Influence of diabetes on the loss of beta cell differentiation after islet transplantation in rats

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Abstract

Aims/hypothesis Hyperglycaemia can impair beta cell function after islet transplantation. Appropriate glucose-induced insulin secretion is dependent on a unique expression pattern of genes. Here we examined the effects of diabetes on gene expression in transplanted islets.

Materials and methods Streptozotocin-induced diabetic or control non-diabetic Lewis rats were transplanted under the kidney capsule with an insufficient number (2,000) of syngeneic islets to normalise blood glucose levels in diabetic rats. Eighteen days after transplantation, islet grafts were retrieved and RT-PCR used to assess expression of selected genes critical for beta cell function. Islet grafts from diabetic rats transplanted with a sufficient number of islets (3,000) to normalise hyperglycaemia were used to assess the effects of correcting blood glucose levels. Additionally, gene expression of transplanted islets from non-diabetic rats was compared with freshly isolated islets.

Results In islet grafts from diabetic rats, mRNA levels of several transcription factors important for the maintenance of beta cell differentiation were reduced (pancreatic and duodenal homeobox 1 [*Pdx1*], neurogenic differentiation 1 [*Neurod1*], NK6 transcription factor related, locus 1 [*Nkx6.1*], paired box gene 6 [*Pax6*]), as were genes implicated in beta cell function (*Glut2* [also known as solute carrier family 2 [facilitated glucose transporter], member 2 [*Slc2a2*], glucokinase, insulin, islet amyloid polypeptide [*Iapp*]). Conversely, mRNA levels of lactate dehydrogenase, which is normally suppressed in beta cells, were increased. The majority of the changes in gene expression were normalised after correction of hyperglycaemia, indicating that the severe loss of beta cell differentiation correlates with continuous exposure to diabetes. Even islet grafts from non-diabetic rats showed a few alterations in beta cell gene expression in comparison with fresh islets.

Conclusions/interpretation Chronic hyperglycaemia contributes to the deterioration of beta cell differentiation after islet transplantation.

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Abbreviations

cDNA complementary DNA
GIIS glucose-induced insulin secretion
NKX6.1 NK6 transcription factor related, locus 1

Introduction

A major defect in insulin secretory function is found in all forms of diabetes and in recipients of islet transplants [1–4].

The most striking defect is a selective loss of glucose-induced insulin secretion (GIIS), whereas responses to other secretory stimuli are less severely impaired [5–7]. Despite its fundamental importance to diabetes pathology, the underlying mechanism(s) for the loss of GIIS remain(s) unknown.

Recent progress in islet transplantation has demonstrated sustained C-peptide production and successful insulin independence in type 1 diabetic patients [8]. Large numbers of islets are needed because of extensive islet cell death in the peri-transplant period and functional impairment in part due to islets residing in an abnormal environment [9–13]. With time, islet function is lost again, such that most patients revert to the use of exogenous insulin within 2 years [14]. It is therefore essential to better understand islet function in the post transplantation period.

Sustained hyperglycaemia has a detrimental impact on the glycaemic outcome of islet transplantation and upon preservation of the graft [5, 7, 10, 15–17]. The usual outcome of islet transplantation in humans is glucose intolerance and the deterioration of glycaemic control over time [9, 12, 14], which may be due to the toxic effects of hyperglycaemia upon islet function, a phenomenon called glucotoxicity. We hypothesised that inadequate insulin secretion in an islet graft is caused by a loss of the unique expression pattern of genes responsible for maintaining GIIS. In this study, we examined the effects of hyperglycaemia on beta cell differentiation in transplanted islets. We also compared gene expression in islet grafts with that in freshly isolated islets.

Materials and methods

Animals and induction of diabetes Male inbred Lewis rats weighing 150 to 200 g were obtained from Taconic Farms (Germantown, NY, USA) and used both as islet donors and recipients. Animals were kept under conventional conditions with free access to water and standard pelleted food. All animal procedures were approved by the Joslin Diabetes Center Animal Care Committee. Graft recipients were either normal or diabetic rats. Rats were made diabetic by a single intraperitoneal dose of streptozotocin (65 mg/kg body weight, dissolved in citrate buffer pH 4.5; Sigma, St Louis, MO, USA). Before transplantation, diabetes was confirmed by the presence of hyperglycaemia (19.4 mmol/l) for at least 1 week. Blood was collected from the tail tip of rats to determine blood glucose levels measured with a portable glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA). To assess the effects of continuous exposure to diabetes on beta cell differentiation in transplanted islets, 2,000 islets (an insufficient number to reverse hyperglycaemia) were transplanted under the kidney

capsule of normal or diabetic rats. To assess the effects of reversing hyperglycaemia after islet transplantation, 3,000 islets were transplanted under the kidney capsule of diabetic rats and compared with those of normal or diabetic rats transplanted with 2,000 islets. To assess the effects of transplantation alone, islet grafts from normal (non-diabetic) rats were compared with freshly isolated islets. Rats were weighed and bled for blood glucose levels on days 7, 14 and 18 after transplantation. Eighteen days after transplantation, islet grafts were retrieved and prepared for analysis.

Islet isolation Islets were isolated from Lewis rats as previously described [12]. Briefly, the pancreatic duct was distended with 10 ml of M-199 medium (Gibco BRL, Grand Island, NY, USA) containing 1.5 mg of collagenase (type P; Boehringer Mannheim, Mannheim, Germany). The pancreas was dissected and incubated in a stationary water bath at 37°C. After digestion, the islets were separated on a density gradient (Histopaque-1077; Sigma, St Louis, MO, USA) and handpicked under a stereomicroscope. Islets were cultured for 24 h in RPMI containing 11.1 mmol/l glucose supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml) in standard humidified culture conditions of 5% CO₂ and 95% air at 37°C.

Transplantation Aliquots of either 2,000 or 3,000 islets were aspirated into pipette tips connected to a 1 ml Hamilton syringe (Hamilton Company, Reno, NV, USA) and then transferred into P-50 polyethylene tubing. Islets were transplanted under the kidney capsule of normal or diabetic recipients as described previously [10]. Grafts were retrieved 18 days after transplantation by excising the portion of the kidney capsule with the graft adhering to it. Using great care, the graft was then removed from the capsule, cut into small fragments and suspended in RNA isolation solution. To assess possible contamination of the grafts with renal parenchymal tissue, expression of the kidney-specific organic anion transporter, solute carrier family 22 (organic anion transporter), member 6 (*Slc22a6*) [18] was measured.

RNA extraction and synthesis of complementary DNA Total RNA was extracted from islet grafts and freshly isolated islets using Ultraspec RNA isolation reagent according to manufacturer-suggested protocols (Biotecx Laboratories, Houston, TX, USA). Following quantification by spectrophotometry, RNA (500 ng) was reverse-transcribed into complementary DNA (cDNA) in a final reaction solution of 25 µl containing the following: 1× Superscript first-strand buffer (50 mmol/l Tris-HCl, 75 mmol/l KCl and 3 mmol/l MgCl₂; Life Technologies, Grand Island, NY, USA), 40 U Rnasin (Promega, Madison, WI, USA), 10 mmol/l dithiothreitol, 1 mmol/l dNTP, 50 ng of random hexamers and 200 U of

Superscript II RNase H⁻ reverse transcriptase (Life Technologies). RT reactions were incubated for 10 min at 25°C, 60 min at 42°C and 10 min at 95°C. Resultant cDNA products were diluted with 50 µl H₂O to a concentration corresponding to 10 ng of starting RNA per 1.5 µl and were stored at -20°C.

Semiquantitative radioactive multiplex polymerase chain reaction Polymerase chain reactions (PCR) were carried out in a volume of 25 µl consisting of 10 to 20 ng of cDNA, 1× GeneAmp PCR Gold buffer (Applied Biosystems, Foster City, CA, USA), 1 to 1.5 mmol/l MgCl₂, 80 to 160 µmol/l dNTP, 100 to 400 nmol of oligonucleotide primers (Sigma Genosys, St Louis, MO, USA), 46.25 kBq of α-³²P-labelled dCTP (111 TBq/mmol; New England Nuclear, Boston, MA, USA) and 2.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems). For specific concentrations of MgCl₂, dNTP and oligonucleotide primers, along with multiplex PCR conditions for each gene tested, see Electronic supplementary material (ESM) Table 1. Reactions were performed in a 9700 Thermocycler (Applied Biosystems), in which samples underwent a 10 min initial denaturing step, followed by the number of cycles indicated (ESM Table 1) for durations of 1 min at 94°C, 1 min at the annealing temperature indicated in ESM Table 1 and 1 min at 72°C. The final extension step was 10 min at 72°C. In each reaction, gene products of interest

Table 1 Comparison of islet-associated transcription factor and beta cell function mRNA levels in islet grafts from normal and diabetic rats

	Normal	Diabetic
Characteristics		
Body weight (g)	284±12	267±13
Blood glucose (mmol/l)	5.7±0.1	18.3±0.8***
Transcription factors		
<i>Neurod1</i>	100±6	39±6***
<i>Pdx1</i>	100±29	39±4*
<i>Pax6</i>	100±25	28±6**
<i>Nkx6.1</i>	100±20	50±4*
Metabolism		
<i>Glut2</i>	100±9	64±9*
Glucokinase	100±16	50±8*
Hexokinase-1	100±27	95±17
<i>Ldha</i>	100±22	260±49*
Glucose-6-phosphatase	100±34	452±203
Ion channel		
<i>Kcnj11</i>	100±39	59±8
Hormones		
Insulin	100±16	16±3***
<i>Iapp</i>	100±2	81±4**

Values are means±SEM determined from three to six experiments and are expressed as a percentage of mRNA levels in islet grafts from normal rats

**p*<0.05 vs islet grafts from normal rats for each gene

***p*<0.01 vs islet grafts from normal rats for each gene

****p*<0.001 vs islet grafts from normal rats for each gene

were amplified with an internal control gene (cyclophilin A [*Ppia*], acidic ribosomal phosphoprotein P0 [*Arbp*], α-tubulin [*Tuba1*], TATA box binding protein [*Tbp*] or 18S rRNA) to correct for experimental variations between samples. Different control genes were used as required to match the amount of expression of the various tested genes. Specifically, *Ppia* was used for neurogenic differentiation 1 (*Neurod1*, also known as *Beta2* in other species), pancreatic and duodenal homeobox-1 (*Pdx1*), glucose transporter 2 (*Glut2*, also known as solute carrier family 2 [facilitated glucose transporter], member 2 [*Slc2a2*]), islet amyloid polypeptide (*Iapp*), glucagon and somatostatin; *Arbp* was used for paired box gene 6 (*Pax6*), NK6 transcription factor related, locus 1 (*Nkx6.1*) and the pore-forming subunit of the ATP-sensitive K⁺ channel (potassium inwardly-rectifying channel, subfamily J, member 11, *Kcnj11*; also known as *Kir6.2*); *Tuba1* was used for glucokinase; *Tbp* was used for hexokinase-1, lactate dehydrogenase A (*Ldha*) and glucose-6-phosphatase; and 18S rRNA was used for insulin. Amplimers were separated on 6% polyacrylamide gels in Tris–borate–EDTA buffer. The gel was dried and the amount of α-³²P-labelled dCTP incorporated into amplimers was measured with a Storm 840 PhosphoImager and quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The average intensity of each product was expressed relative to the internal control gene (ratio of specific product:control gene). These ratios were then expressed as a percentage of the ratio in control samples. Control experiments were performed to adjust the PCR conditions such that the number of cycles used was in the exponential phase of amplification for all products and that each PCR product in a multiplex reaction increased linearly with the amount of starting material.

Immunohistochemistry At 18 days after implantation, grafts were excised under anaesthesia, trimmed of extraneous tissue and fixed in 4% (wt/vol.) paraformaldehyde. After embedding in paraffin, 5 µm sections were stained for insulin (guinea pig anti-human insulin, 1:200; Linco Research, St Charles, MO, USA), GLUT2 (rabbit anti-rat GLUT2, 1:400; Chemicon, Temecula, CA, USA) and NKX6.1 (1:500; gift of P. Serup, Hagedorn, Gentofte, Denmark). The secondary antibodies used for immunofluorescence were: for insulin, Texas Red conjugated affinipure donkey anti-guinea pig IgG (1:400; Jackson ImmunoResearch, West Grove, PA, USA); and for GLUT2 and NKX6.1, donkey biotinylated anti-rabbit IgG (1:400; Jackson ImmunoResearch) followed by streptavidin-conjugated FITC (1:400; Jackson ImmunoResearch). Images of the grafts were taken with a ×25 objective with the same settings in the confocal mode of an LSM 410 microscope (Zeiss, Thornwood, NY, USA). Composite pictures of the grafts were composed in Adobe Photoshop.

Statistical analysis All results are presented as means $\pm$ SEM. Statistical analyses were performed using unpaired Student's *t* test or one-way ANOVA with post hoc test of Fisher's protected least significant difference.

Results

Changes in gene expression in islet grafts from diabetic rats We tested the effects of continuous diabetes exposure on beta cell differentiation in transplanted islets by analysing renal subcapsular grafts of 2,000 syngeneic islets from normal (non-diabetic) or streptozotocin-induced diabetic rats. Eighteen days after transplantation, body weights of diabetic rats did not differ from those of normal rats, whereas blood glucose levels were significantly increased (Table 1). The expression of specialised islet-associated transcription factors, islet hormones and beta cell metabolic enzymes, as well as of other normally suppressed metabolic enzymes was compared in islet grafts retrieved from normal and diabetic rats. After normalisation of the gene of interest to a control gene, mRNA levels in diabetic grafts were quantitated as a percentage of normal grafts (Table 1). In islet grafts from diabetic rats mRNA levels of *Pdx1*, *Neurod1* and *Nkx6.1* were reduced to 40% to 50% of normal and *Pax6* was reduced further. Additionally, in islet grafts from diabetic rats, *Glut2* and glucokinase, the enzyme responsible for the majority of beta cell glucose phosphorylation, were reduced to 64% and 50% of normal, respectively. Interestingly, *Ldha* and glucose-6-phosphatase, enzymes normally expressed at low levels in islets [19, 20], had significantly increased mRNA levels (*Ldha*) or a tendency to be increased (glucose-6-phosphatase; NS) in grafts from diabetic rats. However, hexokinase-1, which is upregulated in islets of other models of diabetes [19], was not altered in islet grafts from diabetic rats. The expression of a gene encoding an ion channel important for glucose-induced insulin release, *Kcnj11*, showed a tendency to have decreased mRNA levels in grafts from diabetic rats. Insulin was markedly downregulated with diabetes; mRNA levels of only 16% of normal denote the most severely reduced transcript of those tested (Table 1), while that of the other beta cell hormone, *Iapp*, was only modestly reduced to 81% of normal (Table 1). Thus, exposure of transplanted islets to a prolonged period of diabetes is associated with a widespread disruption of gene expression in the form of decreased expression of a panel of genes that optimise GIIS and induction of several genes that are normally suppressed.

Effect of reversing diabetes on changes in gene expression in transplanted islets We tested the reversibility of the changes in mRNA levels by analysing islet grafts from

diabetic rats transplanted with either a sufficient (3,000) or an insufficient (2,000) number of islets to normalise hyperglycaemia. Islets (2,000) transplanted into normal rats were used as control. The time course changes in blood glucose levels after transplantation are shown in Fig. 1. Blood glucose levels were high in diabetic recipients on the day of transplantation (Fig. 1). Blood glucose levels of the rats transplanted with 3,000 islets gradually fell after transplantation, but, in contrast, remained elevated in diabetic rats receiving 2,000 islets. Although body weights were slightly lower in diabetic and diabetic-normalised rats than in normal rats, the rats in each group gained weight at a similar rate over the study period (Fig. 2).

Eighteen days after transplantation, mRNA levels were analysed in islet grafts retrieved from normal (2,000 islets), diabetic (2,000 islets) and diabetic-normalised (3,000 islets) rats. Strikingly, the normalisation of diabetes restored several of the islet-associated transcription factors towards the expression levels apparent in islet grafts from normal rats. Thus *Neurod1* (Fig. 3a), *Pax6* (Fig. 3b) and *Nkx6.1* (Fig. 3c) mRNA levels were reduced in diabetic rats, but restored towards normal in diabetic-normalised rats. In contrast, *Pdx1* mRNA levels (Fig. 3d) remained decreased in islet grafts from diabetic-normalised rats.

As shown in Fig. 4, the normalisation of diabetes at least partially reversed the changes in expression of glucose metabolism enzymes; reduced mRNA levels for *Glut2* (Fig. 4a) and glucokinase (Fig. 4b) in islet grafts from diabetic rats were partially restored in diabetic-normalised rats. Downregulation of *Glut2* has been previously identified in transplanted mouse islets [6] and other models of diabetes [19, 21, 22]; here we show that exposure to diabetes clearly contributes to its reduced expression in

Fig. 1 Time-course changes in blood glucose levels after transplantation. Blood glucose levels were measured on days 0, 7, 14 and 18 after transplantation. Normal rats were transplanted with 2,000 islets ($n=4$, squares) and diabetic rats with either 2,000 islets ($n=6$, open circles) or 3,000 islets ($n=6$, closed circles). Results are means $\pm$ SEM. * $p<0.05$, *** $p<0.001$ vs normal at the same time point

Fig. 2 Time-course changes in body weight after transplantation. Body weights were measured on days 7, 14 and 18 after transplantation. Normal rats were transplanted with 2,000 islets ($n=4$, squares) and diabetic rats with either 2,000 islets ($n=6$, open circles) or 3,000 islets ($n=6$, closed circles). Results are means $\pm$ SEM

transplanted rat islets. The mRNA levels of the K^+ channel subunit *Kcnj11* were completely restored by reversing diabetes (Fig. 4c). Furthermore, there was a tendency for the increased mRNA levels of *Ldha* to be partially reversed in diabetic-normalised rats (Fig. 4d). In contrast, glucose-6-phosphatase mRNA levels were not reversed in islet grafts from diabetic-normalised rats (Fig. 4e).

Fig. 3 Reversibility of changes in transcription factor mRNA levels in transplanted islets. mRNA levels were compared by semiquantitative multiplex RT-PCR in islet grafts from normal ($n=4$), diabetic ($n=6$) and diabetic-normalised rats ($n=6$). mRNA levels are shown for the islet-associated transcription factors *Neurod1* (a), *Pax6* (b), *Nkx6.1* (c) and *Pdx1* (d). After normalisation of the specific gene to a control gene, mRNA levels are expressed as a percentage of islet grafts from normal rats. Values are means $\pm$ SEM. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs islet grafts from normal rats for each gene

Fig. 4 Reversibility of changes in glucose metabolism and ion channel mRNA levels in transplanted islets. mRNA levels were compared as in Fig. 3 and are shown for *Glut2* (a), glucokinase (*Gk*) (b), the K^+ channel subunit *Kcnj11* (c), *Ldha* (d), glucose-6-phosphatase (*G6pc*) (e) and hexokinase (*Hk1*) (f). After normalisation of the specific gene to a control gene, mRNA levels are expressed as a percentage of islet grafts from normal rats. Values are means $\pm$ SEM. * $p<0.05$ vs islet grafts from normal rats for each gene

As shown in Fig. 5a, insulin mRNA levels were partially reversed in islet grafts from diabetic-normalised rats, strongly suggesting an influence of the diabetic environment on the marked downregulation of insulin in transplanted islets. Similarly, *Iapp* mRNA levels were reversed in islet grafts from diabetic-normalised rats (Fig. 5b). Glucagon (Fig. 5c) and somatostatin (Fig. 5d) mRNA levels were not different in the grafts from normal, diabetic and diabetic-normalised groups, suggesting that hyperglycaemia specifically altered gene expression of the beta cell. Interestingly, *Myc* (myelocytomatosis viral oncogene homologue [avian], also known as *c-myc*) mRNA abundance, a molecular marker of altered beta cell proliferation and/or apoptosis, was not different in the grafts from normal, diabetic and diabetic-normalised groups (data not shown). Furthermore, based on our previous studies [10], we do not expect variations in cell death among the groups at this time point, although this was not assessed in the present study. The results of this study were not due to variable contamination with kidney tissue in the excised

Fig. 5 Reversibility of changes in islet hormone mRNA levels in transplanted islets. mRNA levels were compared as in Fig. 3 and are shown for insulin (**a**), *Iapp* (**b**), glucagon (*Gcg*) (**c**) and somatostatin (*Sst*) (**d**). After normalisation of the specific gene to a control gene, mRNA levels are expressed as a percentage of islet grafts from normal rats. Values are means $\pm$ SEM. *** p <0.001 vs islet grafts from normal rats for each gene

grafts. Expression of the kidney specific marker, *Slc22a6*, was used to determine the percentage of kidney contamination in each islet graft. *Slc22a6* mRNA levels were low and not significantly different among the groups ($0.8\pm 0.4\%$, $1.3\pm 0.5\%$ and $1.3\pm 0.6\%$ kidney in islet grafts from normal, diabetic and diabetic-normalised rats, respectively).

Sections of 18-day-old islet grafts from diabetic (Fig. 6a) and diabetic-normalised (Fig. 6b) rats were stained for insulin. Immunostaining for insulin was clearly more

Fig. 6 Islet grafts show variable intensities of insulin immunostaining. Grafts from rats exposed to continuous diabetes have partially degranulated beta cells (**a**) as seen by the decreased intensity of the insulin (Texas red) immunostaining, which is far weaker than that in grafts after restoration of normoglycaemia (**b**). Magnification bar=100 μ m

intense in the beta cells of the graft retrieved after restoration of normoglycaemia (Fig. 6b) than in the beta cells that were continuously exposed to diabetes (Fig. 6a). Similarly, immunostaining for GLUT2 was present in the beta cells of the graft retrieved after restoration of normoglycaemia (Fig. 7a,c), compared with minimal staining in the beta cells that were continuously exposed to diabetes (Fig. 7b,d). In addition, typical nuclear NKX6.1 staining was visible in the beta cells of the graft retrieved from diabetic-normalised rats (Fig. 8a), but was virtually absent in beta cells of diabetic rats (Fig. 8b).

Comparison of islet-associated transcription factor and beta cell function mRNA levels between fresh islets and islet grafts RT-PCR analysis was performed to compare gene expression between fresh isolated islets and 18-day-old islet grafts retrieved from under the kidney capsule of normal rats. Expression of specific genes was normalised to their internal control gene and quantified as a percentage of those from fresh islets (Table 2). In comparison with fresh islets, islet grafts from normal rats exhibited markedly decreased mRNA levels of the transcription factor *Pax6* (p <0.05) and a tendency for *Neurod1* and *Nkx6.1* to be reduced (NS). In contrast, *Pdx1* mRNA levels were not different between

Fig. 7 Islet grafts show variable intensities of GLUT2 immunostaining. Immunostaining for GLUT2 (green) (**a–d**) is shown alone (**c**, **d**) or with insulin (red) (**a**, **b**). Grafts retrieved after restoration of normoglycaemia (**a**, **c**) show bright GLUT2 staining, typical of plasma membranes. In comparison, GLUT2 staining was markedly diminished in grafts exposed to continuous diabetes (**b**, **d**). Magnification bar=50 μ m

Fig. 8 NKX6.1 staining (green) is typically nuclear in beta cells of the graft retrieved from diabetic-normalised rats (**a**) compared with an absence of staining in beta cells from diabetic rats (**b**). Red, insulin staining. Magnification bar=50 µm

fresh islets and transplanted islets (Table 2). Of the glucose metabolism enzymes tested, *Glut2* and glucokinase had significantly reduced mRNA levels or a tendency (NS) for mRNA levels to be reduced respectively in islet grafts compared with fresh islets. In contrast, *Ldha* mRNA levels were slightly increased in islet grafts, although non-significantly. Hexokinase-1 and glucose-6-phosphatase mRNA levels were not different between fresh islets and transplanted islets. mRNA levels of the K⁺ channel subunit *Kcnj11* were markedly reduced in islet grafts. Furthermore, insulin ($p=0.06$) and *Iapp* mRNA levels were reduced in islet grafts compared with freshly isolated islets. These data show that in comparison with fresh islets, islets transplanted

Table 2 Comparison of islet-associated transcription factor and beta cell function mRNA levels in fresh islets and islet grafts from normal rats

	Fresh islets	Islet grafts
Transcription factors		
<i>Neurod1</i>	100±28	68±4
<i>Pdx1</i>	100±17	110±37
<i>Pax6</i>	100±20	34±8*
<i>Nkx6.1</i>	100±18	66±21
Metabolism		
<i>Glut2</i>	100±4	67±6**
Glucokinase	100±15	67±11
Hexokinase-1	100±21	97±26
<i>Ldha</i>	100±19	166±36
Glucose-6-phosphatase	100±42	124±42
Ion channel		
<i>Kcnj11</i>	100±6	31±12**
Hormones		
Insulin	100±11	62±10
<i>Iapp</i>	100±4	74±5*
Glucagon	100±15	18±8**
Somatostatin	100±23	18±6*

Values are means±SEM determined from three to four experiments and are expressed as a percentage of mRNA levels in fresh islets

* $p<0.05$ vs isolated islets for each gene

** $p<0.01$ vs isolated islets for each gene

into a normoglycaemic environment display a few significant alterations of beta cell gene expression. Interestingly, both glucagon and somatostatin mRNA levels were markedly reduced in normal grafts compared with fresh islets (Table 2). These findings are consistent with data showing that transplanted rat islets lose many of their non-beta cells within 2 to 3 weeks post transplantation (King et al., unpublished data).

Discussion

Using an islet transplantation model, we show that exposure of beta cells to diabetes is associated with a widespread disruption of gene expression in the form of decreased expression of a panel of genes that optimise GIIS and induction of several genes that are normally suppressed. We also found that islets transplanted into a normoglycaemic environment show some alterations in the expression of genes involved in insulin secretion when compared with fresh islets, whereas a marked deterioration in graft beta cell differentiation was associated with chronic exposure to hyperglycaemia. These data support the hypothesis that hyperglycaemia in the post islet transplantation period leads to a loss of the unique expression pattern of genes responsible for maintaining GIIS, thus adversely influencing transplant outcomes.

The disruption of beta cell gene expression in islet grafts continuously exposed to hyperglycaemia can be correlated with a loss of GIIS from the graft-bearing kidney [6, 7]. Furthermore, the restoration of beta cell gene expression in islet grafts after normalisation of glucose levels correlates with normal biphasic GIIS [6, 7]. In several animal models of hyperglycaemia and type 2 diabetes, such as the *db/db* mouse, Zucker diabetic fatty rat and partially pancreatectomised (Px) rat, studies have found a similar phenomenon of loss of differentiation associated with beta cell dysfunction [19, 21, 23, 24]. Common to these models is a decreased expression of genes important for glucose-stimulated insulin secretion, beta cell development and the regulation of beta cell gene expression. mRNA levels of several transcription factors important for the maintenance of beta cell differentiation (*Pdx1*, *Neurod1*, *Nkx6.1* and *Pax6*) were reduced in transplanted islets in the present study and in endogenous islets from rodent diabetes models [19, 23, 24]. The decreased expression of these islet transcription factors could contribute to the parallel down-regulation of genes essential for GIIS, such as *Glut2*, glucokinase, the K⁺ channel subunit *Kcnj11* and insulin [25–27]. Interestingly, in transplanted islets from diabetic rats, insulin was more severely decreased than the other genes tested (Table 1). This contrasts with findings in *db/db*

mice [24] and Px rats [19, 23], in which insulin mRNA levels were preserved or decreased to a lesser degree, compared with expression changes of numerous glucose-sensing genes and islet transcription factors.

The majority of the gene expression changes in islet grafts from diabetic rats were completely, or at least partially, prevented (or reversed) in transplanted islets from rats in which diabetes was cured. The notable exception was the expression of *Pdx1*, which remained largely suppressed, a finding that could be due to effects of the initial period of hyperglycaemia after transplantation. The delay in normalisation of blood glucose levels may have been due to the time required for vascularisation and recovery from hypoxia and nutrient deprivation [28–30]. In addition, minor residual hyperglycaemia at the time of graft retrieval could have an influence; even minimal levels of hyperglycaemia can have a dramatic impact on beta cell gene expression in the long term [23].

The loss of function of an islet graft is a major obstacle to the success of islet transplantation. We have shown that transplanted islets display an alteration in normal expression of several genes necessary for optimal GIIS. Some alterations were even found in successful transplants, but they were far more severe in grafts exposed to hyperglycaemia. This deterioration in beta cell differentiation may be a major factor in the impaired insulin secretion that accompanies poor transplant outcomes. Thus, hyperglycaemia may produce a dangerous spiral of deficient insulin secretion that leads to even higher glucose levels that can then produce even worse secretion and perhaps even accelerate beta cell death. An understanding of these molecular events should help us develop strategies that could improve transplant outcomes, such as a more aggressive maintenance of normoglycaemia in the peri-transplant period.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. Brunzell JD, Robertson RP, Lerner RL et al (1976) Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests. *J Clin Endocrinol Metab* 42:222–229
2. Porte D Jr (1991) Beta-cells in type II diabetes mellitus. *Diabetes* 40:166–180
3. McCulloch DK, Klaff LJ, Kahn SE et al (1990) Nonprogression of subclinical beta-cell dysfunction among first-degree relatives of IDDM patients. 5-yr follow-up of the Seattle Family Study. *Diabetes* 39:549–556
4. Eisenbarth GS (1986) Type I diabetes mellitus. A chronic autoimmune disease. *N Engl J Med* 314:1360–1368
5. Gray DW, Cranston D, McShane P, Sutton R, Morris PJ (1989) The effect of hyperglycaemia on pancreatic islets transplanted into rats beneath the kidney capsule. *Diabetologia* 32:663–667
6. Ogawa Y, Noma Y, Davalli AM et al (1995) Loss of glucose-induced insulin secretion and GLUT2 expression in transplanted beta-cells. *Diabetes* 44:75–79
7. Korsgren O, Jansson L, Sandler S, Andersson A (1990) Hyperglycemia-induced B cell toxicity. The fate of pancreatic islets transplanted into diabetic mice is dependent on their genetic background. *J Clin Invest* 86:2161–2168
8. Shapiro AM, Lakey JR, Ryan EA et al (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238
9. Alejandro R, Cutfield RG, Shienvold FL et al (1986) Natural history of intrahepatic canine islet cell autografts. *J Clin Invest* 78:1339–1348
10. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC (1996) Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. *Diabetes* 45:1161–1167
11. Mattsson G, Jansson L, Nordin A, Andersson A, Carlsson PO (2004) Evidence of functional impairment of syngeneically transplanted mouse pancreatic islets retrieved from the liver. *Diabetes* 53:948–954
12. Montana E, Bonner-Weir S, Weir GC (1993) Beta cell mass and growth after syngeneic islet cell transplantation in normal and streptozotocin diabetic C57BL/6 mice. *J Clin Invest* 91:780–787
13. Shi CL, Taljedal IB (1996) Dynamics of glucose-induced insulin release from mouse islets transplanted under the kidney capsule. *Transplantation* 62:1312–1318
14. Ryan EA, Paty BW, Senior PA et al (2005) Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060–2069
15. Juang JH, Bonner-Weir S, Wu YJ, Weir GC (1994) Beneficial influence of glycemic control upon the growth and function of transplanted islets. *Diabetes* 43:1334–1339
16. Ar'Rajab A, Ahren B (1992) Prevention of hyperglycemia improves the long-term result of islet transplantation in streptozotocin-diabetic rats. *Pancreas* 7:435–442
17. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E (2002) Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. *Diabetes* 51:66–72
18. Lopez-Nieto CE, You G, Bush KT, Barros EJ, Beier DR, Nigam SK (1997) Molecular cloning and characterization of NKT, a gene product related to the organic cation transporter family that is almost exclusively expressed in the kidney. *J Biol Chem* 272: 6471–6478
19. Jonas JC, Sharma A, Hasenkamp W et al (1999) Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. *J Biol Chem* 274:14112–14121
20. Laybutt DR, Sharma A, Sgroi DC, Gaudet J, Bonner-Weir S, Weir GC (2002) Genetic regulation of metabolic pathways in beta-cells disrupted by hyperglycemia. *J Biol Chem* 277:10912–10921
21. Tokuyama Y, Sturis J, DePaoli AM et al (1995) Evolution of beta-cell dysfunction in the male Zucker diabetic fatty rat. *Diabetes* 44:1447–1457
22. Thorens B, Wu YJ, Leahy JL, Weir GC (1992) The loss of GLUT2 expression by glucose-unresponsive beta cells of db/db

- mice is reversible and is induced by the diabetic environment. *J Clin Invest* 90:77–85
23. Laybutt DR, Glandt M, Xu G et al (2003) Critical reduction in beta-cell mass results in two distinct outcomes over time. Adaptation with impaired glucose tolerance or decompensated diabetes. *J Biol Chem* 278:2997–3005
 24. Kjørholt C, Akerfeldt MC, Biden TJ, Laybutt DR (2005) Chronic hyperglycemia, independent of plasma lipid levels, is sufficient for the loss of beta-cell differentiation and secretory function in the db/db mouse model of diabetes. *Diabetes* 54:2755–2763
 25. Huang HP, Tsai MJ (2000) Transcription factors involved in pancreatic islet development. *J Biomed Sci* 7:27–34
 26. Schwitzgebel VM, Scheel DW, Connors JR et al (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* 127:3533–3542
 27. Servitja JM, Ferrer J (2004) Transcriptional networks controlling pancreatic development and beta cell function. *Diabetologia* 47:597–613
 28. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K (1989) Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 38 (Suppl 1):199–201
 29. Vasir B, Reitz P, Xu G, Sharma A, Bonner-Weir S, Weir GC (2000) Effects of diabetes and hypoxia on gene markers of angiogenesis (HGF, cMET, uPA and uPAR, TGF- α , TGF- β , bFGF and vimentin) in cultured and transplanted rat islets. *Diabetologia* 43:763–772
 30. Dionne KE, Colton CK, Yarmush ML (1993) Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. *Diabetes* 42:12–21