)、インスリンプロセッシング酵素(PC1/3、PC2)、開口放出関連因子（Rab3b、Rab3c、ChromograninA/B）の発現誘導を認めた。また、NeuroDとMafAを同時に導入することによって、インスリン遺伝子の発現誘導が認められた。このインスリン遺伝子の発現誘導は、Isl1によって約8倍に増強された。このように転写因子の導入により、PES細胞の遺伝子発現プロファイルを成熟β細胞に近いところまで誘導することが可能であった。

２）小分子・サイトカインを用いた膵β細胞への分化誘導

BMPs-Smad、MAPK、PI3K、Notchシグナルに関与する10種類のサイトカインおよび小分子をそれぞれPES細胞の培養液中に添加し、インスリン遺伝子発現に与える影響を検討したところ、Notchシグナルの阻害剤であるγ-secretase inhibitorによるインスリン遺伝子の発現誘導を認めた。Notchシグナルは膵内分泌前駆細胞の未分化維持に必須であることが*in vivo*での検討により明らかにされており、こうした反応は、PES細胞が発生学上の生理的な機序により膵β細胞に分化しうる可能性を示唆した。

3）分化誘導したPES細胞の機能評価

NeuroD、MafA、Isl1を同時に導入したPES細胞の電気生理学的興奮性をPatch Clamp法により検討したところ、電位依存性カルシウムチャネルのカレントを確認した。一方、バッチインキュベーション法により同細胞のグルコース応答性インスリン分泌を検討したが、こちらは検出限界以下であった。これらは、PES細胞が膵β細胞に特徴的な性質を部分的に獲得したものの、その分化度がまだ十分でないことを示唆していた。

（倫理面への配慮）

動物実験は、動物愛護管理法および京都大学・動物実験に関する指針に基づき、動物実験計画書を作製し承認を受けている。ヒト組織の使用に関しては、心停止ドナー由来の膵組織は使用せず、膵臓ガン摘出手術時の組織を使用する予定である。本計画は、京都大学・医の倫理委員会から承認を受けている。いずれも両委員会において、厳正かつ慎重な審査が行われており、倫理面には問題がないものと考えられる。

D．考察

本年度は、転写因子導入もしくは 小分子を用いてPES細胞を膵β細胞に分化誘導する試みを中心に行った。この結果、インスリンを全く発現していないPES細胞からインスリン遺伝子を誘導することに成功した。インスリン以外にも、グルコース感知機構関連分子や調節性開口分泌関連分子などの誘導も認められ、成熟膵β細胞に近いプロファイルをもつ細胞に誘導することが出来た。これは、PES細胞が膵β細胞へ分化するポテンシャルを有する証拠であると思われる。しかし一方で、機能的には成熟膵β細胞には劣っており、同時に分化度の低さも明らかとなった。

E．結論

PES細胞が、膵β細胞へ分化する可能性を秘めていることは示唆されたが、転写因子のみの誘導法では限界があると考えられた。より分化度の高い膵β細胞を作製するためには、さらに多くのサイトカインの組み合わせや分化抑制因子の解除による分化誘導法の検討が必要であると考察された。

F．健康危険情報

特になし。

G．研究発表

1. 論文発表

Generation of insulin- secreting cells from pancreatic acinar cells of animal models of type 1 diabetes.
American Journal of Physiology Endocrinology and Metabolism
292(1):E158-65

2. 学会発表

なし。

H．知的財産権の出願・登録状況

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okuno M, Minami K, Okumachi A, Miyawaki K, Yokoi N, Toyokuni S, Seino S.	Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes.	American Journal of Physiology Endocrinology and Metabolism	292	E158-65	2006

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Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes

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Okuno M, Minami K, Okumachi A, Miyawaki K, Yokoi N, Toyokuni S, Seino S. Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes. *Am J Physiol Endocrinol Metab* 292: E158–E165, 2007. First published August 22, 2006; doi:10.1152/ajpendo.00180.2006.—We recently found that pancreatic acinar cells isolated from normal adult mouse can transdifferentiate into insulin-secreting cells in vitro. Using two different animal models of type 1 diabetes, we show here that insulin-secreting cells can also be generated from pancreatic acinar cells of rodents in the diabetic state with absolute insulin deficiency. When pancreatic acinar cells of streptozotocin-treated mice were cultured in suspension in the presence of epidermal growth factor and nicotinamide under low-serum condition, expressions of insulin genes gradually increased. In addition, expressions of other pancreatic hormones, including glucagon, somatostatin, and pancreatic polypeptide, were also induced. Analysis by the Cre/loxP-based direct cell lineage tracing system revealed that these newly made cells originated from amylase-expressing pancreatic acinar cells. Insulin secretion from the newly made cells was significantly stimulated by high glucose and other secretagogues. In addition, insulin-secreting cells were generated from pancreatic acinar cells of Komeda diabetes-prone rats, another animal model of type 1 diabetes. The present study demonstrates that insulin-secreting cells can be generated by transdifferentiation from pancreatic acinar cells of rodents in the diabetic state and further suggests that pancreatic acinar cells represent a potential source of autologous transplantable insulin-secreting cells for treatment of type 1 diabetes.

transdifferentiation; lineage tracing; streptozotocin; Komeda diabetes-prone rat

THERE HAVE BEEN MANY ATTEMPTS to generate and/or expand insulin-secreting cells in vitro. Recent studies have shown that insulin-secreting cells can be generated from non-β-cells, including mouse and human pancreatic duct cells (6, 8, 20), rat hepatic oval cells (33), mouse bone marrow cells (30), putative pancreatic stem/progenitor cells (21, 28, 36), and embryonic stem cells (2, 16, 24). In addition, several studies have suggested that pancreatic acinar cells can transdifferentiate into insulin-secreting cells in vitro (3, 23). We recently found that insulin-secreting cells can be generated from exocrine pancreas of adult mice and provided direct evidence that the cells originated from pancreatic acinar cells using a Cre/loxP-based cell lineage tracing system (17). However, all of these studies used nondiabetic animals.

Hyperglycemia and the other metabolic disorders that characterize diabetes damage various organs and tissues (19). Some patients with type 1 diabetes exhibit considerable reduction in weight and volume of pancreas due to severe atrophy of acinar cells (14). Impairment of exocrine pancreatic function is also found in type 1 diabetes (7, 11, 32). In addition, diabetic conditions might affect the regeneration processes of progenitor cells. Neovascularization capacity of bone marrow-derived mononuclear cells (BM-MNCs) is reduced in streptozotocin (STZ)-induced diabetic mice (29). Such dysfunction is thought to be associated with impaired differentiation of BM-MNCs into endothelial progenitor cells in the diabetic state (29). Dysfunction of endothelial progenitor cells also is found in patients with diabetes (15, 31). Moreover, to our knowledge, there has been no investigation of in vitro generation of insulin-secreting cells from cells of animals in the diabetic state.

In the present study, we use two animal models of type 1 diabetes that exhibit absolute loss of pancreatic β-cells: STZ-injected mice and Komeda diabetes-prone (KDP) rats (9, 34). We find that insulin-secreting cells can be generated from pancreatic acinar cells of these diabetic animals. In addition, pancreatic hormone-producing cells other than β-cells also can be generated from acinar cells. Thus, the present findings represent a step toward cell therapy for type 1 diabetes by autologous transplantation of pancreatic acinar-derived insulin-secreting cells.

MATERIALS AND METHODS

Diabetic animals. Diabetes was induced by intraperitoneal injection of 200 mg/kg STZ to 8- to 12-wk-old male C57Bl/6Cr Slc mice or ROSA26 reporter mice, in which enhanced cyan fluorescent protein (eCFP) transgene is inserted into the ROSA26 locus with a floxed transcriptional stop sequence (R26R-eCFP) (26). Two days after injection of STZ, mice with blood glucose concentration above 19.4 mmol/l were used for isolation of exocrine pancreas. In some experiments, STZ-injected mice were maintained for 3 wk with daily administration of 2–4 U of NPH insulin (Novo Nordisk Pharma, Copenhagen, Denmark). We also used KDP rats, a diabetes-prone substrain of the Long-Evans Tokushima lean (LETL) rat (9, 34), with blood glucose concentration above 19.4 mmol/l ~2 wk after the onset of hyperglycemia. All animal experiments were approved by the animal research committees of the Kyoto University Graduate School of Medicine and the Kobe University Graduate School of Medicine.

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Preparation and culture of pancreatic exocrine cells. Collagenase-digested pancreatic cells were subjected to Ficoll density gradient centrifugation, and the acinar cell-enriched fraction was recovered as a pellet. The absence of mature pancreatic islets in this fraction was confirmed by dithizone staining (22). The acinar cells-enriched fraction was cultured as reported (17). Briefly, the cells were plated onto sticky culture dishes in RPMI 1640 medium containing 10% fetal calf serum (FCS) for 6–8 h. The floating cells were then replated onto 2-methacryloyloxyethyl phosphorylcholine-treated Low-Cell-Binding dishes (Nalge Nunc International, Rochester, NY) in RPMI 1640 medium supplemented with 0.5% FCS, 20 ng/ml epidermal growth factor (EGF), and 10 mmol/l nicotinamide.

Immunocytochemistry. Cryostat sections were prepared from acinar-derived cell pellets fixed in 4% paraformaldehyde. The sections were blocked and permeabilized in phosphate-buffered saline containing 10% normal goat serum and 0.2% Tween 20. The primary and secondary antibodies used were the same as previously reported (17). Images were collected on a fluorescent microscope (Olympus, Tokyo, Japan) with a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

Reverse transcriptase-polymerase chain reaction analysis. Total RNAs were isolated from acinar-derived cells, islets, MIN6-m9 cells (18), or INS-1 cells (1), using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). After treatment with DNaseI (Qiagen), cDNA was prepared from 1 μg of total RNA by ReverTra Ace (Toyobo, Osaka, Japan), and subjected to PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The sequences of the primers, sizes of PCR products, and cycles for each pair are listed in either our previous study (17) or Table 1.

Quantitative real-time PCR was performed using either SYBR Green PCR master mix or TaqMan universal PCR master mix with an ABI-Prism 7300 sequence detector (Applied Biosystems). Measurement of the expression level of 18S ribosomal RNA (rRNA) was used as an internal control.

Cell lineage tracing. In R26R-eCFP reporter mice, an eCFP transgene is inserted into the ROSA26 locus and permanently expressed when a floxed transcriptional stop sequence is excised by Cre-mediated recombination (26). To trace amylase-expressing mature pancreatic acinar cells, pancreatic exocrine cells isolated from STZ-treated R26R-eCFP reporter mice were infected with adenovirus expressing Cre-recombinase under control of amylase-2 promoter (17).

Measurement of insulin secretion. Insulin secretion was measured as reported (17). Briefly, pancreatic acinar-derived cells were harvested and resuspended with Krebs-HEPES buffer containing 0.1% bovine serum albumin. The cell suspension was incubated for 60 min with various secretagogues as indicated, and insulin concentration was measured by ELISA (Shibayagi, Gunma, Japan). The amounts of insulin secretion were normalized by the cellular insulin contents determined by acid-ethanol extraction. Total protein was extracted in 1 N NaOH, and the concentration was determined using Coomasie Brilliant Blue-G250 reagent (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. Data are expressed as means ± SE. The significance of differences between test groups was evaluated by *t*-test or by one-way analysis of variance followed by Scheffé's test.

RESULTS

Induction of genes involved in pancreatic development and β-cell function. An insulin-deficient diabetic state was induced in mice by injection of STZ (200 mg/kg ip). Two days after the injection, the blood glucose levels of the mice were markedly elevated due to disruption of almost all of the pancreatic β-cells. Pancreatic acinar cells were isolated from mice with elevated blood glucose concentration (>19.4 mmol/l), and the absence of native pancreatic islets was confirmed by dithizone staining. We further characterized these isolated acinar cells by quantitative real-time RT-PCR (Fig. 1). Expressions of β-cell specific genes, including insulin-1 and -2, were almost absent, indicating that contamination of pancreatic β-cells in the starting material was negligible. We found reduced expression of amylase in the pancreatic acinar cells of STZ-injected mice compared with that in normal mice, suggesting that the diabetic state affected the function of exocrine pancreas (7, 11, 32). The cells were then cultured with 20 ng/ml EGF and 10 mmol/l nicotinamide in suspension. Morphologically, pancreatic acinar cells from STZ-injected mice formed spherical structures as seen in normal mice (17).

We next investigated expressions of other pancreatic genes during culture. Transcription factors necessary for the development of pancreas, such as Pdx1, NeuroD1, Foxa2, and HNF6, were induced in pancreatic acinar cells of STZ-injected mice (Fig. 2*A*). PGP9.5, a potential marker for endocrine progenitors (35), was induced. Genes of molecules that participate in glucose-induced insulin secretion in pancreatic β-cells, including glucokinase, SUR, Kir6.2, and Cav1.2, also were induced or increased by culture (Fig. 2*B*). Quantitative real-time RT-PCR analysis further confirmed induction of the genes associated with β-cell development and function (Fig. 2*C*). These results indicate acinar-to-endocrine transdifferentiation

Table 1. *List of gene-specific primers for RT-PCR analysis in rats*

Target	Forward	Reverse	Size, bp	Cycle
18S RNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151	20
Insulin-1	AACCCTAAGTGACCAGCTACAATCATAG	GCAGGCTTGGGCTCCC	241	29
Insulin-2	TAAGTGACCAGCTACAGTCGG	GCTCCCCACACACCAGGTAG	307	29
CK20	AATTGGCAATGCAGAACCTC	TCGGGCGTTTTCTATTTGAG	209	25
Pdx1	CATCTCCCCATACGAAGTGC	TTATTCTCCTCCGGTTCTGC	249	32
GLUT2	ATCCACATTCGGAACAGGAC	TCCAGAGGAACACCCAAAAC	208	30
GK	CAGTGGAGCGTGAAGACAAA	CTTGGTCCAATTGAGGAGGA	216	40
Kir6.2	CACAAGAACATCCGAGAGCA	TGGACCTCGATGGAGAAAAG	248	33
SUR1	TGCCACATGTCTTTCTGCTC	GATCTCACACACGAGGACGA	167	35
Cav1.2	TTCCGGAAGATGACTCCAAC	AAAAAGCCCTACAACCACGA	185	35
Cav1.3	TCCGAAGAGCCTGCATTAGT	AGCAGCAGTCCGTACGCTAT	217	35
SNAP25	AAAAAGCCTGGGGCAATAAT	CTCATTGCCCATGTCTAGGG	206	40
VAMP2	CTGCACCTCCTCCAAATCTT	CTTGGCTGCACTTGTTTCAA	191	28
Stx1a	ACCGCTTCATGGATGAGTTC	GAGCTCCTCCAGTTCCTCCT	155	30
PC1/3	GAGATACATGGAGGGCCAGA	ATTGTTGTTCCGCCCATATC	196	35
PC2	CACTCCCAAAGAAGGATGGA	GGCCATTGTGGTAAAAGTGG	232	30

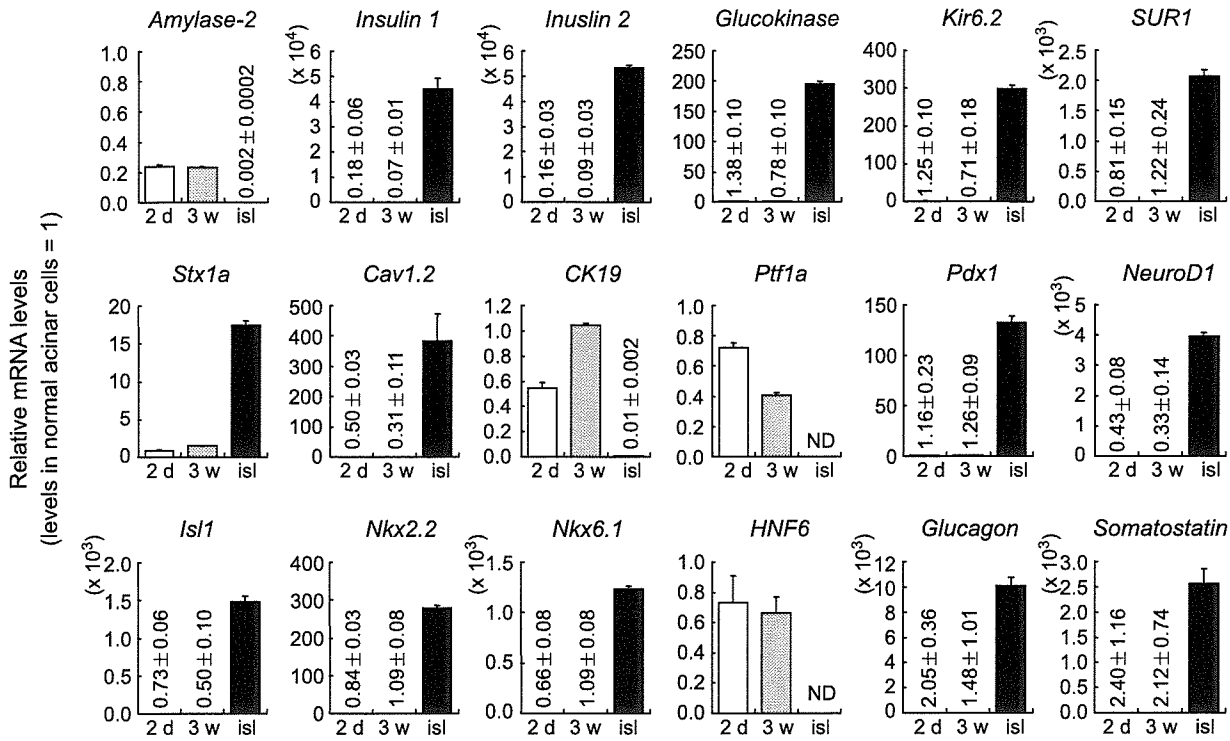

Fig. 1. Expressions of various genes in pancreatic acinar cells of hyperglycemic mice. Quantification of mRNA of genes was done by real-time RT-PCR using TaqMan probe. 18S rRNA was used as an internal control. Expression levels relative to normal acinar cells are shown. 2 d, Acinar cells isolated from acute hyperglycemic mice [2 days after streptozotocin (STZ) injection]; 3 w, acinar cells isolated from chronic hyperglycemic mice (3 wk after STZ injection); isl, mouse pancreatic islets; ND, not detected. Data are means ± SE of 3 independent experiments.

in cells of STZ-injected mice similar to that found in normal mice.

Insulin expression and its secretion in pancreatic acinar-derived cells of STZ-injected mice. Expressions of insulin genes were evaluated by quantitative real-time RT-PCR. mRNA levels of both insulin-1 and insulin-2 genes were gradually increased by culture (Fig. 3*A*). In addition, insulin-positive cells were detected by immunostaining after culture (Fig. 3*B*).

We then examined insulin secretion in pancreatic acinar-derived cells of STZ-injected mice by the batch incubation method. When incubated in the presence of 30 mmol/l KCl, the cells exhibited a significant increase in insulin secretion over basal condition (3 mmol/l glucose; Fig. 3*C*), indicating Ca^{2+}-triggered exocytosis in the cells. Insulin secretion was also increased by glibenclamide (Fig. 3*C*), the sulfonylurea widely used in treatment of diabetes, indicating functional ATP-sensitive potassium channels. Glucose stimulated insulin secretion from the pancreatic acinar-derived cells of STZ-injected mice in a concentration-dependent manner (Fig. 3*D*), indicating that the cells were glucose responsive. In addition, glucagon-like peptide-1 (GLP-1)-(7–36 amide) potentiated insulin secretion (Fig. 3*D*), indicating that the cAMP-mediated potentiation system was also present in the cells. Carbachol, a synthetic acetylcholine derivative that mobilizes intracellular Ca^{2+} through muscarinic receptors, stimulated insulin secretion (Fig. 3*C*), indicating that the phosphatidylinositol signaling system is involved in insulin secretion in these cells. It was noted that no insulin secretion was detected on the day of isolation (Fig. 3*C*). These results demonstrate that glucose-responsive insulin-secreting cells can be generated from pancreatic acinar cells of β-cell-deficient diabetic mice.

Transdifferentiation of pancreatic acinar cells of mice with chronic hyperglycemia. We also attempted to induce insulin-secreting cells from pancreatic acinar cells of mice with chronic hyperglycemia. STZ-injected mice were maintained for 3 wk with daily administration of NPH insulin (2–4 U). By this treatment, the mice were able to survive despite hyperglycemia (~11–28 mmol/l). We found that Ptf1a expression was downregulated by chronic hyperglycemia (Fig. 1). However, pancreatic acinar cells obtained from mice with chronic hyperglycemia began to express β-cell-specific genes by culture (Fig. 4*A*), and the newly generated cells could secrete insulin in response to high KCl and glucose (Fig. 4*B*). These results demonstrate that pancreatic acinar cells retain plasticity in their differentiation capacity even in a chronic diabetic state.

Cell lineage tracing of pancreatic acinar cells of STZ-injected mice. As expressions of pancreatic acinar cell marker genes (amylase and elastase) were decreased, expressions of insulin genes and cytokeratin (CK)19 were induced by the culture (Fig. 5*A*). We have previously established by direct cell lineage tracing that pancreatic acinar cells of normal mice can transdifferentiate into insulin-secreting cells and CK-expressing cells (17). In this study, we utilized STZ-injected R26R-eCFP reporter mice for tracing. As was found in normal mice (17), cells positive for both insulin and eCFP were found in this

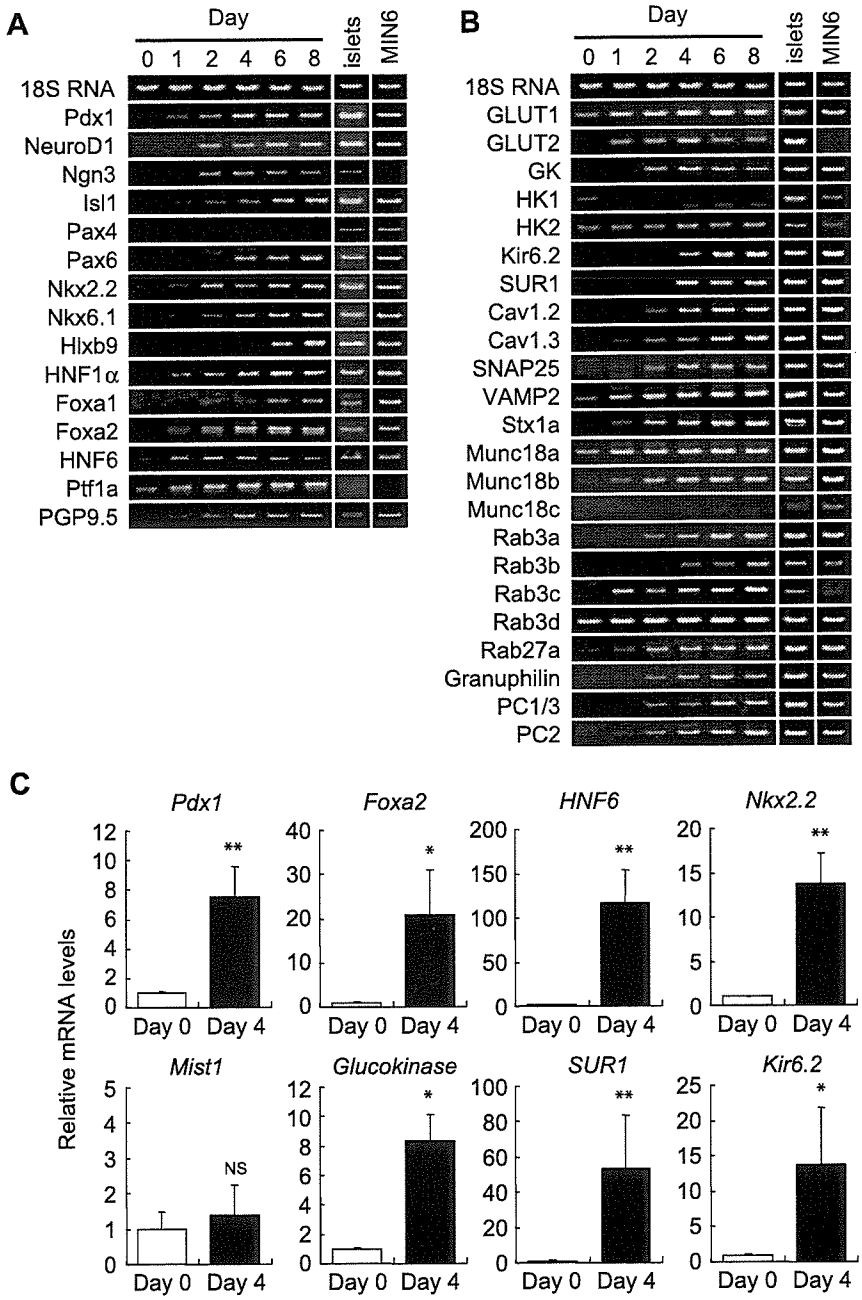

Fig. 2. Changes in expression of genes associated with pancreas development and β-cell function. *A*: RT-PCR analysis of genes involved in pancreatic development. Gene expressions of many transcription factors were induced. Expression of a potential endocrine progenitor marker PGP9.5 was also induced. *B*: RT-PCR analysis for genes involved in glucose-induced insulin secretion. The expression pattern became similar to that of native pancreatic islets and clonal β-cell line MIN6-m9 cells by the culture. *C*: quantitative analysis of gene expression in pancreatic acinar-derived cells. Quantification of mRNA of genes was done by real-time RT-PCR using TaqMan probes. 18S rRNA was used as internal control. Vertical axis represents expression levels of the genes on *day 4* relative to those on *day 0* (as 1). Data are means ± SE of 3–6 independent experiments. $*P < 0.05$; $**P < 0.01$. NS, not significant; GK, glucokinase; HK, hexokinase; Stx1a, syntaxin-1a.

culture system (Fig. 5*B*), indicating that the insulin-secreting cells were transdifferentiated from pancreatic acinar cells of the STZ-injected mice. CK/eCFP double-positive cells also were detected (Fig. 5*B*). In addition to insulin, expressions of other pancreatic hormones were also induced by culture (Fig. 5*A*). Cells positive for eCFP and glucagon, somatostatin, or pancreatic polypeptide (PP) were detected (Fig. 5*B*), indicating that these cells originated from pancreatic acinar cells. These results demonstrate that pancreatic acinar cells of β-cell-deficient diabetic mice can transdifferentiate into all types of pancreatic endocrine cells as well as ductal cells.

Generation of insulin-secreting cells from KDP rats. We then investigated to find whether pancreatic acinar cells of spontaneously diabetic animals could transdifferentiate into insulin-secreting cells. The KDP rat, which was established as a diabetes-prone substrain of the LETL rat, is a model of type 1 diabetes (9, 34). The phenotypic features of the KDP rat include autoimmune destruction of the pancreatic β-cells, and about 80% of them develop diabetes within 220 days of age (9). We used diabetic KDP rats with blood glucose concentrations above 19.4 mmol/l. No dithizone-stained islets or fragmented islets were found in the acinar-enriched fraction from KDP rats, indicating the absence of native pancreatic β-cells in the fraction. RT-PCR analysis revealed that both insulin-1 and insulin-2 genes were induced by the culture in the pancreatic acinar cells of KDP

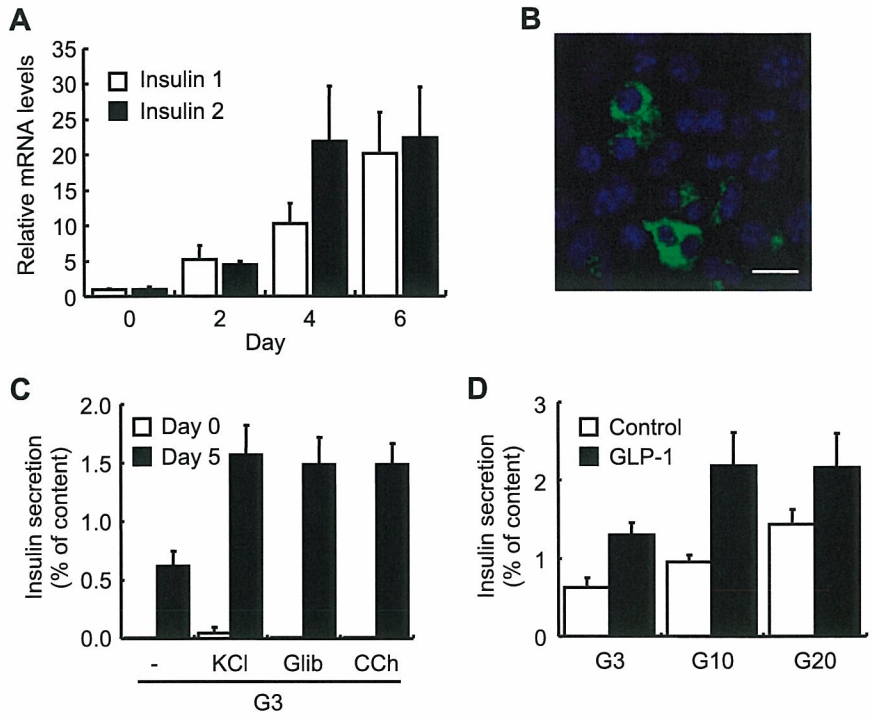

Fig. 3. Insulin expression and secretion in pancreatic acinar-derived cells of STZ-injected mice. *A*: quantitative real-time PCR analysis for insulin genes using SYBR Green PCR master mix. Measurement of the expression level of 18S rRNA was used as an internal control. Insulin expression in the culture was gradually increased. *B*: immunostaining for insulin. Insulin-producing cells (green) were generated from β-cell-deficient mice 6 days after culture. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. *C* and *D*: insulin secretion in acinar-derived cells from STZ-treated diabetic mice. Insulin secretion was measured as accumulation during 60-min incubation. The secretion at 3 mmol/l glucose (G3) represents basal secretion. Addition of 30 mmol/l KCl, 0.1 μmol/l glibenclamide (Glib), or 0.1 mmol/l carbachol (Cch) increased insulin secretion. (*C*). Glucose stimulated insulin secretion in a concentration-dependent manner (G3, 3 mmol/l; G10, 10 mmol/l; G20, 20 mmol/l). Glucagon-like peptide-1 (GLP-1)-(7–36 amide) (100 nmol/l) potentiated insulin secretion in the presence of glucose (*D*). The amount of secreted insulin at 3 mmol/l glucose was 714 ± 143 pg/mg protein. Data are means ± SE of 3–5 independent experiments.

rats as well as in those of normal Wistar rats (Fig. 6, *A* and *B*). In addition, the expression profiles of other pancreatic genes in pancreatic acinar-derived cells of both normal Wistar rats and KDP rats became similar to that of the rat insulinoma cell line INS-1 (1) (Fig. 6, *A* and *B*). We then measured insulin secretion in pancreatic acinar-derived cells of KDP rats by the batch incubation method. Although glucose-induced insulin secretion was not observed, a high concentration of KCl clearly stimulated insulin secretion (Fig. 6*C*). These results demonstrate that insulin-secreting cells can be transdifferentiated from pancreatic acinar cells of spontaneously diabetic animals.

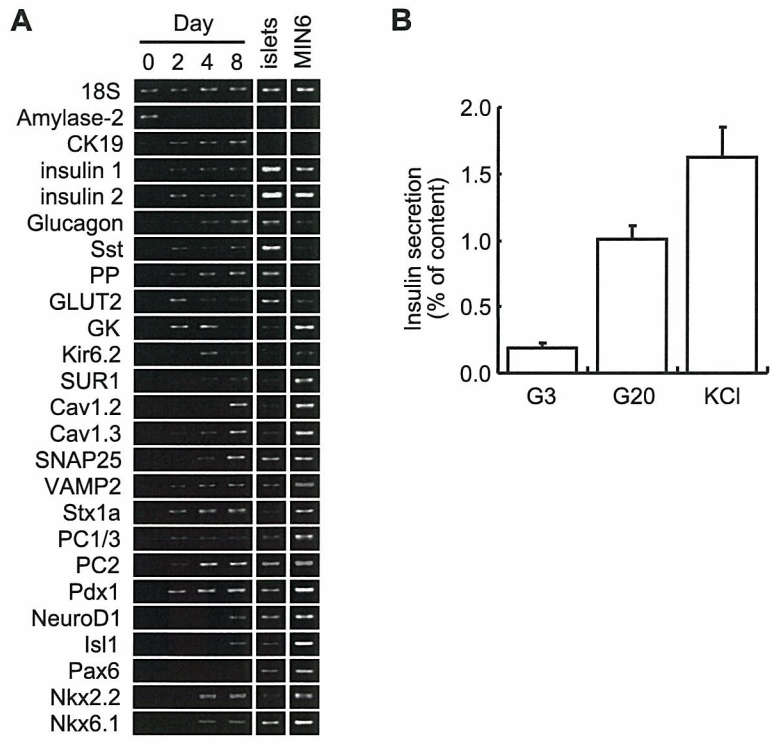

Fig. 4. Transdifferentiation of insulin-secreting cells from pancreatic acinar cells of mice with chronic hyperglycemia. *A*: RT-PCR analysis of genes involved in pancreatic development and β-cell function. Pancreatic β-cell-specific genes were induced by culture. *B*: insulin secretion. Secretion at 3 mmol/l glucose (G3) represents basal secretion. Addition of 30 mmol/l KCl or 20 mmol/l glucose increased insulin secretion.

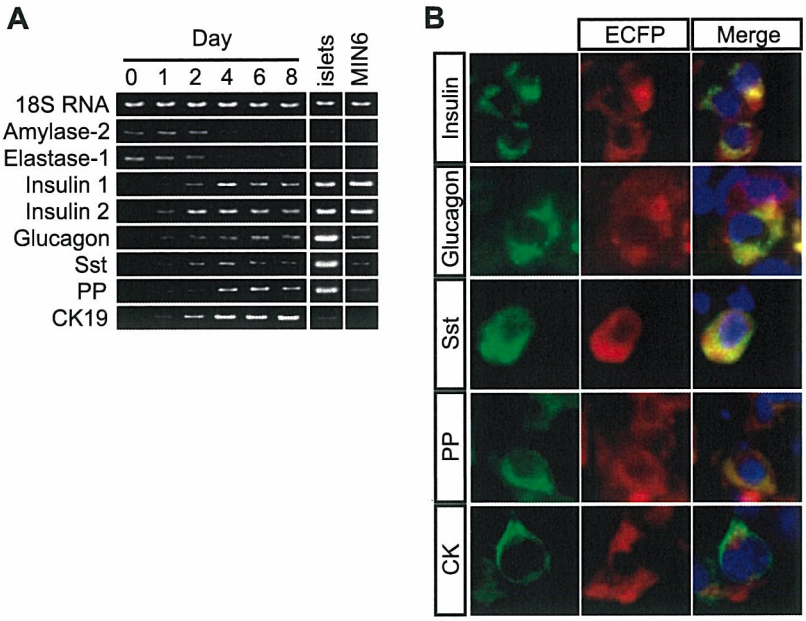

Fig. 5. Generation of endocrine cells from pancreatic acinar cells of STZ-injected mice. *A*: RT-PCR analysis of genes encoding pancreatic hormones and cytokeratin (CK)19. Expressions of glucagon, somatostatin (sst), pancreatic polypeptide (PP), and CK19, as well as insulin, are induced. *B*: cell lineage tracing of pancreatic acinar cells. Pancreatic acinar cells from STZ-injected R26R-eCFP reporter mice were labeled permanently with eCFP by infection of Ad-pAmy-Cre. Double immunostaining was performed after 4 days of culture. eCFP-positive cells (red) also positive for insulin, glucagon, somatostatin, PP, or CK were found. Pancreatic hormones and CK were stained in green, and nuclei were counterstained with DAPI (blue).

DISCUSSION

The present study establishes that pancreatic acinar-to-endocrine transdifferentiation resulting in generation of insulin-secreting cells can occur in β-cell deficient diabetic animals in vitro. Diabetes is characterized by chronic hyperglycemia due to relative or absolute deficiency of insulin action (5). In addition, metabolic disorders in lipids, amino acids, and other nutrients are often found in diabetic patients (4, 27). These abnormalities may influence the cellular function of a variety of tissues (19). Pancreatic exocrine function also is known to be impaired in diabetes (7, 11, 14, 32). We found reduced amylase expression in acinar cells of STZ-injected mice. Similar observations were reported in STZ-injected rats, in which the mRNA level of pancreatic amylase was decreased (10, 13). However, this reduction of amylase expression exerted only a small, if any, effect on transdifferentiation capacity of acinar cells.

Insulin was not detected in pancreatic acinar cell-enriched fractions at the protein level on the day of isolation. Insulin mRNA was detected in pancreatic acinar cell fractions of STZ-injected mice, although the amount was extremely small. This raises the possibility that increased expressions of endocrine-associated genes result from enrichment and/or proliferation of contaminated endocrine cells by culture. However, this can be ruled out by the following considerations: *1*) binucleated insulin-positive cells were often found (Fig. 4*B*); *2*) almost

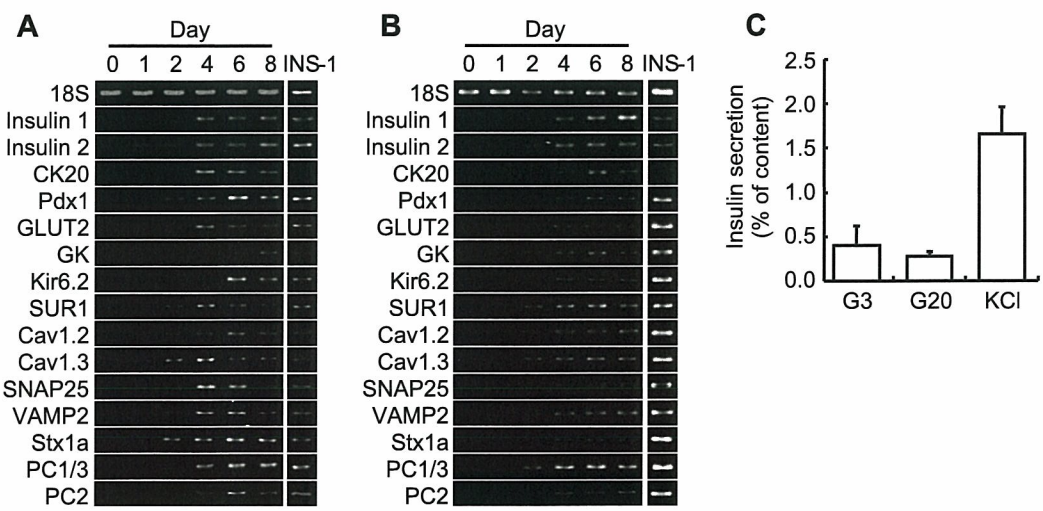

Fig. 6. Generation of insulin-secreting cells from Komeda diabetes-prone (KDP) rats. *A* and *B*: RT-PCR analysis of genes associated with endocrine pancreas. Both insulin-1 and insulin-2 genes were induced in pancreatic acinar cells of normal Wistar rats (*A*) and KDP rats (*B*) by the culture. Expression patterns of other pancreatic genes became similar to that of rat insulinoma cell line INS-1 by the culture in pancreatic acinar-derived cells from normal Wistar (*A*) and KDP rats (*B*). *C*: insulin secretion from pancreatic acinar-derived cells of KDP rats. Insulin secretion was increased significantly by addition of 30 mmol/l KCl. The amount of secreted insulin at 3 mmol/l glucose was 97 ± 56 pg/mg protein.

all of the insulin-positive cells expressed PGP9.5 (17); *3*) cell proliferation was rarely detected, especially in insulin-positive cells, in this culture system (17); and *4*) the expression of pancreatic exocrine specific transcription factor Mist1 showed no significant change by the culture (Fig. 2). Although transdifferentiation of pancreatic acinar cells into endocrine cells occurs in our culture system, because the transdifferentiated insulin-secreting cells have features not found in native β-cells (two nuclei, expressions of PGP9.5 and Mist1), these cells clearly are not fully differentiated endocrine cells. The low insulin production may reflect this immaturity. In addition, we could not detect Pax4 expression at any time point during culture. Since Pax4 has been shown to be essential in development of pancreatic β-cells (25), the absence of Pax4 might indicate that the newly made insulin-secreting cells are incompletely differentiated. Alternatively, the process of transdifferentiation of insulin-secreting cells from acinar cells might differ from that in normal β-cell development.

In the present study, we found that all types of pancreatic endocrine cells can be generated from pancreatic acinar cells by our culture system. We provide clear evidence by direct cell lineage tracing that adult mouse pancreatic acinar cells can transdifferentiate into cells expressing insulin, glucagon, somatostatin, PP, and CK. It has been reported that pancreatic acinar cells from rat can convert into liver cells (12). We also detected mRNAs of albumin and α-fetoprotein in our system (data not shown). These findings indicate that adult pancreatic acinar cells (or dedifferentiated acinar cells) possess multipotentiality in differentiation capacity.

We also found that pancreatic acinar cells of KDP rats undergo transdifferentiation by culture with EGF and nicotinamide. Although the STZ-injected mice diabetes model represents chemically induced acute hyperglycemia with hypoinsulinemia, the diabetic state of KDP rats is genetically established and develops chronically (9). KDP rats show autoimmune destruction of the pancreatic β-cells (9), as is found in human type 1 diabetes. Thus our success in generating insulin-secreting cells from pancreatic acinar cells of KDP rats is of special significance regarding application of these techniques to human subjects in the future. However, the insulin production and secretory responses are even lower in cells from KDP rats than from STZ-injected mice. Apparently, genes involved in glucose sensing (GLUT2 and glucokinase) and metabolism-secretion coupling (ATP-sensitive potassium channels and voltage-dependent calcium channels) are insufficiently induced. Further studies are required to investigate differences between STZ-injected mice and KDP rats in the transdifferentiation capacity of pancreatic acinar cells.

In conclusion, our data demonstrate that insulin-secreting cells can be generated from pancreatic acinar cells of insulin-deficient diabetic animals in vitro. Thus the present study is an important first step toward treatment of type 1 diabetes by autologous transplantation using pancreatic acinar-derived insulin-secreting cells.

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