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ORIGINAL ARTICLE

Reversal of streptozotocin-induced diabetes in rats by gene therapy with betacellulin and pancreatic duodenal homeobox-1

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Ultrasound-targeted microbubble destruction (UTMD) was used to direct betacellulin (BTC) and pancreatic duodenal homeobox-1 (PDX1) to rat pancreas 48 h after islet destruction by streptozotocin (STZ). Sprague–Dawley rats were rendered diabetic by STZ injection. Controls included normal rats, STZ only without UTMD, and UTMD with DsRed reporter gene. Blood glucose increased dramatically in all rats 48 h after STZ, and continued to rise after UTMD with BTC alone. Blood glucose declined from day 3 to day 10 after UTMD with PDX1, but remained elevated (261 ± 8 mg/dl). However, in rats treated with both BTC and PDX1, blood glucose remained below 200 mg/dl throughout day 10. This was accompanied by normalization of blood insulin and C-peptide. Histology demonstrated islet-like clusters of glucagon-staining cells in the rats treated with BTC and PDX1, but these clusters disappeared by 30 days after UTMD treatment. Although regeneration of insulin-producing islets was not seen, diabetes was reversed for up to 15 days after a single UTMD treatment by ectopic insulin production by pancreatic acinar cells. These cells co-expressed amylase and insulin and demonstrated several β-cell markers by reverse transcription-PCR. Gene therapy by UTMD can reverse diabetes in vivo *in adult rats by restoring pancreatic insulin production.*
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Keywords: *diabetes; streptozotocin; betacellulin; insulin; islets*

Introduction

Diabetes affects approximately 200 million people worldwide and is increasing in prevalence.[1] It is estimated to be the fifth leading cause of death in the world,[2] and results in serious complications, including cardiovascular disease, chronic kidney disease, blindness, and neuropathy. Despite a wide variety of pharmacological treatments for diabetes, including insulin therapy, adequate blood sugar control is often difficult, in part because these agents are not able to duplicate the glucose regulatory function of normal islets. Accordingly, new treatment strategies have focused on replenishing the deficiency of β-cell mass common to both major forms of diabetes by either islet transplantation or β-cell regeneration.[3–5] We recently demonstrated that gene therapy could be targeted to pancreatic islets in normal rats, using ultrasound-targeted microbubble destruction (UTMD).[6] Intravenous microbubbles carrying plasmid DNA are selectively destroyed within the pancreatic microcirculation by ultrasound, thereby locally delivering the plasmids. Islet specificity was achieved by incorporating the rat insulin-I promoter (RIP) within the plasmid DNA. We now report the use of UTMD to deliver two genes known to be involved in islet development, *betacellulin* (BTC), and *pancreatic duodenal homeobox-1* (PDX1) in streptozotocin (STZ)-induced diabetes in rats. Primitive islet-like clusters of glucagon-staining cells were seen in the rats treated with a combination of *BTC* and *PDX1*, but these clusters disappeared by 30 days after treatment. Although regeneration of normal islets was not seen, diabetes was reversed for up to 15 days after a single UTMD treatment by ectopic insulin production by pancreatic acinar cells.

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Results

Effect of UTMD gene therapy on blood glucose, insulin, and C-peptide

To evaluate the effects of *BTC* gene therapy on serum glucose and insulin in diabetic rats, 24 rats were treated with intraperitoneal STZ (80 mg/kg) followed by UTMD containing a *DsRed* reporter gene (inactive control, $n=6$), *BTC* ($n=6$), *PDX1* ($n=6$), or *BTC* plus *PDX1* ($n=6$). Six additional controls received STZ alone without UTMD gene therapy ($n=3$) or neither STZ nor UTMD (normal controls, $n=3$). Baseline blood glucose was 113 ± 20 mg/dl and was not significantly different between groups. As shown in Figure 1 (top panel), blood glucose increased

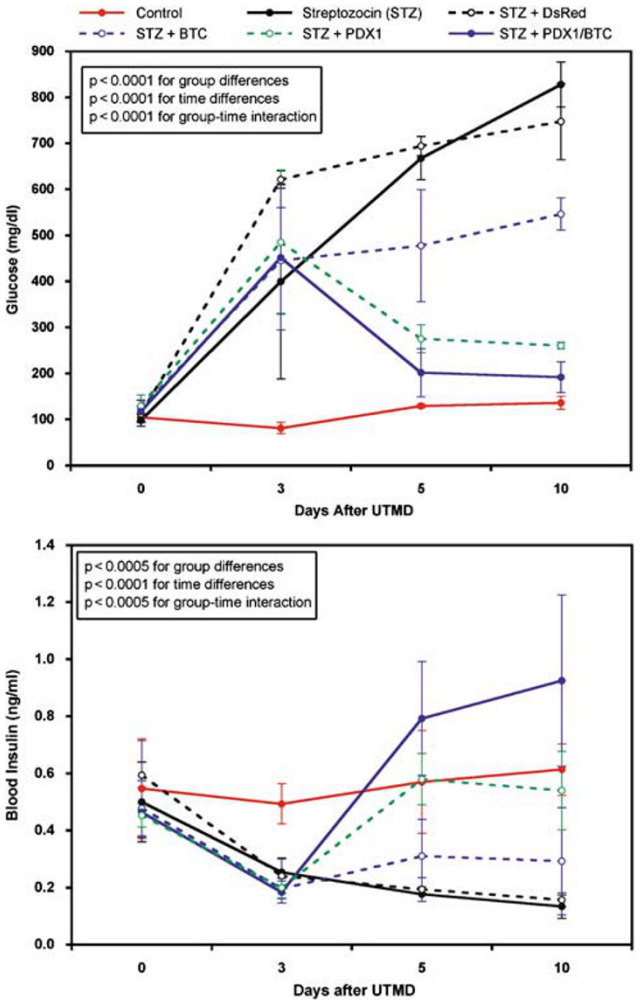

Figure 1 Top panel. Plot of serum glucose levels over time. In normal controls (solid red line), glucose levels are stable. In all STZ-treated rats, glucose rises precipitously by day 3, and continues to rise in rats treated with STZ only (solid black line), *DsRed* (dashed black line), or *betacellulin* alone (dashed blue line). Glucose improves by days 5 and 10 in rats treated with *PDX1* (dashed green line), and is nearly normal in rats treated with both *PDX1* and *betacellulin* (solid blue line). By repeated measures ANOVA, these differences are highly statistically significant both between groups and over time. Bottom panel. Plot of serum insulin levels over time. In normal controls (red line), insulin levels are stable over time. In all STZ-treated rats, insulin declines significantly by day 3, and either decline further (STZ only or UTMD with *DsRed*), or stay low (UTMD with *betacellulin*). In contrast, UTMD with *PDX1* restores insulin levels to normal by day 5 (dashed green line); whereas UTMD with *PDX1* and *betacellulin* results in supranormal insulin levels at days 5 and 10 (solid blue line). By repeated measures ANOVA, these differences are highly statistically significant both between groups and over time (see text for details).

dramatically in all STZ-treated rats by day 2 and continued to rise through day 10 in rats treated with STZ alone, the *DsRed* reporter gene, and *BTC* alone. In rats treated with *PDX1* alone, glucose decreased from day 3 to day 5, but remained elevated (261 ± 8 mg/dl) at day 10. However, in the group treated with both *BTC* and *PDX1*, mean values remained below 200 mg/dl throughout day 10. By repeated measures analysis of variance (ANOVA), differences between treatment groups ($F = 41.4$, $P < 0.0001$) and glucose over time ($F = 95.3$, $P < 0.0001$) were highly statistically significant. By Fisher's *post hoc* tests, blood glucose in the rats treated with STZ alone ($P < 0.0001$), *DsRed* ($P < 0.0001$), *BTC* alone ($P = 0.0003$), or *PDX1* alone ($P = 0.0364$) was significantly higher than control groups. However, blood glucose in the group receiving both *BTC* and *PDX1* was not statistically different from controls ($P = 0.1553$).

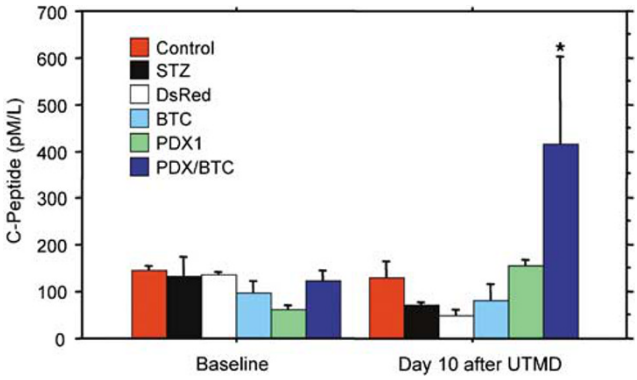

Figure 2 Plot of C-peptide at baseline and day 10. In normal controls, C-peptide is stable over time (red bars). C-peptide decreases in rats treated STZ alone or STZ followed by UTMD with *DsRed* or *BTC*. However, C-peptide roughly doubles in rats treated with *PDX1* alone (green bars, P = NS), and increases significantly in rats treated with STZ followed by UTMD with *BTC* and *PDX1* ($P < 0.03$ vs all other groups at day 10).

Blood insulin levels were 0.51 ± 0.11 ng/ml at baseline and were not significantly different between groups. As shown in Figure 2 (bottom panel), insulin levels decreased at day 3 in all STZ-treated groups relative to normal controls. However, by day 5, insulin levels returned to normal control values in the *PDX1* alone and the *PDX1* and *BTC* combination groups. By repeated measures ANOVA, differences between treatment groups ($F = 15.2$, $P < 0.0001$) and insulin over time ($F = 20.0$, $P < 0.0001$) were highly statistically significant. By Fisher's *post hoc* tests, insulin levels were statistically significantly lower in rats treated with STZ alone ($P = 0.001$), *DsRed* ($P = 0.003$), or *BTC* alone ($P = 0.0026$) compared to controls. However, insulin levels in the rats treated with *PDX1* ($P = 0.16$) or the combination of *PDX1* and *BTC* ($P = 0.89$) were not significantly different from control values.

To confirm that the detected insulin was produced by the UTMD treatment, C-peptide levels were measured at baseline and day 10. As shown in Figure 2, C-peptide remained stable in the normal controls, and decreased in rats treated with STZ alone or STZ with *DsRed* or *BTC*. However, C-peptide roughly doubled in rats treated with *PDX1* and increased markedly in rats treated with both *BTC* and *PDX1* at day 10. By repeated measures ANOVA, differences in C-peptide between treatment groups were statistically significant ($F = 10.5$, $P = 0.0004$). By *post hoc* test, the day 10 C-peptide level was higher in the *BTC/PDX1* group compared to all other groups ($P < 0.03$).

A glucose tolerance test was performed in order to determine whether the UTMD treatments resulted in glucose-regulatable insulin production. As shown in Figure 3, STZ-treated animals had markedly abnormal glucose tolerance curves, as did rats treated with *BTC* alone. In contrast, rats treated with *PDX1* or the

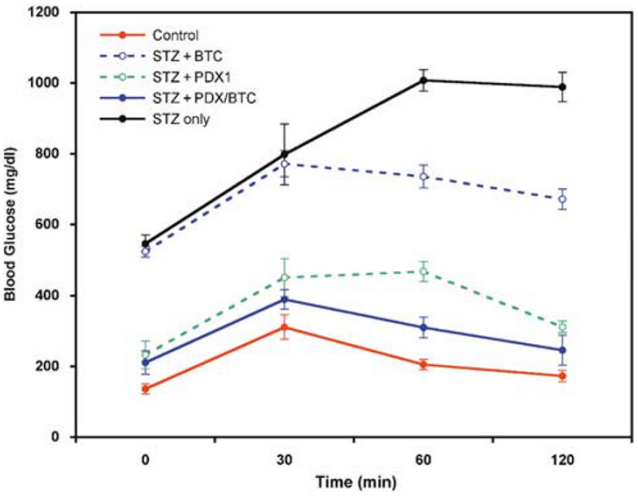

Figure 3 Results of a glucose tolerance test performed 10 days after UTMD. The rats treated with STZ alone (black line) have markedly elevated blood glucose levels at baseline that increase further after glucose challenge. The glucose response is slightly improved by *BTC* alone (dashed blue line). The *PDX1* alone (dashed green line) and *betacellulin* and *PDX1* (solid blue line) have nearly normal glucose responses that are similar to normal control rats (red line).

combination of *BTC* and *PDX1* had a nearly normal glucose tolerance response.

Immunohistology of islet morphology

Figure 4 shows representative histological samples of rat pancreas at day 10, stained with fluorescein isothiocyanate (FITC)-labeled anti-insulin (green) and CY5-labeled anti-glucagon (blue). The left upper panel shows a typical normal islet characterized by central green anti-insulin staining and peripheral blue anti-glucagon staining. At low power ($\times 100$), normal controls had 3–4 islets per field. Rats treated with STZ alone had virtually no detectable anti-insulin staining at day 10, although occasional faint anti-glucagon staining in presumed islet remnants was present (not shown). Similar findings were seen in rats treated with *DsRed* (not shown). In rats treated with *BTC* alone, 1–2 islet-like clusters were seen per field, mostly consisting of glucagon-positive (blue) cells, with little insulin staining. Rats treated with *BTC* and *PDX1* demonstrated 3–4 islet-like clusters per field, with predominant anti-glucagon staining, shown at high power ($\times 400$) in the right upper panel. In addition, there appeared to be substantial anti-insulin staining in the exocrine pancreas at $\times 100$, a finding not seen in controls. On higher power ($\times 400$, left

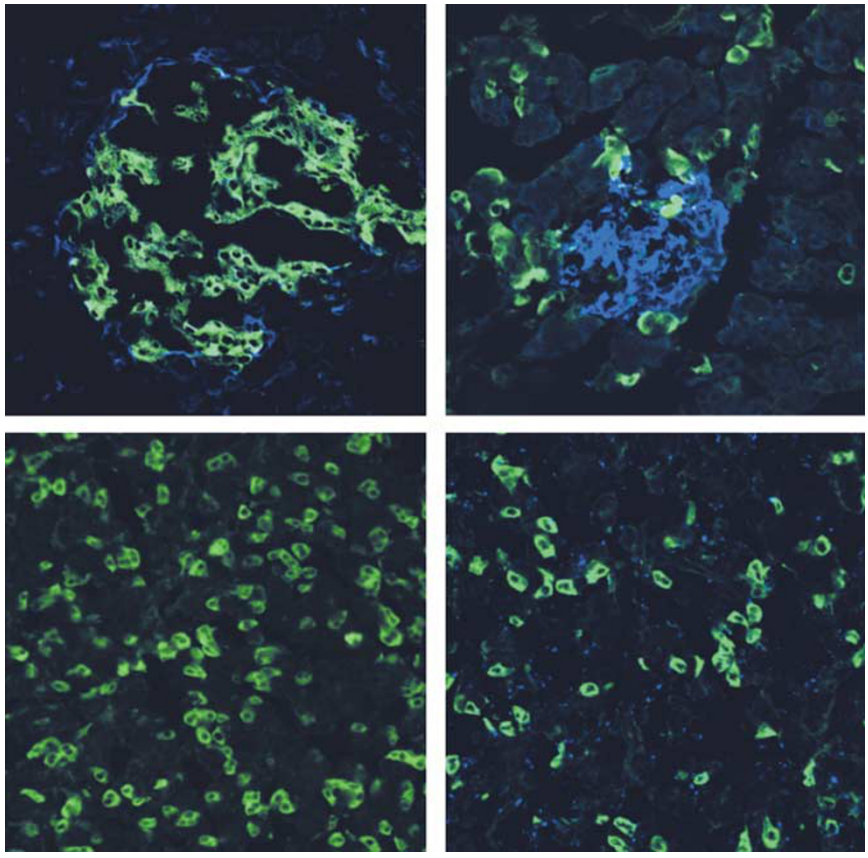

Figure 4 Representative histological sections of rat pancreas (day 10) stained with FITC-labeled anti-insulin (green) and CY5-labeled anti-glucagon (blue) antibodies. Left upper panel. High power ($\times 400$) section from a normal control rat showing a typical islet with β-cells in the center (green) and α-cells on the periphery (blue). There is some background autofluorescence in both colors. Right upper panel. High power section ($\times 400$) from a rat treated with *PDX1* and *betacellulin* plasmids by UTMD. Atypical islet-like clusters of cells stain mostly with glucagons (blue). In addition, anti-insulin staining (green) appears to be present in the exocrine pancreas. Left lower panel. High power ($\times 400$) image from a rat treated with *PDX1* and *betacellulin* plasmids showing prominent insulin staining in what appear to be acinar cells. Right lower panel. High power ($\times 400$) image from a rat treated with *BTC* and *PDX1*. FITC-labeled anti-insulin staining (green) shows insulin present in acinar cells. CY5-labeld anti-BTC staining (blue) shows BTC present in and around these cells within the exocrine. CY5-labeled anti-PDX1 had a nearly identical appearance (not shown).

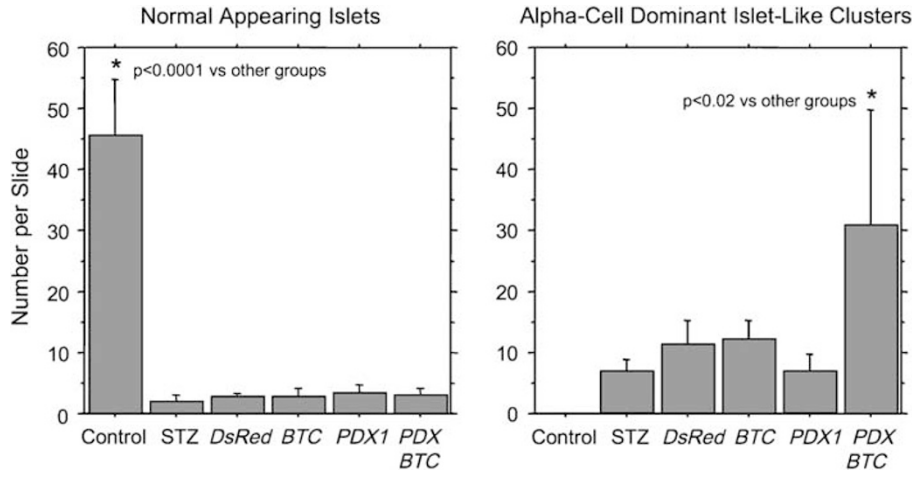

Figure 5 Plot showing number of islets per slide at day 10 for normal islet morphology (left panel) and islet-like clusters of predominantly glucagon-positive cells (right panel). Normal islets were common in controls (46±9 islets per slide), but rare in STZ rats, regardless of treatment group ($P<0.0001$). Islet-like clusters of glucagons-positive cells were absent in controls, but present in modest numbers in STZ rats, particularly in those treated with both *BTC* and *PDX1* (19±8 per slide, $P<0.02$ vs other groups).

lower panel), numerous non-islet cells are seen to show cytoplasmic anti-insulin staining. Anti-BTC and anti-PDX1 staining (CY5-labeled) demonstrated proximity of both BTC and PDX1 to the insulin-positive non-islet cells (right lower panel). Western blot (data not shown) clearly demonstrated increased BTC (21 kDa) and PDX1 (31 kDa) in the pancreas of rats that received these genes by UTMD compared to controls.

Figure 5 shows the differences in islet morphology between treatment groups. Normal islets were common in the control group (46±9 islet per slide), but rare in all STZ-treated groups (<3 islets per slide) ($P<0.0001$ vs control). The unusual islet-like clusters of predominantly glucagon-staining cells were absent in normal controls, being only seen after STZ treatment. These abnormal appearing islets were more prevalent in the rats treated with *BTC* and *PDX1* (19±8 clusters per slide) than in STZ alone (7±2), *DsRed* (11±4), *PDX1* alone (7±3), or *BTC* alone (12±3) ($P<0.02$). Moreover, these islet-like clusters of glucagon-staining cells were associated with higher blood glucagon levels in the rats treated with *BTC* and *PDX1* than in other groups. At baseline, blood glucagon was 95±12 pg/ml and it was not different between groups. At day 10, glucagon had increased to 263±145 pg/ml in the rats treated with *BTC* and *PDX1* ($F=4.6$, $P<0.014$), but had not changed significantly in the other groups.

Immunohistology of insulin-producing cells in the exocrine pancreas

To further evaluate the insulin-producing cells in exocrine pancreas seen in Figure 4, we performed confocal microscopy with both FITC-labeled anti-insulin with DsRed-labeled anti-amylase (Figure 6). As shown in the left panel at ×1000, there is prominent anti-insulin activity (FITC, green) in the cytoplasm of these cells. The middle panel shows anti-amylase (DsRed, red) in the same cells. The right panel shows colocalization of anti-insulin (green) and anti-amylase (red), indicating ectopic insulin production by acinar cells. To further confirm that acinar cells were producing insulin, acinar cells were

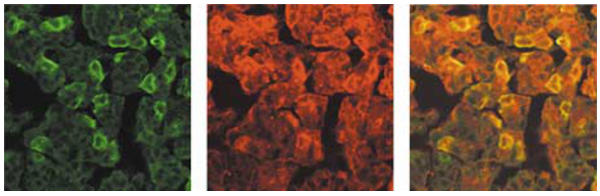

Figure 6 High power images (×1000) from a rat treated with *PDX1* and *betacellulin* by UTMD. The left image is stained with FITC-labeled anti-insulin and shows what appear to be acinar cells producing insulin. The middle panel is stained with Cy5-labeled anti-amylase and shows expected cytoplasmic red staining typical of acinar cells. The right panel is a confocal image showing colocalization of FITC-labeled anti-insulin and Cy5-labeled anti-amylase, confirming that these are acinar cells. Images were obtained at day 10 after STZ.

isolated from all six treatment groups; normal controls, STZ alone, and STZ followed by UTMD with *BTC* alone, *PDX1* alone, and both *BTC* and *PDX1*. The isolated acinar cell fraction was then subjected to reverse transcription (RT)-PCR for a number of β-cell markers (Figure 7). β-Actin, a positive control, was present in all groups. Insulin 1 and 2 were not seen in the control, STZ, or *DsRed* groups, confirming the purity of the acinar cell isolation. A number of markers were detected only in the groups receiving *BTC* and/or *PDX1*, including INS-1, INS-2, glucagon, somatostatin, MIST-1, VMAT, GLP-1 receptor, neurogenin-3, and Nkx2.2, suggesting that these acinar cells were co-expressing β-cell markers after gene therapy with *BTC* and *PDX1*.

Time course of acinar cell production of insulin

In a separate experiment, six rats were treated with STZ, followed by UTMD with *BTC* and *PDX1*, and followed for 30 days. Serum glucose and insulin were measured every 5 days, and three rats each were killed at days 20 and 30, respectively. As shown in Figure 8, the cytoplasmic anti-insulin staining of acinar cells had disappeared by day 30, with marked reduction of serum insulin and elevation of serum glucose. In addition, none

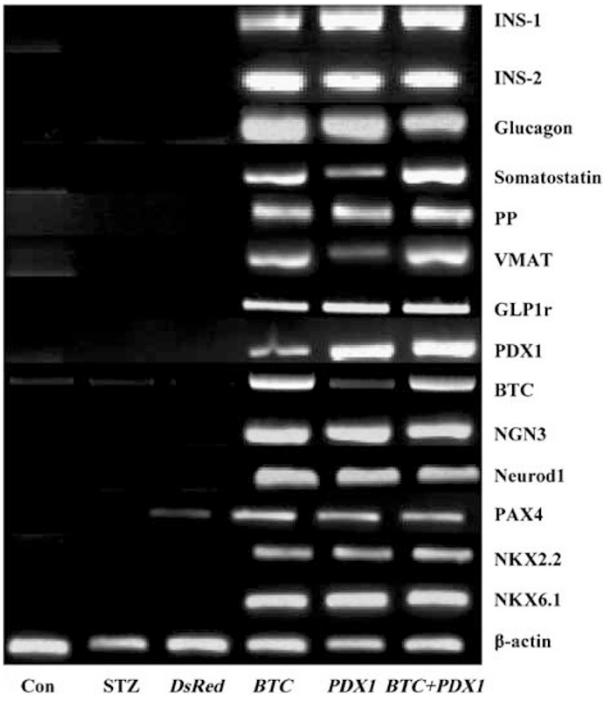

Figure 7 RT-PCR of isolated acinar cells after UTMD treatment. The vertical columns are normal control, STZ-treated control, UTMD with *DsRed*, UTMD with *betacellulin*, UTMD with *PDX1*, and UTMD with *PDX1* and *betacellulin*. A number of β-cell markers are upregulated in these UTMD-treated acinar cells. β-Actin is used as a positive control.

of the islet-like clusters of glucagon-positive cells were present at day 30.

Discussion

Although pharmacological therapy for diabetes has continued to improve, 'tight' glucose control has not eliminated the devastating complications of diabetes,[7,8] primarily because the available drugs do not reconstitute the glucose-regulatory function of normal islets. Pancreas or islet transplantation can achieve this goal, but are limited by an inadequate donor supply, the need for immunosuppression, and loss of function of the transplanted islets. Thus, current research efforts have focused on creating new sources of β-cells for transplantation, or regeneration of functioning β-cells within the pancreas or other tissues.[3–5] In the present study, we have adopted the latter approach, attempting to use ultrasonic destruction of plasmid-carrying microbubbles to direct gene therapy to the pancreas. Our data demonstrate that in rats with STZ-induced diabetes, gene therapy with *BTC* and *PDX1* results in ectopic insulin production by pancreatic acinar cells *in vivo* with subsequent restoration of normal blood glucose.

The origin of β-cell mass expansion, which is known to occur after partial pancreatectomy,[9] STZ,[10] or interferon-γ,[11] has been a subject of recent controversy. Dor *et al*.[12] concluded that new islets could only be formed by replication of existing β-cells after birth or partial pancreatectomy in adult mice. However, a recent study by Hao *et al*.,[13] showed that isolated human pancreatic epithelial cells could regenerate β-cells *in vitro* or when injected along with fetal pancreatic tissue into the renal capsule of immunodeficient mice. The present study supports the notion that the nonendocrine pancreas can be stimulated to produce insulin. The insulin-producing cells observed in our model showed colocalization of insulin and amylase by immunohistochemistry. Furthermore, when the acinar cell fraction was isolated, it was shown to contain several β-cell markers in the rats treated with *PDX1* and *BTC*, but not in controls. These observations, along with the fact that gene therapy by UTMD was introduced after complete destruction of existing β-cells by high dose STZ treatment, makes β-cell replication a highly unlikely explanation for these findings. However, the transient nature of this phenomenon suggests that these cells were not truly 'transdifferentiated' into β-cells.

UTMD has been shown previously to target genes to the pancreas *in vivo*, using an insulin promoter to achieve selective expression in islets.[4] In the present study, both cytomegalovirus (CMV) and RIP promoters were used because the latter was not expected to work after islet destruction by STZ. Instead, we reasoned that CMV-driven *BTC* and/or *PDX1* would initiate insulin production in nonendocrine pancreas,[14] which would in turn drive RIP-driven gene expression, and enhance the process. Ferber *et al*.[15] previously used both CMV and RIP promoters in driving insulin production in liver using adenoviral vectors. We chose to use *BTC*, alone and in combination with *PDX1* to attempt to regenerate β-cell mass. *BTC* is a mitogen and β-cell-stimulating hormone[16] that has been shown to induce insulin production in intestinal cells[17] and hepatocytes.[18] We used a 21 kDa membrane-anchored form of *BTC* that has been shown to induce insulin production in AR421 rat pancreatic cells.[19] *PDX1* is a transcription factor that is considered a master switch for fetal pancreatic development. Although this combination of genes restored normal insulin and C-peptide levels by acinar cell insulin production, it did not promote regeneration of normal islets. Instead, we observed the transient appearance of islet-like clusters of cells that produced glucagon. We suspect that this represents α-cell hyperplasia since blood glucagon was elevated and GLP-1 was induced in the acinar cell fraction of the treated rats.[20] Nevertheless, this study demonstrates that UTMD is a feasible method of introduction of other genes, alone or in various combinations that might be effective in islet regeneration *in vivo*. This is particularly relevant in the adult animal because genes involved in islet regeneration could differ from those known to regulate embryological development. Potential candidate genes, as reviewed recently by Samson and Chan,[4] include *INGAP*, *neurogenin*, *neuroD*, *GLP-1*, *exendin*, *gastrin* and *EGF*, *Mafa*, *PAX6*, *Nkx2.2*, *Nkx6.1*, and others. It was far beyond the scope of this study to attempt to test all of the potential gene combinations that could reconstitute insulin production or islet formation.

Acinar cell transformation to a β-cell phenotype has been reported previously. Investigators in the Bouwens laboratory[21–23] have shown that isolated exocrine cells treated *in vitro* with EGF and either gastrin or leukemia inhibitory factor were transformed into insulin-producing cells that restored normoglycemia when injected into diabetic rodents. Similarly, infusion of gastrin into

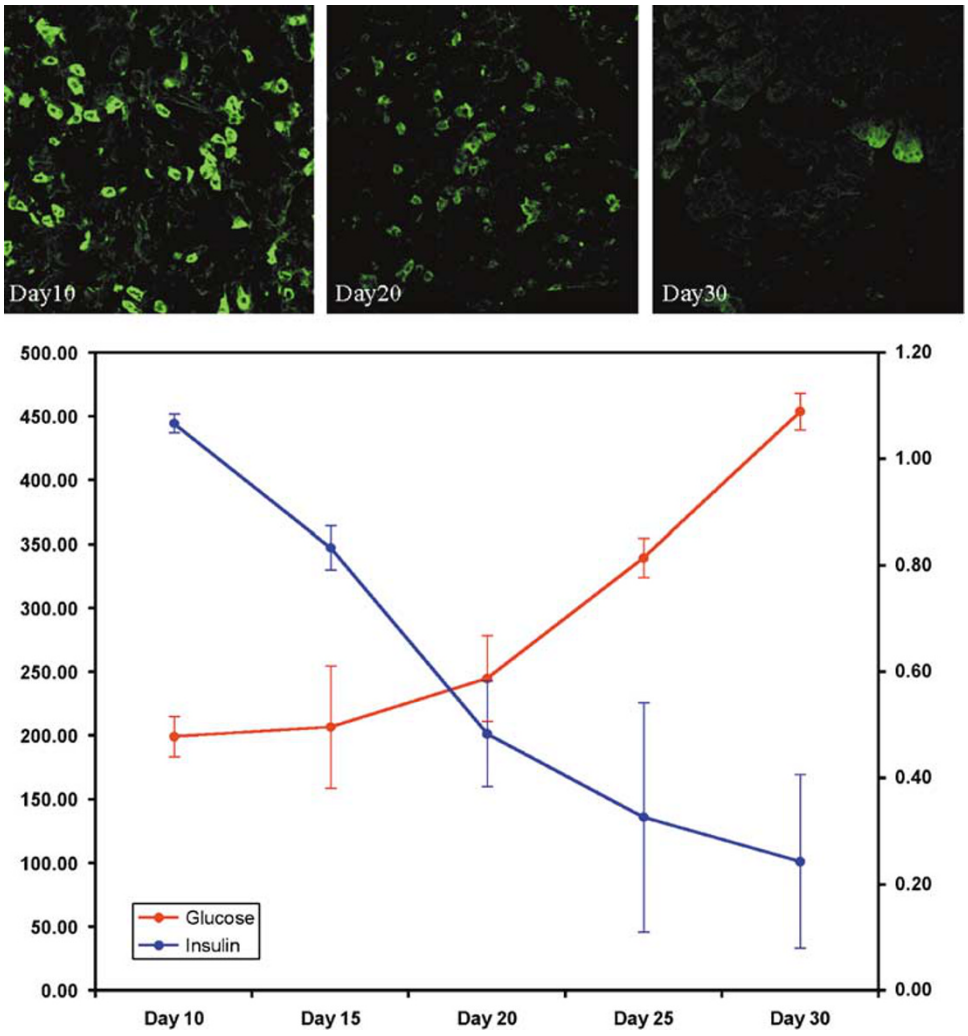

Figure 8 Time course of acinar cell transdifferentiation after UTMD with *PDX1* and *betacellulin*. Top panels. Histological images showing FITC-labeled insulin production in acinar cells, which is prominent at day 10, reduced at day 20, and nearly absent at day 30 after UTMD. Bottom panel. Glucose (left vertical axis) increases between days 10 and 30, whereas insulin (right vertical axis) decreases.

rats, after ligation of the pancreatic duct, induced β-cell regeneration with colocalization of amylase and insulin, indicating acinar cell origin.[24] In a subsequent study in alloxan-treated mice, the same group concluded that β-cell regeneration originated from ductal cells.[25] The present study indicates that acinar cell transformation to a β-cell phenotype is feasible with restoration of normal insulin production for up to 15 days. Loss of endocrine function of these acinar cells by 30 days suggests that these cells have not fully 'transdifferentiated.' We have not studied that mechanism by which these cells have lost their insulin-producing capability, but it may relate to the limited duration of effect of the plasmids *in vivo*. The use of other vectors such as lentivirus or helper-deficient adenovirus may enhance longevity.

Finally, other investigators have been successful in using gene therapy approaches to produce insulin in intestinal or liver cells.[15,18,26,27] A potential advantage to our approach is that the pancreas might be the ideal milieu for islet regeneration. This concept is supported by the recent study of Hao *et al*.,[13] in which cultured human pancreatic endothelial cells produced islets when injected into the renal capsule of mice along with fetal pancreatic tissue. It is presumed that the latter contains the appropriate stimuli for islet regeneration. In our study, we found that gene therapy with *PDX1* and *BTC* produced primitive islet-like clusters that contained predominantly α-cells and disappeared by 30 days. This finding, though disappointing at first glance, indicates that there may be a potential to regenerate normal islets by this approach if the appropriate stimuli or the right combination of genes can be identified. UTMD offers a non-invasive *in vivo* method for testing candidate genes for islet regeneration in adult animal models of diabetes.

Materials and methods

Animal protocol and UTMD

Animal studies were performed in accordance with National Institute of Health (NIH) recommendations and the approval of the institutional animal research committee. Male Sprague–Dawley rats (200–250 g) were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (5 mg/kg). Hair was shaved from left abdomen and neck, and a polyethylene tube (PE 50,

Becton Dickinson, Franklin Lakes, TN, USA) was inserted into the right internal jugular vein by cut-down. In a first experiment, 30 rats received one of six treatments: (1) no treatment (normal control rats, $n=3$); (2) STZ (80 mg/kg/i.p., Sigma, St Louis, MO, USA) alone without UTMD ($N=3$); (3) STZ and UTMD with plasmids encoding a *DsRed* reporter gene ($n=6$); (4) STZ and UTMD with plasmids encoding the 21 kDa membrane-anchoring rat *BTC gene*[19] ($n=6$); (5) STZ and UTMD with plasmids encoding the rat *PDX-1* gene ($n=6$); STZ and UTMD with plasmids encoding both *BTC* and *PDX1* ($n=6$). For all plasmid preparations, half of the genes contained an RIP promoter and the other half a CMV promoter. Microbubble or control solutions (0.5 ml diluted with 0.5 ml phosphate-buffered solution (PBS)) were infused over 20 min via pump (Genie, Kent Scientific, Torrington, CT, USA).

During the infusion, ultrasound was directed to the pancreas using a commercially available ultrasound transducer (S3, Sonos 5500, Philips Ultrasound, Bothell, WA, USA). The probe was clamped in place. Ultrasound was then applied in ultraharmonic mode (transmit 1.3 MHz/receive 3.6 MHz) at a mechanical index of 1.4. Four bursts of ultrasound were triggered to every fourth end-systole by electrocardiogram using a delay of 45–70 ms after the peak of the *R* wave. These settings have shown to be optimal for plasmid delivery by UTMD using this instrument.[28] Bubble destruction was visually apparent in all rats. After UTMD, the jugular vein was tied off, the skin closed, and the animals allowed to recover. Blood samples were drawn after an overnight 10-h fast at baseline, and at 3, 5, and 10 days after treatment. Animals were killed at day 10 using an overdose of sodium pentobarbital (120 mg/kg). Pancreas, liver, spleen, and kidney were harvested for histology and assessment of PDX1 and betacellulin proteins by Western blot. Blood glucose level was measured with blood glucose test strip (Precision, Abbott, Abbott Park, IL, USA); blood insulin, C-peptide, and glucagon level were measured with RIA kit from Linco Research, Radioimmunoassay, Billerica, MA, USA. A glucose tolerance test was performed by measuring fasting blood glucose, then giving 20% glucose solution (2 g/kg) orally, and then measuring blood glucose 30, 60, and 120 min later.

In a second protocol designed to assess time course of acinar cell transdifferentiation, six rats with STZ-induced diabetes were treated with UTMD using microbubbles containing both *PDX1* and *betacellulin* plasmids. Blood samples for glucose, insulin, C-peptide, and glucagon were obtained at days 5, 10, 15, 20, 25, and 30 after UTMD. Half the rats were killed at day 20 for histology (days 25 and 30 blood samples not obtained in these), and the other half killed at day 30.

Manufacture of plasmid-containing lipid-stabilized microbubbles

Lipid-stabilized microbubbles were prepared as previously described in our laboratory.[6] Briefly, 250 μl of DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, Sigma), 2.5 mg/ml and DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine, Sigma, St Louis, MO, USA) 0.5 mg/ml solution, and 50 μl of pure glycerol were added to 1.5 ml vials containing 2 mg of dried plasmid and mixed well and 10 μg of dexamathasone and incubated on room temperature for 30 min, the remaining headspace was filled with the perfluoropropane gas (Air Products, Inc., Allentown, PA, USA) and then mechanically shaken for 30 s by a dental amalgamator (VialmixTM, Bristol-Myers Squibb Medical Imaging, N. Billerica, MA, USA).The mean diameter and concentration of the microbubbles were measured by a particle counter (Beckman Coulter Multisizer III, Fullerton, CA, USA).

Plasmid constructs

RIP fragment (from −412 to +165; gene bank#:J00747) was PCR amplified from Sprague–Dawley DNA by using following PCR primers:

primer 1 (*Xho*I) 5′-CAACTCGAGGCTGAGCTAAGAA TCCAG-3′;
primer 2 (*Eco*RI) 5′-GCAGAATTCCTGCTTGCTGATGG TCTA-3′.

Following rat *PDX1* and *betacellulin* cDNA preparation: New born rat pancreatic samples were flash frozen in liquid nitrogen and stored at −86°C. The frozen samples were thawed in 1 ml of RNA-STAT solution and immediately homogenized using a polytron 3000 homogenizer at 10 000 r.p.m. for 30s × 2.Total RNA was prepared from the specimens using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA (30 ng) was reverse-transcribed in 20 μl by using a Sensiscript RT Kit (Qiagen) with oligo$(dT)_{16}$. The reaction mixture was incubated at 42°C for 50 min, followed by a further incubation at 70°C for 15 min. PCR was performed for all samples using a GeneAmp PCR System 9700 (PE ABI, Foster City, CA, USA) in 50 μl volume containing 2 μl cDNA, 25 μl of HotStarTaq Master Mix (Qiagen), and 20 pmol of each primer: rat pdx1 cDNA primer (from gene bank#NM022852): 5′-AAGAATTCCCATGAATAGT GAGGAGCA-3′ (sense); 5′-AAGCGGCCGC TCAGCCTG CGGTCCTCACC-3′ (antisense). Rat *betacellulin* cDNA primers (from Gene bank#NM022256): 5′-AAGAATTCCG GTTGATGGACTCGACT-3′(sense); 5′AAGCGGCCGCCA TTAAGTTAAGCAATAT(antisense).

The corresponding PCR products were purified by agarose gel electrophoresis and QIAquick Gel Extraction kit (Qiagen). PCR products were sequenced with dRhodamine Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) on an ABI 3100 Genomic Analyzer. RIP fragments were digested with *Xho*I and *Eco*RI and then ligated into the *Xho*I–*Eco*RI sites of pDsRed-Express-1 vector (BD Biosciences, San Jose, CA, USA). *PDX1* and *betacellulin* cDNA fragments digested with *Eco*R1 and *Not*1 and ligated into the *Eco*R1 and *Not*1 sites of RIP or CMV driving vectors and ligation reactions were carried out in 20 ml of 20 mM Tris-HCL, 0.5 mM ATP, 2 mM dithiothreitol, and 1 unit of T4 DNA ligase. Cloning and isolation of plasmids were performed by standard procedures.

Isolation of acinar cells and RT-PCR

Acinar cells were isolated as previously described.[8] In brief, 1 gram of rat pancreas tissue was placed in a 100 ml beaker with 20 ml RPMI-1640 medium containing

200 U/ml collagenase, 10 mM Hepes, 5% fetal bovine serum, penicillin 100 U/ml, streptomycin 50 μg/ml, and soybean trypsin inhibitor 0.2 mg/ml. It was cut into very small pieces with a scissor and transferred to a sterile flask, and incubated at 37°C with reciprocal shaking at 150 cycles/min for 40 min. Acinar cells suspensions were filtered with 100 μm mesh nylon gauze. The acinar cells were cultured with RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 4 mM streptozocin (depleted of residual β-cells) at 37°C, 5% CO_2 for 2 h. The cells were harvested and total RNA were extracted and reversed into cDNA pool. PCR was performed for all samples using a GeneAmp PCR System 9700 (PE ABI) in 50 μl volume containing 2 μl cDNA, 25 μl of HotStarTaq Master Mix (Qiagen), and 20 pmol of each prime. After an initial hold at 94°C (1 min), the PCR was carried out for 34 cycles (94°C for 30 s, 57°C for 30 s, and 72°C for 1 min). At the end, a single incubation at 72°C was added for 5 min. Amplification products were analyzed on 1.5% agarose gels. PCR products were confirmed by sequencing.

Immunohistochemistry

Cryostat sections 5 μm in thickness were fixed in 4% paraformaldehyde for 15 min at 4°C and quenched for 5 min with 10 mM glycine in PBS. Sections were then rinsed in PBS three times, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Sections were blocked with 10% goat serum and 10% bovine serum at 37°C for 1 h and washed with PBS three times. The primary antibody (anti-mouse insulin, 1:10 000 dilution from Sigma; anti-rabbit glucagon, 1:500 dilution, Chemicon, Temecula, CA, USA; anti-rabbit pdx1 and anti-rabbit betacellulin, 1:500 dilution, Chemicon Co.; anti-α amylase, 1:500 dilution, Abcam, Cambridge, MA, USA) was added and incubated at 4°C overnight. After washing with PBS three times for 5 min, the secondary antibody (anti-mouse immunoglobin G (lgG) conjugated with FITC, 1:250 dilution, Sigma Co., anti-rabbit lgG conjugated with Cy5, 1:250 dilution, Chemicon) was added and incubated for 1 h at 37°C. Sections were rinsed with PBS for 10 min, three times, and then mounted. Confocal microscopy was used to detect FITC signal (488 nm/510 nm) and Cy5 signal (633/710 nm).

Data analysis

Data was analyzed with Statview software (SAS, Cary, NC, USA). The results are expressed as mean $\pm$ one standard deviation. Differences were analyzed by repeated measures ANOVA with Fisher's *post hoc* test and considered significant at $P<0.05$.

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