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**PARTIAL PANCREATIC TRANSDIFFERENTIATION OF PRIMARY HUMAN
HEPATOCYTES IN THE LIVERS OF A HUMANISED MOUSE MODEL**

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Running Title: Pancreatic differentiation of human hepatocytes

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Keywords: diabetes, liver, gene – expression, gene – therapy, immunohistochemistry,
vector – viral.

Abstract:

Background: Gene therapy is one treatment that may ultimately cure Type 1 diabetes. We have previously shown that introduction of furin-cleavable human insulin (INS-FUR) to the livers in several animal models of diabetes has resulted in reversal of diabetes and partial pancreatic transdifferentiation of liver cells. This study assessed if streptozotocin (STZ)-diabetes could be reversed in FRG mice in which chimeric mouse-human livers can be established, and if pancreatic transdifferentiation occurred in the engrafted human hepatocytes.

Methods: Engraftment of human hepatocytes was verified by measuring human albumin levels. Following delivery of the empty vector or the INS-FUR vector to diabetic FRG mice, mice were monitored for weight and blood glucose levels. Intraperitoneal glucose tolerance tests (IPGTTs) were performed. Expression levels of pancreatic hormones and transcription factors were determined by reverse transcriptase PCR (RT-PCR) and immunohistochemistry.

Results: Diabetes was reversed for a period of 60 days (experimental endpoint) after transduction with INS-FUR. IPGTTs of the insulin-transduced animals were not significantly different from nondiabetic animals. Immunofluorescence microscopy revealed expression of human albumin and insulin in transduced liver samples. Quantitative RT-PCR showed expression of human and mouse endocrine hormones and β -cell transcription factors, indicating partial pancreatic transdifferentiation of mouse and human hepatocytes. Non-fasting human C-peptide levels were significantly higher than mouse levels, suggesting that transdifferentiated human hepatocytes made a significant contribution to diabetes reversal.

Conclusions: These data show that human hepatocytes can be induced to undergo partial pancreatic transdifferentiation *in vivo*, indicating that the technology holds promise for treatment of Type 1 diabetes.

INTRODUCTION

Type I diabetes (T1D) is caused by the autoimmune destruction of pancreatic beta (β)-cells¹. Current treatment requires multiple daily injections of insulin to control blood glucose levels. Gene therapy is one strategy that holds promise to cure the disease. Of the accessible primary cells available, our laboratory, and others, have shown that hepatocytes are suitable target cells for the genetic engineering of artificial β -cells²⁻¹³. Most importantly, liver cells express the high capacity glucose transporter, GLUT 2¹⁴, and the high capacity phosphorylation enzyme, glucokinase¹⁵, which constitute the key elements of the “glucose sensing system” that regulates insulin release from pancreatic β -cells in response to small extracellular nutrient changes. Studies have also shown that these insulin-secreting liver cells are resistant to the detrimental effects of classical β -cell cytotoxins and pro-inflammatory cytokines that play principal roles in the autoimmune process of diabetes^{10, 16-18}. Additionally, no infiltrates of immune cells were found in nonobese diabetic (NOD) mice engineered to express insulin in their livers^{10, 17}. Thus, liver cells are attractive candidates for the creation of artificial β -cells to cure diabetes.

In order to obtain high transduction rates in diabetic animal livers using a lentiviral vector, we developed a microsurgical procedure of intervallic infusion in full flow occlusion (FFO), which involves clamping the major veins and arteries to stop blood flow to the liver whilst the vector is injected into the portal circulation. This allowed the vector, containing the insulin gene, to remain isolated within the liver, with resultant high transduction rates [60% in streptozotocin (STZ)-diabetic rats⁹ and 42% in spontaneously-diabetic NOD mice¹⁰]. Using this technique, similar results were seen in a pancreatectomised pig model¹¹. Diabetes was reversed in these animal models, with accompanying normal glucose tolerance and partial endocrine pancreatic transdifferentiation of the liver resulting from the expression of several β -cell transcription factors. Indeed, it is the normal glucose tolerance displayed by these animals that sets our studies using the FFO technique apart from other regimes that have expressed insulin alone in the liver with no pancreatic transdifferentiation^{10, 13}. Attempts to induce pancreatic transdifferentiation using delivery of pancreatic transcription factors (notably *Pdx1*) have resulted in concomitant exocrine transdifferentiation and significant tissue damage^{5, 7}. To apply this form of gene therapy to the clinical situation we must be able to show that primary human hepatocytes can undergo transduction with the insulin gene, and that these hepatocytes can undergo pancreatic transdifferentiation, as seen in livers of the animal models studied to date⁹⁻¹¹.

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3 In the current study, we have utilised the FRG mouse model in which chimeric mouse-
4 human livers can be readily established^{19, 20}. FRG mice have Tyrosinaemia type I (Fah^{-/-}) and
5 lack both humoral and cell-mediated immunity as a consequence of *Rag2* and *Ii2rg*^{-/-}
6 deficiency (*Rag2*^{-/-}/*Ii2rg*^{-/-}). The tyrosinaemic phenotype leads to the accumulation of
7 hepatotoxic metabolites in host mouse hepatocytes, such that healthy human hepatocytes
8 have an engraftment and growth advantage. We have used the FFO technique to deliver
9 furin-cleavable human insulin (INS-FUR)²¹, via a lentiviral vector (HMD)²², to the livers of
10 STZ-diabetic FRG mice. This has resulted in reversal of diabetes, restoration of glucose
11 tolerance, and partial endocrine pancreatic transdifferentiation of both mouse and human
12 hepatocytes *in vivo*.
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For Peer Review

Materials and Methods

Vector production

The HIV/ MSCV (HMD)/ INS-FUR lentiviral vector, which expresses both INS-FUR and enhanced green fluorescent protein (EGFP), was constructed as previously described⁹. The HMD lentiviral vector²² has a HIV/ murine stem cell virus hybrid long terminal repeat as the promoter and an internal ribosomal entry site, allowing bicistronic gene expression of both EGFP and INS-FUR. The vectors were produced by $\text{Ca}_3(\text{PO}_4)_2$ precipitation in 293T cells using conditioned medium, as described previously⁹. Culture medium was harvested 48h after transfection and subjected to tangential flow filtration⁹, followed by pelleting of the vector (75 000g/ 2 h). Virus titre was determined by transducing 293T cells (5×10^5) with serially diluted vector stocks and quantifying numbers of EGFP-positive cells by flow cytometry, as previously described²³. Viral replication-competency was also assessed by a p24 ELISA kit (CVPK-107, Cell Biolabs, San Diego, CA), following the manufacturer's instructions.

Transduction of Liver Tissue

Male and female, FRG mice were obtained from the Children's Hospital, Westmead where they had been bred following approval from the Westmead Hospital Animal Care and Ethics Committee (ACEC) (Approval No. C268). Maintenance and experimental manipulations were performed in accordance with the National Health and Medical Research Council of Australia principles of laboratory care and the relevant regulations of the Australian Research Council and were approved by the University of Technology Sydney (UTS) ACEC (Approval No. 2011-035A). Twenty-four hours prior to engraftment of human hepatocytes, 8-11 week old FRG mice received an intravenous injection of 6.4×10^9 plaque forming units of human urokinase adenoviral vector. Each animal received 1×10^6 human hepatocytes (Lonza, Basel, Switzerland) via intrasplenic injection. Cycles of (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC, Yechuris, Tualatin, OR, USA) were initiated 48 h after engraftment, as previously described¹⁹. NTBC cycling was continued until initiation of diabetes.

Liver repopulation was monitored by a human albumin ELISA kit and goat anti-human albumin HRP antibody (Bethyl Laboratories, Montgomery, TX, USA), as described^{19, 24}. It has been shown that the level of humanisation of the liver roughly correlates with human albumin levels in the blood, where a concentration of 1 mg/ml corresponds to ~20% human hepatocytes²⁴. Engrafted hepatocytes were allowed to expand for ~150 days before experimentation. Livers of animals used for further experimentation had approximately 50--62% human hepatocytes, based on albumin measurements. Diabetes was induced by a

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3 single intraperitoneal injection of STZ (170 mg/kg). Mice that were assessed as diabetic
4 (blood glucose >12mmol/L on 3 separate occasions) were used for experimentation.
5 Animals were randomly allocated into groups of six and HMD/INS-FUR (INS-FUR carrying
6 HMD vector) and HMD (empty vector) (3.5×10^8 transduction units [TDU]) were delivered to
7 the liver via the portal circulation, by FFO, as previously described^{9, 10}.
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10 11 12 **Functional Analyses**

13 After treatment, animals were monitored for body weight and blood glucose concentrations.
14 IPGTTs (2g glucose/ kg after a 6 h fast) were carried out on the treated animals at 60 days after
15 transduction together with diabetic and normal/nondiabetic controls (n=6).
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19 Non-fasting blood samples were collected from: HMD/INS-FUR-transduced FRG mice, non-
20 diabetic FRG mice engrafted with human hepatocytes, and untransduced diabetic FRG mice
21 engrafted with human hepatocytes. The levels of mouse and human C-peptide in the sera of
22 mice were measured using a human C-peptide ELISA kit (E7HCP-20K) (Merck Millipore,
23 Bayswater, VIC, Australia), and rat/mouse C-peptide ELISA kit (EZRMCP2-21K) (Merck
24 Millipore). The insulin content of the liver and pancreas were measured following acid-
25 ethanol extraction². Levels of human and mouse insulin were measured using the EZ-14K
26 (Merck Millipore) and the 90080 kit (Crystal Chem, Downer's Grove, IL), respectively.
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32 To assess liver function, serum levels of alanine transaminase (ALT, Abcam, Cambridge,
33 MA, ab105134) and aspartate aminotransferase (AST, Abcam 105135) were sampled at 2
34 and 10 days following the surgical delivery of INS-FUR and at sacrifice. As the model is quite
35 fragile, it is not possible to take more frequent blood samples.
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40 41 42 **Immunofluorescence Microscopic Analyses**

43 For immunofluorescence studies, serial frozen sections (15 μm) of liver and pancreas were
44 cut from all animals in the experimental groups. For triple staining of insulin, glucagon and
45 somatostatin, the primary antibodies were mouse anti-human insulin (1:100, BioGenex,
46 Fremont, CA), goat anti-glucagon (1:200 Santa Cruz Biotechnology, Dallas, TX), and goat
47 anti-somatostatin (1:200, Santa Cruz). The secondary antibodies were anti-mouse IgG and
48 anti-goat IgG (1:200, Vector Laboratories, Burlingame, CA). The fluorochromes were
49 fluorescein avidin D, AMCA avidin D, and rhodamine avidin D (1:200, Vector Laboratories).
50 For double staining of insulin and PDX1, the antibodies were mouse anti-insulin (1:100,
51 BioGenex) and goat anti-PDX1 (1:100, Santa Cruz). The secondary antibodies used were
52 anti-mouse IgG and anti-goat IgG (1:200, Vector Laboratories). The fluorochromes were
53 fluorescein avidin D and rhodamine avidin D (1:200, Vector Laboratories). The staining
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3 procedure followed the fluorescein M.O.M kit (FMK–220, Vector Laboratories,). Between
4 application of the primary antibodies against insulin and glucagon, glucagon and
5 somatostatin and insulin and PDX1, the avidin/ biotin blocking kit (sp-200, Vector
6 Laboratories) was used, following the manufacturer's instructions. Mounting medium
7 containing DAPI (Vector Laboratories) was used to visualise nuclei.
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11 For double staining of insulin and EGFP, insulin and human albumin, or FAH and human
12 albumin, the liver sections were incubated for 1 h with antibodies against EGFP (1:100
13 Santa Cruz), mouse anti-human insulin (1:100 BioGenex, CA and goat anti-human albumin
14 (Abcam, Cambridge, MA), or mouse anti-human FAH (1:100, Sapphire Bioscience, Redfern,
15 NSW, Australia) and goat anti-human albumin (Abcam), diluted 1:100 in 10% v/v donkey
16 serum at room temperature (RT). The secondary antibodies were then incubated for 1 h at
17 RT with FITC-conjugated donkey anti-mouse IgG (for insulin or FAH), or rhodamine red
18 conjugated donkey anti-goat IgG (Jackson ImmunoResearch, Westgrove, PA) for human
19 albumin or EGFP, diluted 1:100 in 10%v/v donkey serum for 1h at RT. Sections were
20 mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Ten sections
21 were analysed from each animal for immunofluorescent studies. Digital images were
22 acquired on a fluorescent microscope (Olympus BX60, Olympus Imaging, Macquarie Park,
23 NSW, Australia) and captured with an Olympus camera. To count the number of double or
24 triple positive-labelled cells on the acquired images, 6 random fields per section were
25 analysed using the regions of interest analysis available in the Image Pro plus 6.2 software
26 (Media Cybernetics, Inc, Rockville, MD). Data were analysed using Excel and a two-tailed
27 Student's t-test was used to evaluate the results. Representative images are shown in the
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40 **Reverse transcriptase-PCR analyses**

41 For RT-PCR analysis, liver, pancreas and spleen were harvested at 60 days after vector
42 infusion and snap-frozen in liquid nitrogen. Control pancreas was also obtained from non-
43 diabetic FRG mice. Expression levels of transgenic insulin were examined in all tissues.
44 Total RNA was extracted using Trizol (Invitrogen/ ThermoFisher Scientific, Waltman, MA),
45 and samples were treated with DNase I (Invitrogen/ ThermoFisher Scientific) prior to RT-
46 PCR analyses. Expression of human and mouse insulin, other pancreatic hormones
47 (glucagon, somatostatin and pancreatic polypeptide), β -cell transcription factors, and the
48 exocrine marker, p48, were examined in liver and pancreas from untreated/nondiabetic FRG
49 mice, and HMD/ INS-FUR-treated STZ-diabetic FRG mice (at 60 days, experimental
50 endpoint). Primer concentrations and other PCR parameters were optimised for each primer
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3 set using DNA derived from normal control animals. All primers used in the study were
4 species specific.
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7 Real-time quantitative RT-PCR (RT-qPCR) using SYBR GreenER RT-qPCR Supermix
8 (Invitrogen/ ThermoFisher Scientific) and a real-time detection system (Eppendorf
9 Realplex2, Eppendorf South Pacific Pty Ltd, North Ryde, NSW, Australia) was used to
10 determine the likelihood that both human and mouse cells contributed to the pancreatic
11 transdifferentiation of the FRG livers by assessing the expression levels of a number of β -
12 cell transcription factors and pancreatic hormones. RT-qPCR reactions contained either
13 primers specific to the gene of interest, or the reference gene, β -actin. RT-PCR/ RT-qPCR
14 primer sequences are available (Table S1). Melt curve analysis was performed to ascertain
15 primer specificity. Pre-optimised TaqMan® Gene expression Assays (Applied Biosystems/
16 ThermoFisher Scientific, Waltman, MA) were also used for the quantitation of the expression
17 of mouse *Pdx1*, *NeuroD1*, *Pax4*, *Ngn3*, *Nkx6.1*, and *MafA*. TaqMan® gene expression
18 assays identification numbers are available (Table S2). TaqMan assays for the reference
19 gene, mouse β -actin, were also obtained and used for the quantitation of expression levels
20 of the genes of interest. Taqman RT-qPCR assays were performed using the TaqMan® Fast
21 Advance Master Mix (Applied Biosystems/ ThermoFisher Scientific), according to the
22 manufacturer's protocol. The reactions were performed using the QuantStudio™ 6 Flex Real
23 Time PCR System (ThermoFisher Scientific). Data was represented using the $2^{-\Delta\Delta t}$ method
24 where $\Delta\Delta Ct = (C_{t, Target} - C_{t, Reference})_{control\ tissue} - (C_{t, Target} - C_{t, Reference})_{sample\ tissue}$. The fold change
25 in the target gene expression levels normalised to β -actin and measured relative to the
26 expression levels in the control tissue, was calculated for each sample. The mean fold
27 change \pm standard error of the mean (SEM) in the expression levels for each gene was then
28 determined. Applied Biosystems QuantStudio™ 6 & 7 Flex Real-Time PCR System software
29 was used to obtain the melting curves, the Ct values for the target and reference genes, the
30 ΔCt , the $\Delta\Delta Ct$ values, as well as fold change differences in target gene expression levels in
31 the different samples. Reactions were carried out in triplicate and experiments for each
32 target gene were performed at least five times.
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46 47 **Statistical Analysis**

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50 Differences between groups in Fig. 3-2 were determined by a repeated measures analysis of
51 variance. Log transformation of data was used where variances were heterogeneous. All other
52 data was analysed using 2-tailed Student's-T-tests. Data were expressed as means \pm SEM.
53 Differences were considered significant if $p < 0.05$.
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Results

Engraftment and Repopulation of Human Hepatocytes in the Livers of FRG mice

~~Figure 1 shows a top view (Fig. 1A) and the underside (Fig. 1B) of a mouse liver 5 months after engraftment of human hepatocytes was commenced and prior to transduction with INS-FUR. Significant engraftment of human hepatocytes (distinguished by the orange colouration) was observed throughout the entire liver. In particular, engraftment was pronounced on the underside of the liver lobes (Fig 1B).~~ Hepatocyte engraftment was also monitored by measurement of blood human albumin concentration which increased throughout the engraftment period from 0.05 ± 0.01 mg/ml at 50 days, 2.1 ± 0.1 mg/ml at 100 days and 2.8 ± 0.3 mg/ml at 150 days ($n=6$), when transduction of the mouse livers took place.

~~In liver sections taken from areas observed to be heavily repopulated with human hepatocytes (Fig. 1),~~ Serial frozen sections of livers were cut from the animals and the identity of the cells was confirmed by performing triple immunofluorescence staining of ~~serial~~ the sections with human-specific FAH and albumin, as well as DAPI staining of the nuclei (Fig 21). Fig. 2A-1A shows the expression of human FAH and human albumin in a representative section from the liver of an engrafted FRG mouse treated with INS-FUR, together with DAPI nuclear staining. Analysis of images from areas heavily repopulated with human hepatocytes indicated that human FAH was detected in $89.6 \pm 9\%$ of cells, human albumin in $92.9 \pm 10\%$ of cells and $88.6 \pm 9.8\%$ of cells stained positive for both human FAH and albumin. As expected, staining for human FAH and albumin in non-engrafted mice was negative (Fig. 2B1B). Figure 2C-1C represents a no antibody and no DAPI control of the mouse in Fig. 2A1A, where no staining is visible.

Reversal of Diabetes in FRG mice

Blood glucose levels of diabetic FRG mice decreased to normal levels within 24 h of transduction with the HMD/INS-FUR vector (Fig. 3A2A), and reversal of diabetes was maintained for 60 days (experimental endpoint). Diabetic FRG mice treated with HMD alone remained hyperglycaemic until day 2 (experimental endpoint). The body weights of diabetic mice treated with HMD/INS-FUR at day 0 were 23.6 ± 0.7 g ($n=6$), and this weight was maintained until day 60 (24.2 ± 1.3 g). With the exception of the 30 min time point (when blood glucose concentrations of FRG mice treated with INS-FUR were significantly higher than those of control animals), there was no significant difference between the ability of diabetic FRG mice transduced with the HMD/INS-FUR vector and non-diabetic FRG mice to normalise blood glucose levels during intraperitoneal glucose tolerance tests (IPGTTs) (Fig. 3B2B). Diabetic FRG mice were unable to decrease blood glucose levels during IPGTTs

(Fig. [3B2B](#)). In INS-FUR treated FRG mice, non-fasting human C-peptide levels were 5.61 ± 0.9 pmol/L, and by comparison non-fasting mouse C-peptide levels were significantly lower (1.8 ± 0.2 pmol/L; $p < 0.05$). Human and mouse C-peptide were not detectable in untreated diabetic animals. Human insulin storage was 2.4 ± 0.1 nmol in liver isolated from HMD/INS-FUR treated animals ($n=4$) and mouse insulin was 0.6 ± 0.1 nmol, as compared to 4.4 ± 0.2 nmol in normal mouse pancreas ($n=4$). Mouse insulin was not detectable in acid ethanol extracts from the pancreas of HMD/INS-FUR-treated mice or diabetic controls.

There was a significant elevation of AST (526.4 ± 24.6 U/l) and ALT levels (330 ± 7.4 U/l) 2 days after surgical delivery of INS-FUR, compared to normal untreated animals (AST: 325.4 ± 2.5 U/l; ALT: 259.8 ± 5.4 U/l). This elevation was transient with AST (337.3 ± 2.4 U/l) and ALT (270.8 ± 5.9 U/l) levels having returned to normal at 10 days after vector transduction and remaining normal until sacrifice (AST: 331.6 ± 2.5 U/l; ALT 268 ± 5.4 U/l) ($n=6$).

Immunofluorescence staining for pancreatic hormones in liver tissue from FRG mice

Serial sections of FRG mouse livers were stained for human albumin (Fig. [4A3A](#)), human insulin (Fig. [4B3B](#)) and with DAPI for nuclei (Fig. [4C3C](#)). Analysis of merged images of combined human albumin and DAPI (Fig. [4D3D](#)), human albumin and insulin (Fig. [4E3E](#)) and human albumin, insulin and DAPI (Fig. [4F3F](#)) indicated that $41.4 \pm 13.3\%$ of the cells were positive for human albumin, $34.7 \pm 6.8\%$ were positive for insulin, and $19 \pm 3\%$ were positive for both human albumin and insulin (Fig. [4F3F](#)). The expression of EGFP ($32.6 \pm 2.4\%$) in the animals was not significantly different from insulin. We also examined FRG mouse livers for the expression of other pancreatic hormones which revealed extensive staining for insulin ($35.4 \pm 2.9\%$) with limited expression of glucagon ($1.0 \pm 0.2\%$) and somatostatin ($0.7 \pm 0.2\%$) (Fig. [5A4A](#)). Pancreatic hormones were not detected in normal engrafted FRG livers (Fig. [5B4B](#)) or animals treated with empty vector (data not shown). Figure [5C-4C](#) shows extensive insulin staining in the pancreas of normal (non-diabetic) FRG mice engrafted with human hepatocytes, however insulin-positive cells were rare, with $0.8 \pm 0.3\%$ of the total cells staining positive for insulin in the pancreas of STZ-diabetic FRG mice treated with INS-FUR (Fig. [5D4D](#)), indicating that β -cell regeneration had not occurred. Staining for insulin together with PDX1, once again showed marked staining for insulin ($37.5 \pm 4.1\%$) and PDX1 ($33.5 \pm 3.5\%$), that were not significantly different from each other, (Fig. [5E4E](#)). By comparison insulin and PDX1 were not detected in normal engrafted FRG livers (Fig. [5F4F](#)).

Detection and quantification of expression levels of β -cell transcription factors and pancreatic hormones in FRG mouse livers transduced with INS-FUR.

As expected from previous studies employing the FFO technique for the delivery of INS-FUR to animal livers⁹⁻¹¹, INS-FUR expression was exclusively restricted to the livers of the FRG mice (Fig. 6A5A). INS-FUR was not detected in livers of normal, FRG mice engrafted with human hepatocytes (Fig. 6B5B) or in animals transduced with empty vector (data not shown). In an attempt to further confirm that both human and mouse cells contributed to the pancreatic transdifferentiation of the FRG livers, the expression levels of a number of β -cell transcription factors and pancreatic hormones were determined by RT-PCR analysis. It can be seen from Figure 6B that the human β -cell transcription factors, *Pdx1*, *Neurod1*, *Neurog3*, *Nkx2-2* and *Pax4*, were expressed in HMD/INS-FUR treated FRG livers, as were MAFA and MAFB, the insulin proconvertase, PC1, and synaptophysin. GLUT2 (*Sica2*) and glucokinase were also expressed in transduced and normal liver and pancreas, as expected. The human pancreatic hormones, glucagon and somatostatin, were also expressed in HMD/INS-FUR transduced tissue, but not in normal liver. The pattern of expression of mouse transcription factors and hormones was similar to that observed for human transcription factors, with the exception that mouse *Neurog3* was not detectable in FRG mouse pancreas or HMD/INS-FUR-transduced liver, mouse *Nkx6-1* was detected in HMD/INS-FUR treated FRG livers as were mouse insulin 1 and 2. Surprisingly, mouse glucagon and somatostatin were detected in both normal and transduced liver as was synaptophysin. The exocrine marker, p48, and pancreatic polypeptide (human and mouse) were not detected in any of the liver samples.

The expression levels of both human and mouse β -cell transcription factors and pancreatic hormones were examined by RT-qPCR and all expression results provided are relative to normal engrafted liver (Table S3). The results showed upregulation of several β -cell transcription factors (Fig. 6C5C) and pancreatic hormones (Fig. 6D5D). Marked upregulation of human *Pdx1*, synaptophysin, insulin (as expected), glucagon and somatostatin and murine insulin 1 was observed (Fig. 6D5D). However, there was no discernible trend indicating higher levels of human transcription factors and pancreatic hormone expression when compared to the murine genes or *vice versa*. Whilst these RT-qPCR results do not definitely show the relative contributions of transdifferentiated human versus transdifferentiated murine liver cells to the reversal of diabetes and the normal glucose tolerance observed (Fig. 3B2B), the data suggest that there was contribution from both human and murine transdifferentiated hepatocytes.

Discussion

In the current study, we have employed the chimeric FRG mouse model, which allows the engraftment and proliferation of human hepatocytes in mouse livers, to demonstrate the ability of INS-FUR (delivered by the lentiviral vector HMD and the FFO technique) to transduce human hepatocytes *in vivo*. Importantly, this resulted in the expression of human β -cell transcription factors and pancreatic hormones in the human hepatocytes *in vivo*. Whilst we have previously reported partial pancreatic transdifferentiation of rodent and porcine liver tissue using this methodology⁹⁻¹¹, this is the first report of partial pancreatic transdifferentiation of primary human hepatocytes *in vivo* by the delivery of INS-FUR alone.

The engraftment success rate of human hepatocytes was high (50-62%) without the requirement for serial transplantation of hepatocytes, as described in other studies²⁰. This was likely related to the fact that engrafted hepatocytes were allowed to expand for 150 days prior to diabetes induction and experimentation. As seen in other studies^{19, 20}, serial sectioning and immunohistochemical analysis showed that in areas of liver heavily repopulated with human hepatocytes a high proportion of the cells (89%) showed dual positivity for human FAH and human albumin confirming their human origin, as the murine hepatocytes in the FRG livers have an FAH deletion.

As we previously described in studies using the FFO technique to deliver the HMD/INS-FUR vector to diabetic animals^{9, 10}, blood glucose levels of STZ-diabetic FRG mice decreased to normal within the first day following transduction with INS-FUR, and remained not significantly different to normal animals for the period of experimentation (60 days). By comparison, animals treated with HMD alone remained hyperglycaemic, and were sacrificed 2 days after transduction. The immediate reduction in blood glucose concentrations may have been attributable to the expression of the INS-FUR transgene, with long term maintenance of normoglycaemia due to pancreatic transdifferentiation with insulin storage, as seen previously⁹. The reduction in blood glucose was not an effect of the surgical procedure or the lentiviral vector alone, as empty vector-treated animals remained hyperglycaemic. Definitive proof would require analysis of INS-FUR expression 24 h after gene transfer, which was not carried out in the current study. The body weights of the HMD/INS-FUR-treated animals remained relatively constant throughout the period of experimentation. Also, similar to our previous studies⁹⁻¹¹, the glucose-responsiveness of INS-FUR-treated animals (Fig. [3B2B](#)) was not significantly different from the normal control animals. The one exception was the 30 min time point in the IPGTTs when the transduced animals had significantly higher blood glucose as compared to non-diabetic controls. This normal glucose tolerance is likely related to the storage of insulin in the mouse livers (as

opposed to constitutive expression and unregulated secretion¹³), as the transduced hepatocytes had a reservoir of intracellular insulin, which allowed immediate responsiveness to increases in blood glucose concentrations, and insulin production was not reliant upon constitutive secretion¹³ or the delayed response from a glucose-responsive promoter to stimulate transcription, not translation or secretion⁴. Insulin storage was approximately half that of a normal mouse pancreas, which is within clinical limits for islet transplantation²⁵. The presence of the other pancreatic hormones glucagon and somatostatin in close proximity to the cells that stained positive for insulin may have assisted with the normal response to glucose; however they were expressed at very low levels as assessed by immunohistochemical staining (Fig. 5A4A), which would limit the possibility of a significant biological effect. Future in-depth determination of pancreatic transdifferentiation could be ascertained by RNA-seq analysis as described²⁶.

Due to the coexistence of both human and murine hepatocytes in the humanised mice and the significant storage of human and mouse insulin in transduced livers, it is a reasonable assumption that both populations of hepatocytes underwent a degree of pancreatic transdifferentiation and contributed to the amelioration of diabetes. We used RT-PCR and RT-qPCR to determine if human hepatocytes expressed β -cell transcription factors and pancreatic hormones, as observed in rodent and porcine cells following expression of HMD/INS-FUR⁹⁻¹¹. The key human and mouse transcription factors, *Pdx1*, *Neurod1* and *Nkx.2*, were significantly upregulated in transduced livers. Extensive staining of PDX1 was also shown in INS-FUR-transduced livers (Fig. 5E4E), with no expression in untransduced livers (Fig. 5F4E). Human *Pax4* was also significantly upregulated and human *Ngn3* was detected, however mouse *Pax4* and *Ngn3* were not detected in the transduced liver and *Ngn3* was absent from normal mouse pancreas. These results are not all together surprising as it is well documented that *Pax 4* and *Ngn3* are mainly expressed during the embryonic stage, during the differentiation of embryonic progenitor cells, and are rarely detected in the mature mouse pancreas²⁷. *MAFB* was expressed in all murine samples, which corroborates our previous study using HMD/INS-FUR-transduced NOD mice, and other studies, which have observed its expression at the mRNA level in liver tissue^{10, 28}. Human and murine *MAFA* were only detected in transduced liver tissue, which may have been associated with the significant upregulation of mouse insulin 1 and 2 as *MAFA* is functionally linked to insulin gene transcription²⁹. Similar to our previous studies using this technique pancreatic transdifferentiation was not complete, as human and mouse *Pax6* and *Pc2* were not detected. Fortunately, the exocrine marker, p48, was also not detected indicating exocrine differentiation was therefore prevented, and thus no destruction of transduced hepatocytes was seen, as observed in studies that induced transdifferentiation via the expression of

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3 *Pdx1*^{5, 7}. Whilst human glucagon, somatostatin and synaptophysin were detected in
4 transduced liver tissue alone, the corresponding murine factors were expressed, at the
5 mRNA level, in normal engrafted liver. However, immunofluorescence staining of normal
6 engrafted liver did not detect any protein expression of either glucagon or somatostatin,
7 likely due to extremely low levels of expression of the proteins. RT-qPCR indicated that
8 expression of human and mouse glucagon and synaptophysin and human somatostatin
9 were significantly upregulated in the HMD/INS-FUR-treated livers, as compared to normal
10 tissue. Whilst synaptophysin is known to modulate secretory pathways in pancreatic
11 endocrine cells³⁰, synaptophysin protein has been detected in the liver perisinusoidal stellate
12 cells, in human and rat livers, and its expression levels increased in pathological conditions
13 involving liver damage³¹. Thus, the expression of murine synaptophysin in the untransduced
14 engrafted FRG livers may have resulted from tissue damage following hepatocyte
15 engraftment, as may the expression of glucagon and somatostatin, which are normally
16 expressed in the developing pancreas prior to insulin expression³². Indeed, the exposure of
17 liver cells to toxins and cellular insults has been shown previously to induce liver-to-pancreas
18 transdifferentiation³³. In the untransduced chimeric liver, the human hepatocytes were
19 exposed to less damage than the mouse hepatocytes, which were FAH deficient and were
20 further damaged by urokinase treatment, prior to human liver cell engraftment.

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30 This is the first study to show clear transdifferentiation of human hepatocytes with storage of
31 human insulin and the expression of human β -cell transcription factors and pancreatic
32 hormones in an *in vivo* setting using a lentiviral system to deliver INS-FUR alone. However,
33 the results of this study do not provide a definitive answer as to which transduced liver cell
34 type, mouse or human, made the greatest contribution to the reversal of diabetes and
35 normal glucose tolerance observed in the FRG mouse.

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40 It has been established in our previous study utilising NOD mice¹⁰ and a study by Elsner *et*
41 *al* using diabetic rats¹³, that the simple injection of a lentiviral vector containing a modified
42 insulin transgene was sufficient to reduce daily blood glucose levels to near normal.
43 However, no storage of insulin in the livers was detected and glucose tolerance was
44 abnormal. As pancreas and liver come from the same endodermal origin, it has been
45 previously documented that certain conditions such as insults to the cells or high glucose
46 conditions can prime hepatocytes for pancreatic transdifferentiation³³. It therefore seems
47 likely that the insult provided by the FFO clamping technique and the delivery and
48 expression of the INS-FUR transgene in the lentiviral vector collectively has led to the liver-
49 to-pancreas transdifferentiation in the current study. The transient rise seen in the liver
50 enzymes AST and ALT following surgical delivery of the INS-FUR vector, which returned to
51 normal within 10 days would seem to support this argument. Unpublished current work in our
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laboratory has found that delivery of INS-FUR by FFO using an adeno-associated vector reduces diabetic mouse blood glucose, but does not induce expression of β -cell transcription factors or storage of insulin. Thus, some facet of the HMD lentiviral vector was likely responsible; possibly the expression of the Tat protein in this generation of vector, combined with the FFO technique to deliver the transgene has initiated the process. Work is underway to better understand the transdifferentiation process and what component of the vector is responsible. This knowledge will be particularly important for clinical application, which would require the use of a self-inactivating (SIN) lentiviral vector, which uses 4 plasmids, rather than the three vector system utilised in the current study. These SIN vectors also lack some components of the 2nd generation vector system used in this study, and demonstrate a much improved insertion profile which has shown promise in recent clinical applications³⁴. However problems such as immunogenicity of vector particles and fears of insertional mutagenesis still remain prominent areas for consideration³⁴, which may require further vector development. Despite possible limitations of the vector system that was used, this study has shown for the first time, that it is possible to transduce primary hepatocytes *in vivo* with a lentiviral vector, with partial pancreatic transdifferentiation resulting in human insulin storage and expression of human β -cell transcription factors. Further development of such technology holds promise for clinical application.

Acknowledgements: A.M.S and I.E.A initiated the concept of the study and A. M. S wrote the article. B.R. produced the vectors, performed the animal experiments, immunohistochemistry, RT-PCR, qRT-PCR analysis and data analysis. Q. L performed RT-PCR and qRT-PCR analysis and data analysis. N.T.N. performed primer design and data analysis. Y. T, A. P. D. and R.M-W contributed to the animal experiments. D. G. and B. A. O'B contributed to experimental design and data analysis. F. T. designed the statistical analysis. All authors contributed to the interpretation of the data. The authors have no conflicts of interest.

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20 vectors in gene therapy. *Curr Gene Ther.* 2013;13: 453-68.
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23 **List of Table Legends**

24 Table S1: RT-PCR Primers

25 Table S2: TaqMan assay identification of murine genes

26 Table S3; Results of RT-qPCR analysis of human and mouse β -cell transcription factors and
27 pancreatic hormones in INS-FUR-treated FRG mouse liver tissue compared to untransduced
28 FRG mouse liver.
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Figure Legends

Figure 1 — **Engraftment and repopulation of human hepatocytes in adult FRG mice.** Large areas of human hepatocytes (orange) are shown in all liver lobes (A) top view of the liver and (B) underside of the lobes of the liver in a FRG mouse 5 months after commencement of the engraftment procedure and prior to the delivery of the HMD/INS-FUR vector. Selected areas of human hepatocyte repopulation are indicated with arrows.

Figure 21 **Expression of human FAH and human albumin in liver tissue from FRG mice treated with INS-FUR.** Representative photomicrographs of immunofluorescence staining for human FAH, human albumin and DAPI stained nuclei, together with a merged image, in (A) liver tissue from an FRG mouse 60 days after treatment with INS-FUR; (B) liver tissue from a non-engrafted, non-diabetic FRG mouse of the same age and (C) a no antibody, no DAPI control of liver tissue from the mouse in A. Scale bar= 40 μ m.

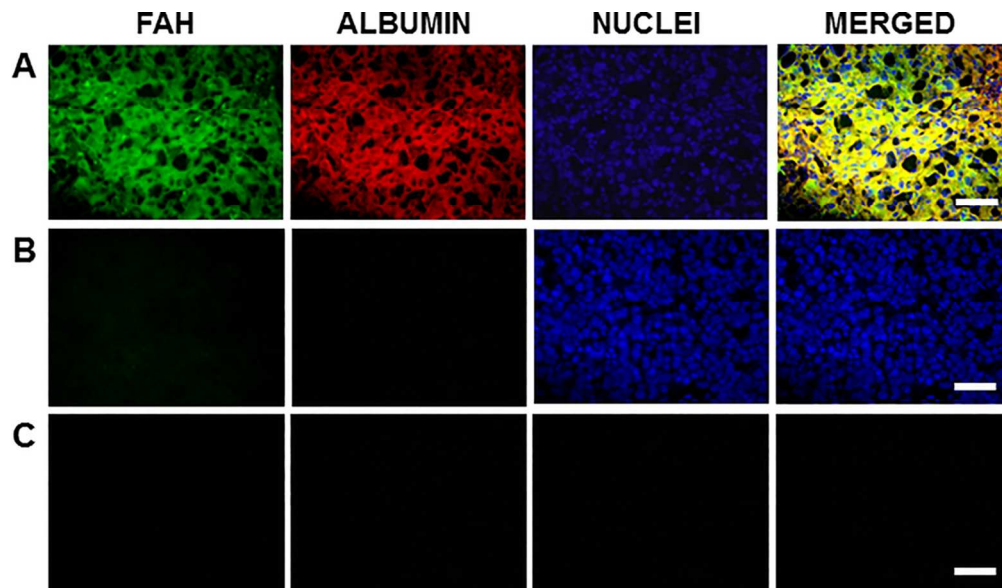
Figure 32 **Expression of INS-FUR ameliorated STZ-induced diabetes in FRG mice.** (A) Blood glucose concentrations of non-diabetic engrafted FRG mice and diabetic engrafted mice treated with HMD/INS-FUR and HMD alone. (B) Blood glucose levels following IPGTTs of nondiabetic engrafted FRG mice, diabetic mice treated with HMD/INS-FUR, and diabetic controls 60 days after vector infusion (n=6; means \pm SEMs).

Figure 43 **Expression of human albumin and insulin in liver tissue from FRG mice treated with INS-FUR.** Representative photomicrographs of immunofluorescence staining for (A) albumin, (B) insulin and (C) DAPI nuclei staining in liver tissue from an FRG mouse 60 days after treatment with INS-FUR. (D) merged image of A and C; (E) merged image of A and B; (F) is a merged image of A, B, and C. Scale bar= 40 μ m.

Figure 54 **Expression of pancreatic hormones and PDX1 following reversal of STZ-diabetes after treatment with INS-FUR.** Representative photomicrographs of triple immunofluorescence staining for insulin (INS), somatostatin (SST), glucagon (GLUC) and a merged image in: (A) liver from a diabetic FRG mouse treated with HMD/INS-FUR 60 days previously; (B) liver from an untreated FRG mouse; (C) pancreas from an untreated non-diabetic FRG mouse and (D) pancreas from a diabetic FRG mouse treated with HMD/INS-FUR 60 days previously. (E) and (F) are representative photomicrographs of

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3 immunofluorescence staining for insulin (INS), PDX1, DAPI stained nuclei
4 and a merged image in: liver from a diabetic FRG mouse treated with
5 HMD/INS-FUR 60 days previously and liver from an untreated FRG mouse
6 respectively. Scale bar= 40 μ m.
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10 **Figure 65** **Expression of β -cell transcription factors and pancreatic hormones in**
11 **FRG mice 60 days after transduction with HMD/INS-FUR. (A)** RT-PCR
12 analyses of tissues for expression of INS-FUR and β -actin (*Actb*) in animals
13 transduced with HMD/INS-FUR : spleen (lane 1), pancreas (lane 2), and liver
14 (lane 3) **(B)** RT-PCR analysis for human and mouse β -cell transcription
15 factors (*Pdx1*, *Neurod1*, *Neurog3*, *Nkx2-2*, *Nkx6-1*, *Pax 4*, *Pax 6*, *MAFA*,
16 *MAFB*), insulin proconvertase 1 and 2 (*Pc1* and *Pc2*), GLUT 2 (*Slc2a2*), and
17 glucokinase (*Gck*); synaptophysin (*Synap*), exocrine marker p48; the
18 pancreatic endocrine hormones: glucagon (*Gcg*), somatostatin (*Sst*), mouse
19 insulin 1 and 2 (*M.Ins1* and *M.Ins2*), *human insulin (H.Ins)*, pancreatic
20 polypeptide (*Ppy*) and *Actb* in normal liver (lane 1), liver transduced with
21 HMD/INS-FUR (FFO) (lane 2) and either human islets or normal mouse
22 pancreas (lane 3). Results of RT-qPCR analysis of human and mouse β -cell
23 transcription factors **(C)** and pancreatic hormones **(D)** that were significantly
24 upregulated in INS-FUR-treated FRG mouse liver tissue compared to normal
25 liver. Five replicates of each treatment, each consisting of at least 3
26 methodological replicates were used. Expression data for each gene of
27 interest are presented as the average and SEM of data obtained for each
28 gene, compared to untransduced FRG liver.
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26 Figure 1 Expression of human FAH and human albumin in liver tissue from FRG mice treated with INS-FUR.
27 Representative photomicrographs of immunofluorescence staining for human FAH, human albumin and DAPI
28 stained nuclei, together with a merged image, in (A) liver tissue from an FRG mouse 60 days after
29 treatment with INS-FUR; (B) liver tissue from a non-engrafted, non-diabetic FRG mouse of the same age
30 and (C) a no antibody, no DAPI control of liver tissue from the mouse in A. Scale bar= 40 μ m.

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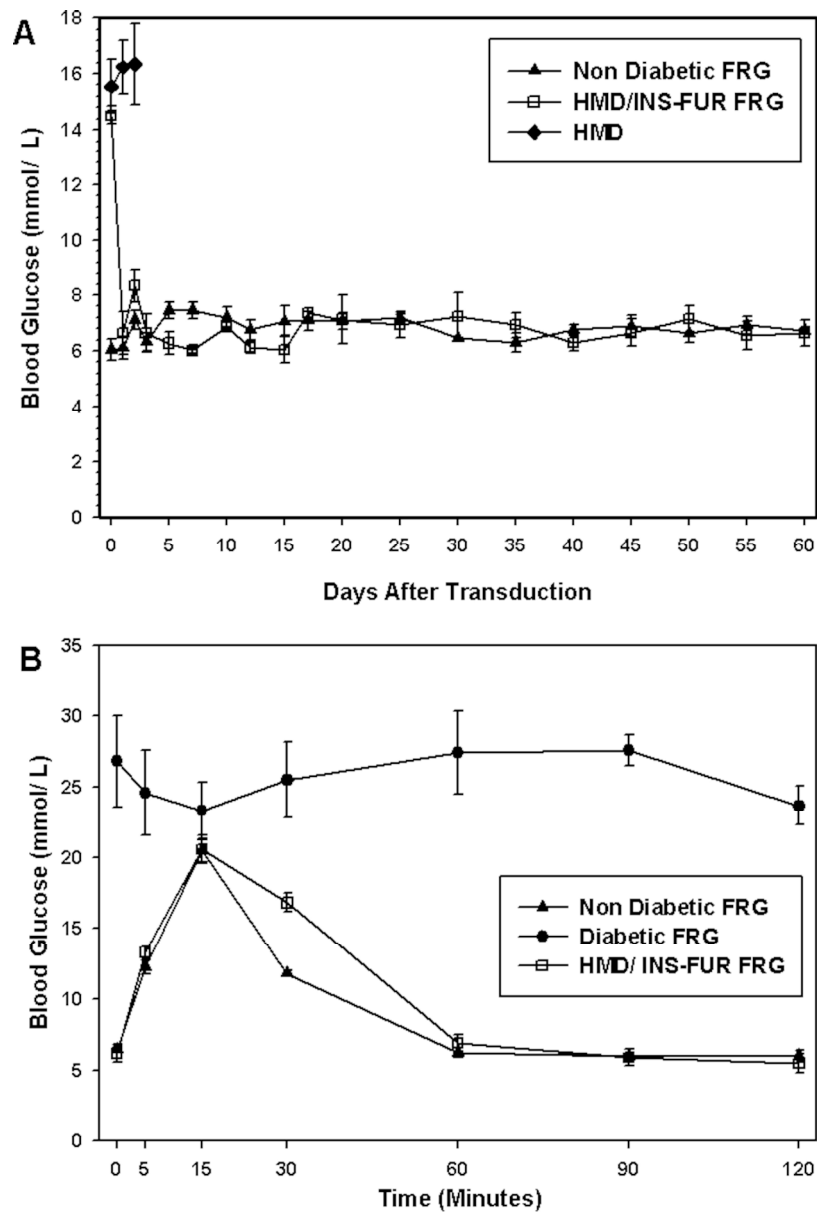


Figure 2 Expression of INS-FUR ameliorated STZ-induced diabetes in FRG mice. (A) Blood glucose concentrations of non-diabetic engrafted FRG mice and diabetic engrafted mice treated with HMD/INS-FUR and HMD alone. (B) Blood glucose levels following IPGTTs of nondiabetic engrafted FRG mice, diabetic mice treated with HMD/INS-FUR, and diabetic controls 60 days after vector infusion ($n=6$; means \pm SEMs).

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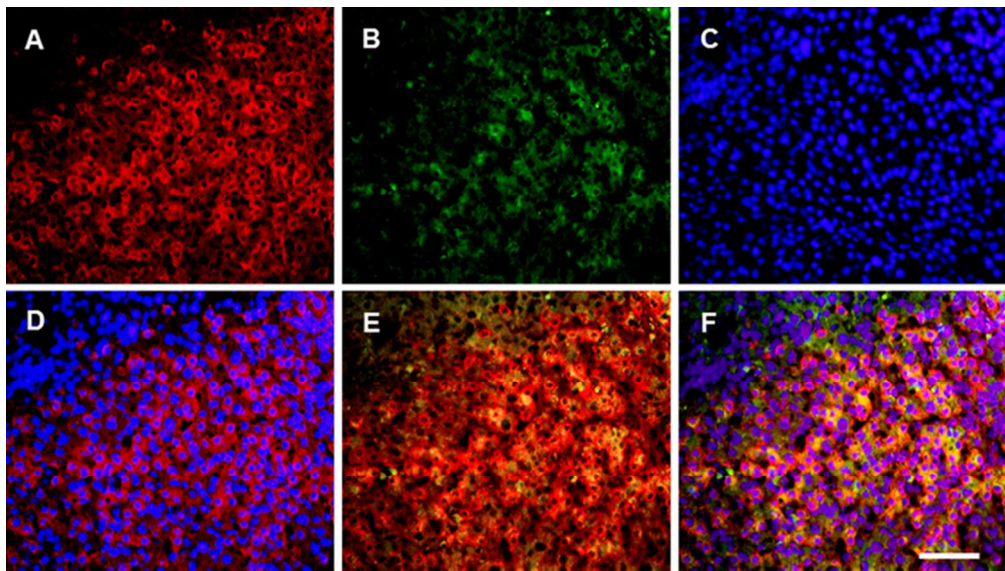


Figure 3 Expression of human albumin and insulin in liver tissue from FRG mice treated with INS-FUR. Representative photomicrographs of immunofluorescence staining for (A) albumin, (B) insulin and (C) DAPI nuclei staining in liver tissue from an FRG mouse 60 days after treatment with INS-FUR. (D) merged image of A and C; (E) merged image of A and B; (F) is a merged image of A, B, and C. Scale bar= 40 μ m.

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Review

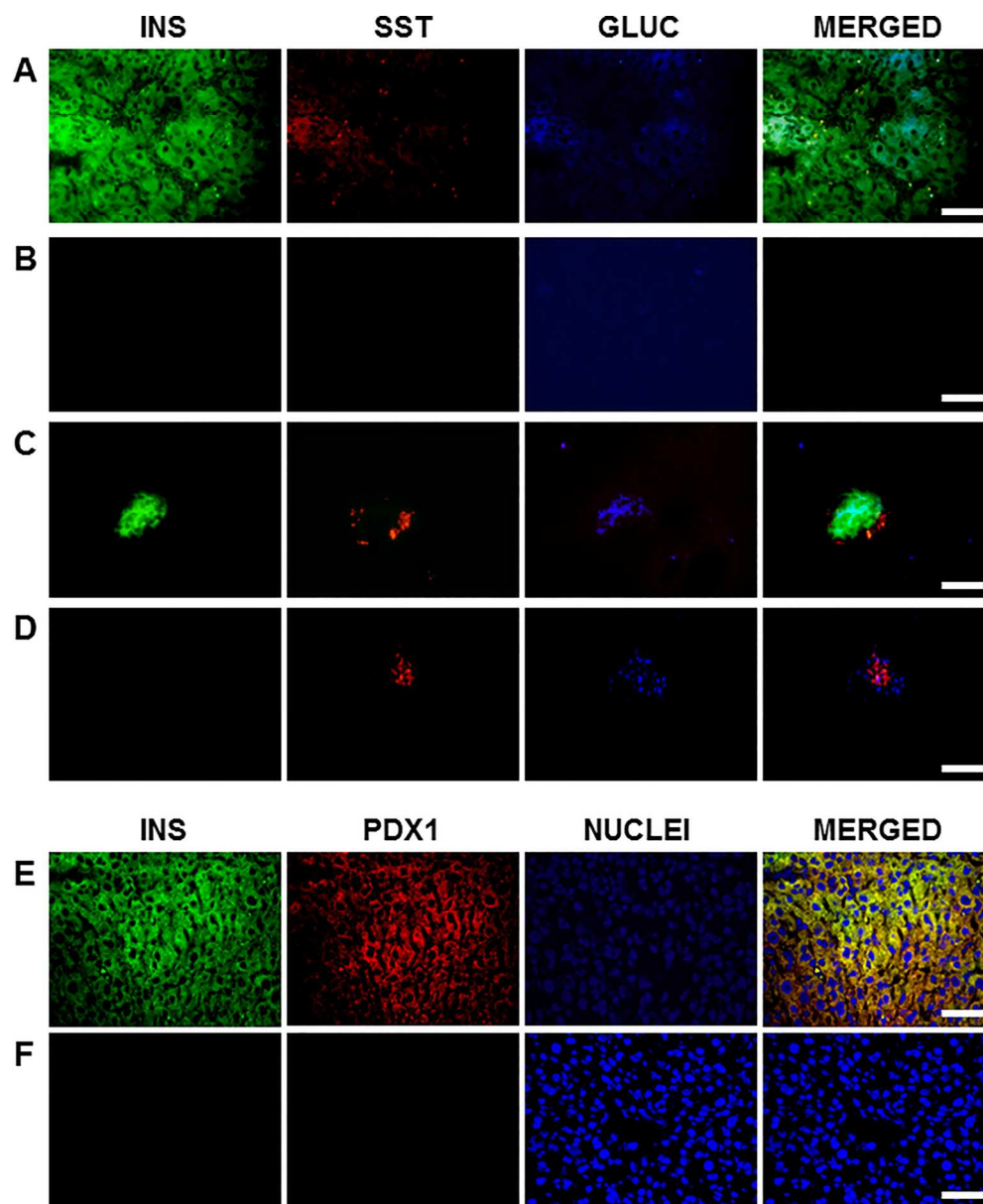


Figure 4 Expression of pancreatic hormones and PDX1 following reversal of STZ-diabetes after treatment with INS-FUR. Representative photomicrographs of triple immunofluorescence staining for insulin (INS), somatostatin (SST), glucagon (GLUC) and a merged image in: (A) liver from a diabetic FRG mouse treated with HMD/INS-FUR 60 days previously; (B) liver from an untreated FRG mouse; (C) pancreas from an untreated non-diabetic FRG mouse and (D) pancreas from a diabetic FRG mouse treated with HMD/INS-FUR 60 days previously. (E) and (F) are representative photomicrographs of immunofluorescence staining for insulin (INS), PDX1, DAPI stained nuclei and a merged image in: liver from a diabetic FRG mouse treated with HMD/INS-FUR 60 days previously and liver from an untreated FRG mouse respectively. Scale bar= 40 μ m.

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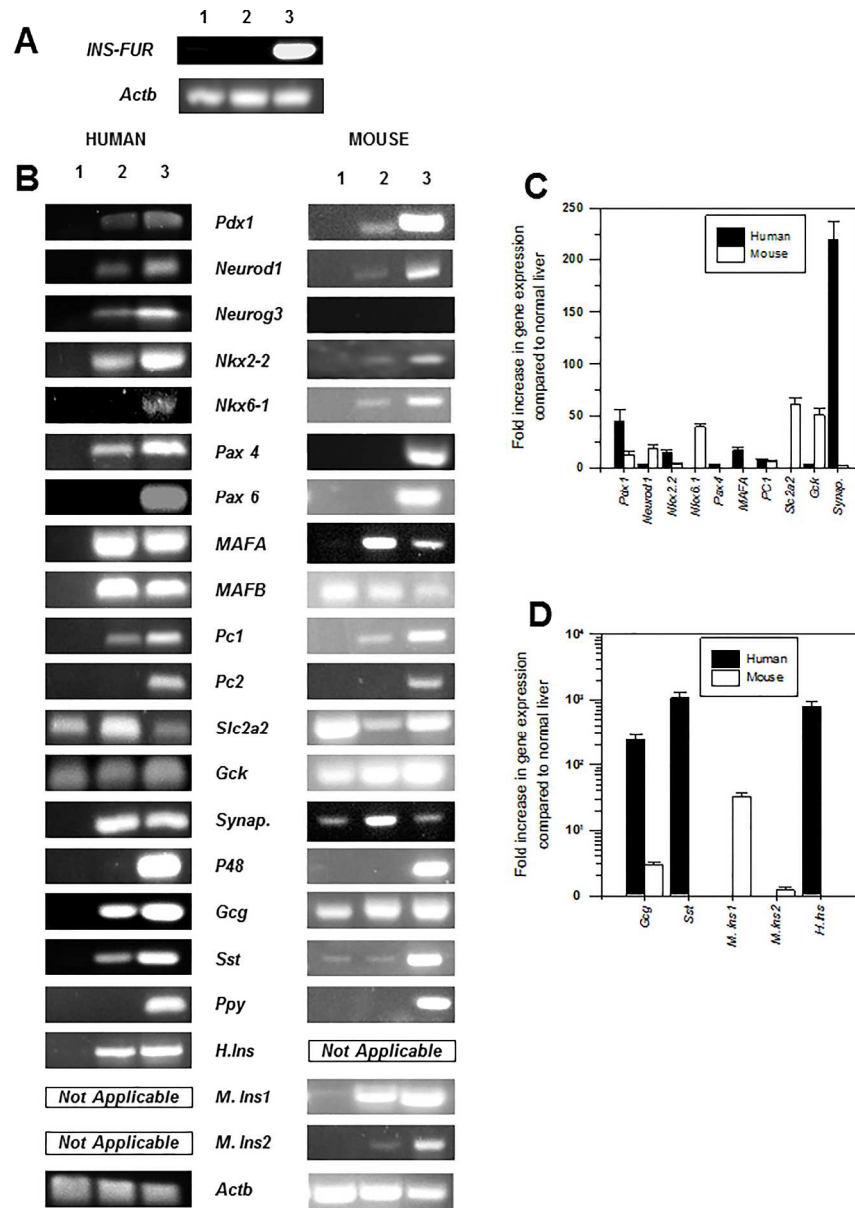


Figure 5 Expression of β -cell transcription factors and pancreatic hormones in FRG mice 60 days after transduction with HMD/INS-FUR. (A) RT-PCR analyses of tissues for expression of INS-FUR and β -actin (*Actb*) in animals transduced with HMD/INS-FUR : spleen (lane 1), pancreas (lane 2), and liver (lane 3) (B) RT-PCR analysis for human and mouse β -cell transcription factors (*Pdx1*, *Neurod1*, *Neurog3*, *Nkx2-2*, *Nkx6-1*, *Pax 4*, *Pax 6*, *MAFA*, *MAFB*), insulin proconvertase 1 and 2 (*Pc1* and *Pc2*), GLUT 2 (*Slc2a2*), and glucokinase (*Gck*); synaptophysin (*Synap.*), exocrine marker *p48*; the pancreatic endocrine hormones: glucagon (*Gcg*), somatostatin (*Sst*), mouse insulin 1 and 2 (*M.Ins1* and *M.Ins2*), human insulin (*H.Ins*), pancreatic polypeptide (*Ppy*) and *Actb* in normal liver (lane 1), liver transduced with HMD/INS-FUR (FFO) (lane 2) and either human islets or normal mouse pancreas (lane 3). Results of RT-qPCR analysis of human and mouse β -cell transcription factors (C) and pancreatic hormones (D) that were significantly upregulated in INS-FUR-treated FRG mouse liver tissue compared to normal liver. Five replicates of each treatment, each consisting of at least 3 methodological replicates were used. Expression data for each gene of interest are presented as the average and SEM of data obtained for each gene, compared to untransduced FRG liver.

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For Peer Review

Table S1: RT-PCR Primers

Gene	Human		Murine	
	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer
Pdx1	ACGCGTGGAAAGGCCAGTGGG	GGCGCGGCCGTGAGATGTACT	CCAGTGGGCAGGAGGTGCTTACA	GGGGCCGGGAGATGTATTTGTTA
Neurod1	GAGGAATTCGCCACGCAGGA	TTGGAGACCTTGGGGCTGAGG	GCTCCAGGGTTATGAGATCG	CTCTGCATTCATGGCTTCAA
Neurog3	AACTTGGCGACCAGAAGCCCG	GGGTCACCTGGACAGTGGGCG	CCGGATGACGCCAAACTTA	CATAGAAGCTGTGGTCCGCTATG
Nkx2-2	TCAGTACTCCCTGCACGGTC	CGGGGTCTCCTTGTCAATTGT	ACTTGGAGCTCGAGTCTTGG	AGCCCTTTCTACGACAGCAG
Nkx6-1	AGAGGGCTCGTTTGGCCTATTGCG	CGGCAGCGTGCTTCTTCCTCC	CCGAGTCTGCTTCTTCTTG	TATTCTCTGGGGATGACGGA
Pax 4	GCCCGTGGAAAGCTGGCTACT	ACAGTCAGCCCCTGGGAAGCA	CCACCTCTCTGCCTGAAGAC	CCCACAGCATAGCTGACAGA
Pax 6	AGCTGAAGCGGAAGCTGCAA	TGGCTGCTAGTCTTCTCGGGC	CTTCATCCGAGTCTTCTCCG	AGTTGGTGTCTCTCCCCCT
MAFA	GCTCTGGAGTTGGCACTTCT	CTTCAGCAAGGAGGAGGTCA	CTCTGGAGCTGGCACTTCTC	AGGAGGTCATCCGACTGAAA
MAFB	AAGCCTCTCACCTTAGGAGC	GGAAAACAGATCCTCCCCTC	CACACGTAGCAAGAGGTGGA	AGACAGGCTTTGCGTCCTAA
Pc1	GCGTGCCTGAGAAGAAAGAGTGTG	GTACATGCTTCCAGGGACTTGACAGC	TTTCCAACCTGGGTTACTGG	GGCTGCTGGTATCTTTGCTC
Pc2	TTCCCTTTGCTGAAGGTCTG	TTTCGGTCAAATCCTTCCTG	AAATCCTTCTTGTTCAGCG	TTGCAGAAGGCCTGTATCAC T
Slc2a2	ATCCAAACTGGAAGGAACCC	CATGTGCCCACTCACACAA	TCTTCACGGTGTCTCTGTGCT T	AGCAGCACAAAGTCCCACCGA C
Gck	GCACTGCTGAGATGCTCTTC	TGTTCCCTTCTGCTCCTGAG	GTCGCAGGTGGAGAGCGACT C	GCGCACAATGTCGCAGTCCG G
Snapophysin	CGAGGTCGAGTTCGAGTACC	TGACGAGGAGTAGTCCCCAA	ACCTCGGTGGTGTGGCTTC C	CCTGGAGGTGCGCGCATGA A
P48	AAATGTACGGGAGCGCGGCGCA	GGAAGTTGATGTATCCAATGG	GAAGGTTATCATCTGCCATCG	GGGTGGTTCGTTCTCTATGT T
Gcg	GGGCTTGCTCTCTTTCACCTGCT	ACGTTGCCAGCTGCCTTGTACC	ACCCAGATCATTCCAGCTT CCC	AATCTTGGGCACGGCGGGA G
Sst	CGGACCCCAGACTCCGTCAGT	GGTTGGGTTCCAGACAGCAGCTC	CCAGGGCATCATTCTCTGTC	CCCAGACTCCGTCAGTTTCT

Ppy	TCGCTTGGTCTAGTGCCCATTT AC	GCACCCAGCAGTGGCTGTAGT	GCATAGTCGCCTGGGTACAT	CCAACACTCACTAGCTCAGC A
INS-FUR	CTTACCATGGCCCTGTGGAT	TAGAAGAAGCCTCGTTCCCC		
Mouse Ins 1	N/A	N/ A	TGGCTTCTTCTACACACCCAA G	ACAATGCCACGCTTCTGC
Mouse Ins 2	N/ A	N/ A	GAGTCCCACCCACCCAG	TCCACTTCACGGCGGGACA
Actb	CTGAGGCACTCTTCCAGCCTT CCTT	AGGTCTTTGCGGATGTCCACGT CA	ATGGAGGGGAATACAGCCC	TTCTTTGCAGCTCCTTCGTT

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Table S2: TaqMan® assay for quantitation of murine gene expression

Gene	TaqMan Assay Identification No.
Murine Actb	Mm02619580_g1
Murine Mafa	Mm00845206_s1
Murine Pax4	Mm01159036_m1
Murine Pdx1	Mm00435565_m1
Murine NeuroD1	Mm01946604_s1
Murine Ngn3	Mm01173349_g1
Murine Nkx6.1	Mm00454961_m1

For Peer Review

Table S3

Results of RT-qPCR analysis of human and mouse β -cell transcription factors and pancreatic hormones in INS-FUR-treated FRG mouse liver tissue compared to normal liver.

Fold increase in gene expression compared to normal liver		
	Human	Mouse
<i>Pdx1</i>	44.7 \pm 10.6*	12.34 \pm 3.4*
<i>Neurod1</i>	2.4 \pm 0.6*	18.5 \pm 3.3*
<i>Neurog3</i>	3.6 \pm 0.7	Not detected
<i>Nkx2-2</i>	14.7 \pm 2.3*	4.2 \pm 0.6*
<i>Nkx6-1</i>	Not detected	39.16 \pm 2.6*
<i>Pax4</i>	3.4 \pm 0.5*	Not detected
<i>MAFA</i>	17.1 \pm 2.2*	85.8 \pm 37.9
<i>MAFB</i>	196.1 \pm 19.8	5.3 \pm 4.6
<i>PC1</i>	8.6 \pm 0.3*	6.6 \pm 0.4*
<i>Slc2a2</i>	No change	61.2 \pm 5.6*
<i>Gck</i>	3.2 \pm 0.3*	50.8 \pm 6.9*
<i>Synaptophysin</i>	219.5 \pm 18.5*	2.4 \pm 0.3*
<i>Glucagon</i>	2460.3 \pm 422.3*	29.2 \pm 2.6*
<i>Somatostatin</i>	10680.5 \pm 2324.9*	0.1 \pm 0.0
<i>Mouse insulin 1</i>	N/ A	322.4 \pm 54.1*
<i>Mouse insulin 2</i>	N/ A	12.2 \pm 1.2*
<i>Human insulin</i>	7870.2 \pm 1437.9*	N/A

Five replicates of each treatment, each consisting of at least 3 methodological replicates were used. Expression data for each gene of interest are presented as the average and SEM of data obtained for each gene, compared to untransduced FRG liver. Asterisks indicate a statistically significant fold change in expression compared to normal engrafted liver at alpha = 0.05.