厚生労働科学研究費補助金

再生医療研究事業

膵上皮幹細胞による糖尿病細胞治療に関する研究

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主任研究者　宮脇　一真

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（主任）研究者　宮脇　一真　神戸大学医学系研究科　助教

研究要旨

わが国における糖尿病患者数は約740万人、その予備軍も含めると約1620万人にも達するとされ、国民健康上の深刻な問題となっている。本研究では、糖尿病再生医療を実現するための基盤研究として、*in vitro*で自己複製可能な体性幹細胞を同定し、その細胞から膵β細胞を分化誘導する方法の開発を行った。

マウスの新生児膵から単離されたPES細胞は、未分化状態を維持したまま長期間増殖し、膵前駆細胞に特徴的な遺伝子プロファイルを有していた。遺伝子導入や低分子化合物添加によって膵内分泌ホルモンを発現し、膵内分泌細胞への分化能を示した。本研究は、糖尿病細胞治療の実現へ向け重要な進捗をもたらした。

分担研究者氏名・奥野　正顕
所属機関・神戸学院大学薬学部
職名・　助教

分担研究者氏名・片山　弘一
所属機関・京都大学医学部附属病院
探索医療センター
職名・　実験助手

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、長期的な糖尿病の罹患により重篤な合併症（網膜症、腎症、神経障害など）を発症する患者も年々増加しており、糖尿病は国民健康上の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療法、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することは困難であることから、糖尿病患者に対し失われた内分泌組織を補充、もしくは復元する移植・再生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、国内でも膵島移植が実施されるようになり、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ている。しかしながら、慢性的なドナー不足が解消される見通しは立たないことから、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、糖尿病移植医療におけるドナー不足の問題を解決するため、*in vitro*で長期間自己複製可能な体性幹細胞を同定し、その幹細胞からインスリン産生細胞への分化誘導法を開発することである。本研究の遂行により、*in vitro*で膵幹細胞からインスリン分泌細胞を分化誘導する基盤技術が提供できれば、将来的に1型糖尿病や重度２型糖尿病患者への移植ソースとして、膵幹細胞を臨床応用することが可能になると考えられ、より多くの糖尿病患者が移植・再生医療の恩恵を享受できる。

B．研究方法

Pancreatic Epithelial Stem(PES)細胞の樹立および培養

生後1日から2日のマウス新生児からピンセットにより膵臓を摘出し、コラゲナーゼD（Roche）20 mgを3 ml のHBSSに溶した溶液中で20分間、37℃で振盪し消化した。分散した細胞は、PES細胞に最適化した培地中で、35 mm dishあたり10000細胞になるように播種した。10日後にはPES細胞のみが出現してくるので、TrypLE（GIBCO）を用いて継代した。その後は1週間おきに、35 mm dishあたり50000細胞になるように継代を続けた。PES細胞の凍結保存にはセルバンカー（日本全薬工業）を用い、-130℃のディープフリーザーで保存した。

テロメア長の測定

テロメア長の測定にはテロメアレングスアッセイ(Roche)を用いた。PES細胞の精製ゲノムDNAを、制限酵素ミックスで切断し、DNA断片をゲル電気泳動で分離した。電気泳動したDNAをナイロンメンブレンにトランスファー後、DIG標識TTAGGGプローブとハイブリダイズし、AP標識抗DIG抗体とインキュベートした。固相化されたテロメアプローブをアルカリホスファターゼ化学発光基質CDP-Starで発光させ検出した。

アデノウイルスベクターの作製および感染

マウス膵ラ氏島からtotal RNAをRNeasy Mini Kit（QIAGEN）を用いて抽出し、Random Primer法を用いてcDNAを作製した。作製したcDNAをテンプレートとして、膵発生上重要な役割を担う11種類の転写因子（Pdx1、NeuroD1、Ngn3、Pax4、Pax6、Isl-1、MafA、Hlxb9、Nkx2.2、Nkx6.1、Ptf1a）のcDNAをPlatinum Pfx DNA Polymerase（Invitrogen）を用いてPCRで増幅し、pENTRベクターにサブクローニングした。シークエンスを確認後、ViraPowerアデノウイルス発現システム（Invitrogen）を用いてアデノウイルスを作製した。得られたアデノウイルスは、-80℃のディープフリーザーで保存し、必要量を溶解して100 moiになるようにPES細胞に感染させた。

GeneChip解析

Mouse insulin promoter-1下でEGFPを発現するトランスジーンをホモに持つトランスジェニックマウス（ICRバックグラウンド）を野生型マウスのC57/BL6と一度戻し交配してヘテロトランスジェニックマウスを作製し、前述の方法によりPES細胞を樹立した。数回継代したのちtotal RNAをRNeasy Mini Kit（QIAGEN）を用いて抽出した。他方、同じヘテロトランスジェニックマウスの膵島を、コラゲナーゼP（Roche）を用いて単離し、Trypsin-EDTA（GIBCO）で単細胞化した。単細胞化した細胞をフィルターにかけた後、FACS Aria(Becton Dickinson)でEGFPを発現する膵β細胞のみを回収し、同様にtotal RNAをRNeasy Mini Kit（QIAGEN）を用いて抽出した。このように回収したPES細胞と膵β細胞（コントロール）のtotal RNAを用いてGeneChip解析（Affymetrix）を行った。

siRNAの作製および導入

siRNAは17種類の遺伝子（HES1、Id1、Id2、Id3、RBP-J、Jagged1、Jagged2、delta1、Notch2、Notch3、Shh、Ihh、Dhh、HNF6、Cdx2、Sox17、Smo）対してデザインした。1つのターゲット遺伝子につきデザイン済みsiRNA（Dharmacon）100 pmolをLipofectamine siRNA MAX(Invitrogen)により$1x10^5$細胞にトランスフェクションした。siRNA導入によるターゲット遺伝子発現抑制効果は、Taqman probe（ABI）を用いたReal-time RT-PCR法により検討し、70%以上の抑制効果が得られたものについて解析した。

分化誘導細胞の生理的解析

PES細胞を35 mmディシュに播種し、分化誘導の後に、インスリン分泌能の検討を行った。3 mMグルコースを含むKRBバッファーで30分間、37℃でインキュベーションした後、3 mMもしくは20 mMグルコース存在下で、2時間、37℃でインキュベーションし、インスリン分泌を測定した。インスリンの測定には、レビスインスリンキット（シバヤギ）を用いた。

電気生理学的検討では、Patchクランプ法によりPES細胞と電極とのギガシールを形成後、Whole cell configurationにより電位依存性カルシウムチャネルの活性を測定した。

免疫染色

PES細胞を４％パラホルムアルデヒドにて20分間固定後、10%ヤギ正常血清、0.2% Tween20を含むPBSで20分間インキュベートし膜の透過性を確保した。次に、rabbit-anti-Pdx1抗体（1次）を室温で2時間、続いてgoat-anti-IgG-Alexa488標識抗体（2次）を室温で1時間反応させることにより免疫染色を行った。核染色にはDAPIを用いた。

C．研究結果

膵上皮幹細胞の未分化性の解析

主任研究者らがマウス新生児膵から単離したPES細胞は、最適化された培養条件下で長期にわたり旺盛な増殖力を保持した。このPES細胞は非常に小型で、高い核／細胞質比を持ち、未分化細胞に共通してみられる形態学的特徴を有する。この細胞は膵発生において中心的な役割を担う転写因子Pdx1が強く発現していることが免疫染色で確認されたほか、HNF1α、HNF3β、Nkx2.2、Nkx6.1といった生体内の膵前駆細胞と同じ転写因子発現プロファイルを示した。加えて、膵前駆細胞のマーカーとして知られているHes1やHNF6、ATP感受性カリウムチャネルを形成するKir6.2、インスリン顆粒の開口分泌機構であるVAMP2やSyntaxin 1A、膵内分泌ホルモンの一つであるソマトスタチンのなどを発現しており、これらの遺伝子プロファイルはPES細胞が膵発生の系譜上にあることを示すものであると考えられた。次に、マウス胎児膵由来PES細胞とマウス成体膵由来PES細胞の成長曲線を描いたところ、両細胞とも約48時間のダブリングタイムを持ち、これを少なくとも30週間以上維持していた。テロメアの長さを胚性幹（ES）細胞や膵外分泌細胞と比較したところ、PES細胞のテロメアはES細胞とほぼ同等の長さを有し、また膵外分泌細胞のテロメアより長かった。以上のことから、PES細胞は未分化な膵幹（前駆）細胞であると考えられた。

低分子化合物、siRNAを用いた分化誘導

PES細胞と膵β細胞のGeneChip解析比較を行った結果、PES細胞ではNotchシグナル（Notch、Hes1、Jagged1、Jagged2）やヘッジホックシグナル（Shh、Ihh、Dhh、Smo、Patched）などの分化抑制機構が非常に強く働いていることが明らかとなった。そこでNotchシグナルの阻害剤であるγ-secretase inhibitorを添加したところ、インスリン２遺伝子のわずかな発現誘導を認め、またShhのシグナルをsiRNAによって遮断することによりグルカゴン遺伝子の発現誘導を認めた。このことは、これらの分化抑制機構がPES細胞の未分化状態維持に重要な役割を担っていることを示す証拠であると考えられた。

転写因子を用いた膵β細胞への分化誘導

膵発生学上重要な役割を果たす転写因子（Pdx1、NeuroD1、Ngn3、Pax4、Pax6、Isl-1、MafA、Hlxb9、Nkx2.2、Nkx6.1、Ptf1a）11種類を、PES細胞に様々な組み合わせで導入し遺伝子発現変化を検討した。

PES細胞と膵β細胞の遺伝子プロファイル比較の結果、膵β細胞の発生に必要なNeurogenin3、NeuroD、Pax4、Hlxb9、Isl1等の発現が欠如していることが明らかとなった。そこでアデノウイルスによりNeuroDをPES細胞に導入したところ、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（Rab3、Chromogranin A/B）の発現が誘導された。さらに、NeuroDに加えて、MafA、Isl1を同時に導入することにより、インスリン２遺伝子が強く誘導された。しかしながら、インスリン１遺伝子は誘導されなかった為、さらなる組み合わせを検討した結果、NeuroD、MafA、Isl1、Hlxb9、Nkx2.2、Nkx6.1、Pax6を同時

に導入することにより、インスリン１、２の遺伝子が誘導され、より膵β細胞に近い遺伝子プロファイルを示す細胞に誘導することが可能であった。

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することができた。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るものと考えられた。

ヒトPES細胞樹立の試み

ヒト組織からPES細胞を樹立するため、膵ガン患者のオペ時に摘出されるガン組織に含まれる僅かな正常組織を材料として、培養条件の検討を行った。しかしながら、高齢者からの組織であったことが原因と考えられるが、３回の樹立の試みはすべて不首尾に終わった。

D．考察

本研究では、マウス新生児膵より膵発生学上の系譜に乗ったPES細胞を樹立し、膵発生に重要な転写因子導入や、シグナルを遮断する低分子化合物、分化を抑制する因子をsiRNAなどによって膵内分泌細胞に分化誘導する試みを中心に行った。この結果、インスリンやグルカゴンといった膵分泌ホルモンをPES細胞から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、分化刺激によってカスケード的に膵β細胞を特徴づける因子が発現することが明らかとなった。このことはPESが未分化な膵幹細胞としての特徴を有していること、PES細胞が膵β細胞へ分化するポテンシャルを有していることを示す証拠であると思われる。しかし一方で、今回の検討では完全に機能な成熟膵β細胞への誘導には至っておらず、またヒトPES細胞の樹立においては高齢者からの組織では困難であった。

（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て承認を受けている。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。

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

Masaaki Okuno,[1]* Kohtaro Minami,[1]* Akinori Okumachi,[1] Kazumasa Miyawaki,[1] Norihide Yokoi,[2] Shinya Toyokuni,[3] and Susumu Seino[1,2]

[1]*Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto;* [2]*Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Kobe; and* [3]*Department of Pathology and Biology of Diseases, Kyoto University Graduate School of Medicine, Kyoto, Japan*

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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.

* M. Okuno and K. Minami contributed equally to this work.
Address for reprint requests and other correspondence: S. Seino, Division of Cellular and Molecular Medicine, Kobe Univ. Graduate School of Medicine, Kobe 650-0017, Japan (e-mail: seino@med.kobe-u.ac.jp).

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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	GTAACCGTTGAACCCCATT	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

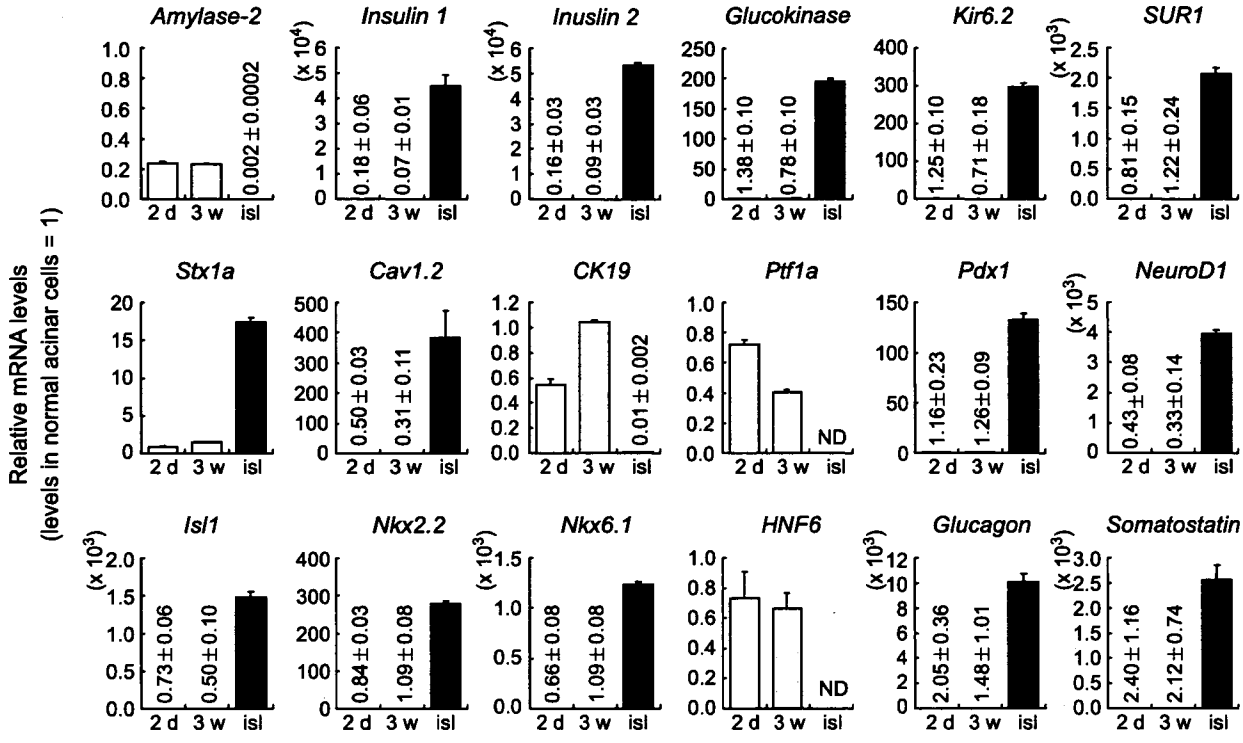

Fig. 1. Expressions of various genes in pancreatic acinar cells of hyperglycemic mice. Quantification of mRNA of genes was done by real-time RT-PCR using TaqMan probe. 18S rRNA was used as an internal control. Expression levels relative to normal acinar cells are shown. 2 d, Acinar cells isolated from 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

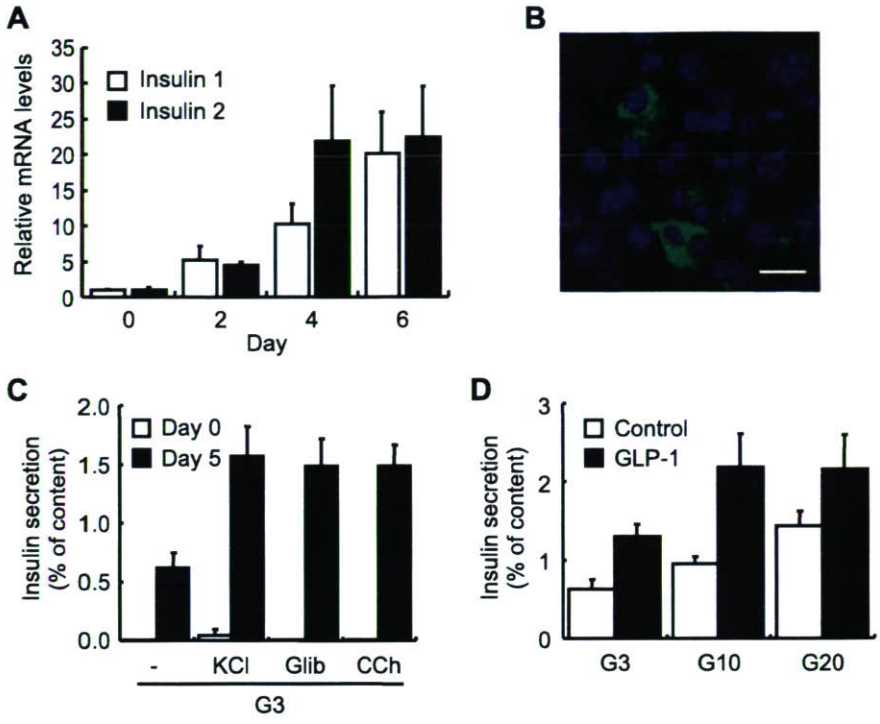

Fig. 3. Insulin expression and secretion in pancreatic acinar-derived cells of STZ-injected mice. *A*: quantitative real-time PCR analysis for insulin genes using SYBR Green PCR master mix. Measurement of the expression level of 18S rRNA was used as an internal control. Insulin expression in the culture 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.

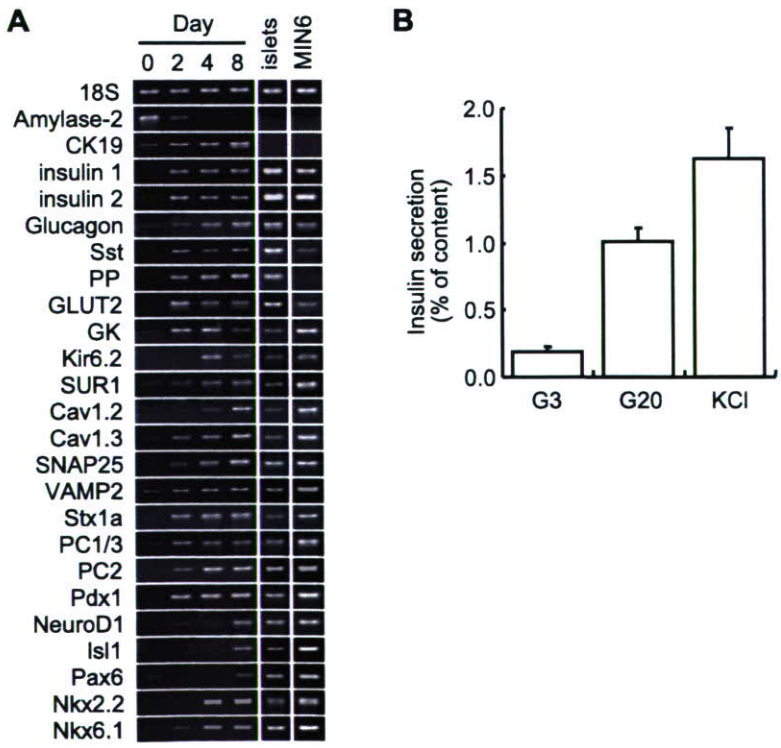

Fig. 4. Transdifferentiation of insulin-secreting cells from pancreatic acinar cells of mice with chronic hyperglycemia. *A*: RT-PCR analysis of genes involved in pancreatic development and β-cell function. Pancreatic β-cell-specific genes were induced by culture. *B*: insulin secretion. Secretion at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

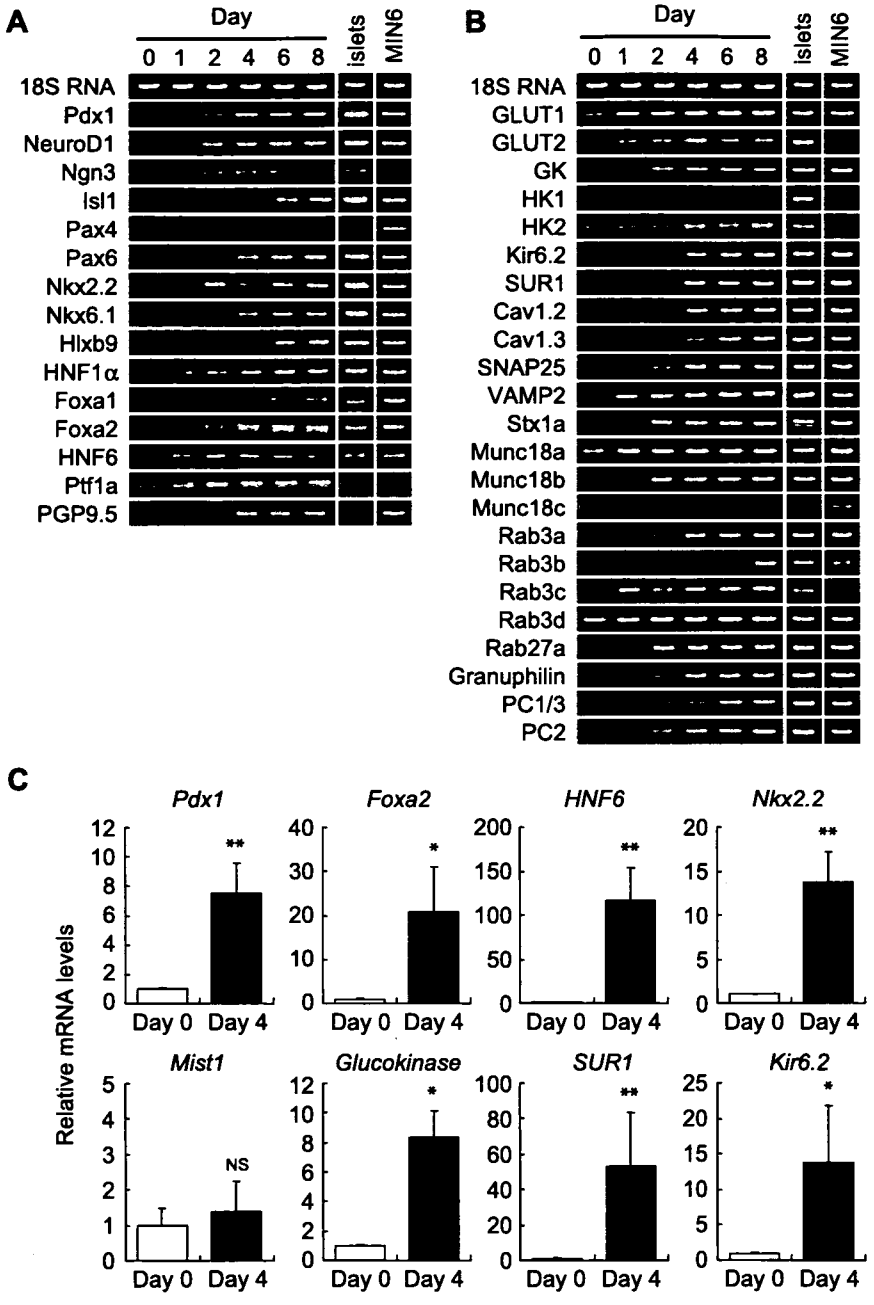

Fig. 2. Changes in expression of genes associated with pancreas development and β-cell function. *A*: RT-PCR analysis of genes involved in pancreatic development. Gene expressions of many transcription factors were induced. Expression of a potential endocrine progenitor marker PGP9.5 was also induced. *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

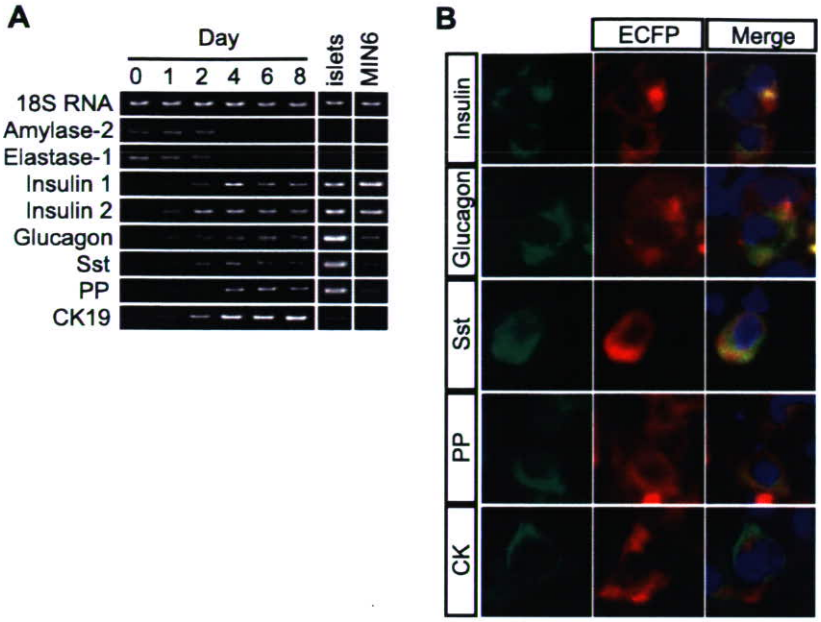

Fig. 5. Generation of endocrine cells from pancreatic acinar cells of STZ-injected mice. *A*: RT-PCR analysis of genes encoding pancreatic hormones and cytokeratin (CK)19. Expressions of glucagon, somatostatin (sst), pancreatic polypeptide (PP), and CK19, as well as insulin, are induced. *B*: cell lineage 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

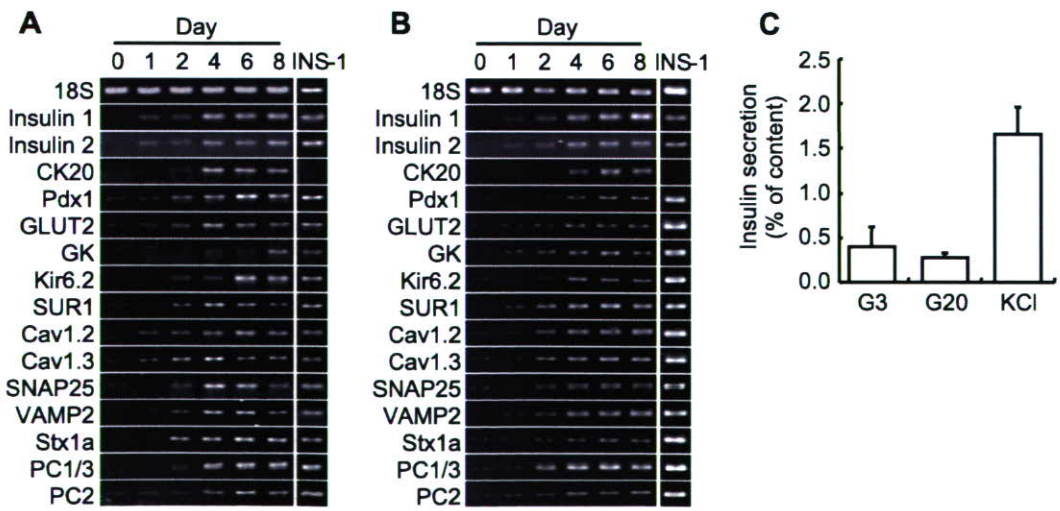

Fig. 6. Generation of insulin-secreting cells from Komeda diabetes-prone (KDP) rats. *A* and *B*: RT-PCR analysis of genes associated with endocrine pancreas. Both insulin-1 and insulin-2 genes were induced in pancreatic acinar cells of normal Wistar rats (*A*) and KDP rats (*B*) by the culture. Expression 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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