***In vivo* studies on non-viral transdifferentiation of liver cells towards pancreatic β cells**

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Abstract

Transdifferentiation *in vivo* is an attractive option for autologous replacement of pancreatic β cells in patients with type 1 diabetes. It has been achieved by adenoviral delivery of genes for transcription factors in the liver and pancreas of hyperglycaemic mice. However, these viral approaches are not clinically applicable. We used the hydrodynamic approach to deliver genes *Pdx1*, *Ngn3* (*Neurog3*) and *MafA* singly and in combination to livers of normoglycaemic rats. Five expression plasmids were evaluated. Livers were removed 1, 3, 7, 14 and 28 days after gene delivery and assayed by quantitative PCR, semi-quantitative PCR and immunohistology. Functional studies on hyperglycaemic rats were performed. The highest and most sustained expression was from a CpG-depleted plasmid (pCpG) and a plasmid with an in-frame scaffold/matrix

attachment region ((pEPI(CMV))). When *Pdx1*, *Ngn3* and *MafA* were delivered together to normoglycaemic rats with these plasmids, insulin mRNA was detected at all time points and was ~ 50 -fold higher with pCpG. Insulin mRNA content of livers at days 3 and 7 was equivalent to that of a pancreas, with scattered insulin-positive cells detected by immunohistology, but levels declined thereafter. Prohormone convertase 1/3 was elevated at days 3 and 7. In hyperglycaemic rats, fasting blood glucose was lower at days 1, 3 and 7 but not thereafter, and body weight was maintained to day 28. We conclude that hydrodynamic gene delivery of multiple transcription factors to rat liver can initiate transdifferentiation to pancreatic β cells, but the process is reversible and probably requires more sustained transcription factor expression.

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Introduction

The introduction of insulin therapy for type 1 diabetes in the 1920s represented a monumental advance (Banting *et al.* 1922). However, in spite of increasingly sophisticated approaches to regulating blood glucose with exogenous insulin (Hovorka *et al.* 2010, Renard 2010), optimal blood glucose control remains difficult. As a consequence, serious late complications (e.g. renal failure) are common in diabetic patients.

The only approach currently able to achieve physiological blood glucose control is the transplantation of pancreatic β cells, either as islet or as whole pancreas allografts. However, the risks of lifelong immunosuppression (Matas *et al.* 2004) and the paucity of organ donors (Daneman 2006, Johnson *et al.* 2010) mean that allotransplantation cannot be regarded as a definitive therapy for type 1 diabetes. The possibility of xenogeneic pancreatic β cells for transplantation has not been realised, mainly because of the risks of interspecies viral

transfer (Patience *et al.* 1997, Cunningham *et al.* 2004) and the vigour of the rejection response to xenografts (McGregor *et al.* 2005). Various stem cell types are being evaluated for *in vitro* differentiation to pancreatic β cells (Takahashi & Yamanaka 2006, Byrne *et al.* 2007, Jiang *et al.* 2007, Karnieli *et al.* 2007), but establishing optimal conditions for differentiation, the allogenicity of embryonic stem cells and risks of neoplasia from non-differentiated cells (Piscaglia 2008) remain serious issues.

In situ transdifferentiation of some of the patient's own cells into pancreatic β cells (Graf & Enver 2009) bypasses the phase of pluripotency involved in most stem cell studies, thereby eliminating the risk of neoplasia. Moreover, there are neither issues with allogenicity nor requirements for expensive facilities. Based on modern knowledge of the embryonic differentiation of pancreatic β cells (Habener *et al.* 2005), several groups have demonstrated the feasibility of transducing hepatocytes towards a pancreas phenotype *in vivo*.

Ferber *et al.* (2000) gave standard E1/E4-deleted adenoviral vectors encoding rat *Pdx1* i.v. to mice and found insulin-producing endocrine cells in the liver. These cells produced multiple pancreatic endocrine hormones but nevertheless were able to regulate blood glucose in streptozotocin-treated mice. When the experiments were repeated using 'gutless' adenoviral vectors encoding mouse *Pdx1* (Kojima *et al.* 2003), the mice rapidly developed a lethal hepatitis. This was probably a consequence of transdifferentiation of transfected hepatocytes to an exocrine (as well as endocrine) pancreas phenotype, with the secretion of digestive enzymes. Expression of the down-stream transcription factor *NeuroD1* avoided exocrine transdifferentiation (Kojima *et al.* 2003).

More recently, transdifferentiation to insulin-producing cells, without exocrine differentiation, has been achieved in mice with adenovirus delivery of multiple transcription factors: *Ngn3* (*Neurog3*), *NeuroD1* and *MafA* to liver (Song *et al.* 2007) and *Pdx1*, *Ngn3* and *MafA* to pancreatic acinar cells in immune-deficient mice (Zhou *et al.* 2008). With the pancreatic acinar cells, expression of the transcription factors for only a few weeks was sufficient for irreversible transdifferentiation to morphologically distinct pancreatic β cells with insulin granules (Zhou *et al.* 2008).

For a chronic disease like type 1 diabetes, with excellent short-term and medium-term therapy, risks must be low for gene therapy to be a viable option in the early phase of the disease. Given the risks posed by viral vectors (Raper *et al.* 2003, McCormack & Rabbitts 2004), non-viral approaches offer significant advantages. Moreover, the liver is a much safer target organ than the pancreas. The risk of pancreatitis in fact essentially excludes the pancreas as an acceptable target in the clinic. The hydrodynamic approach requires neither viral nor non-viral vectors. It is currently the most promising non-viral approach for the clinical application of liver gene therapy, primarily because of lack of immunogenicity (Sawyer *et al.* 2009). Moreover, surgical techniques have been developed in large animals, which enable adequate pressurisation of individual liver segments (corresponding to $\sim 25\%$ of the liver mass) using small volumes (equivalent to $\sim 0.6\%$ of body weight) without reducing venous return to the heart or obstructing blood flow through the intestines (Fabre *et al.* 2011). However, the requirements (in terms of levels and duration of expression of the pancreatic transcription factors in liver) for the irreversible transdifferentiation of liver cells to pancreatic β cells are not known, other than that provided by adenoviruses is sufficient.

The level and duration of gene expression from DNA plasmids varies with the nature of the promoter, the presence of enhancer elements, plasmid copy number per cell, the propensity for DNA hypermethylation, the capacity of the plasmid to replicate in mammalian cells, the stability of the DNA plasmid in cells and other factors (Jackson *et al.* 2006). A major challenge for the non-viral gene therapy of type 1 diabetes is to identify gene delivery systems and expression plasmids that produce the required levels and duration of gene expression in the key liver cells.

Materials and Methods

Animals

Inbred male DA strain rats (Harlan, Oxon, UK) were used at 180–220 g weight. All procedures were approved by the King's College London Ethics Committee and by the UK Home Office.

Normoglycaemic rats

Initial studies were performed on normal rats to avoid the potentially confounding effects of hyperglycaemia on insulin expression in liver (Kojima *et al.* 2004).

Studies on hyperglycaemic rats

Streptozotocin (Sigma–Aldrich) was prepared as a 4% solution by dissolving 20 mg in 0.5 ml of 10 mM citrate, 0.15 M NaCl, pH 4.5 (citrate buffer), at 4 °C and was administered within 10 min of preparation. Rats were given 25 mg/kg i.p. daily, after a 3 h fast. Blood glucose was measured using a portable glucose meter (Accu-check Aviva; Roche) from blood obtained by pricking the tail with a needle.

Because rats feed mainly at night, non-fasting blood glucose measurements were obtained at ~ 0900 h and fasting blood glucose at ~ 1500 h after removing food for 6 h. Non-fasting and fasting blood glucose in normal rats was 6.3 ± 1.2 and 6.2 ± 0.8 mM respectively (mean $\pm$ s.d., $n = 6$ per group). Non-fasting blood glucose was measured daily during streptozotocin administration. Streptozotocin was continued on the first day that non-fasting blood glucose reached 20 mM (usually day 5 or 6) and then stopped. Rats were entered into the study if the non-fasting blood glucose was > 20 mM also on the following day and on the day of gene delivery (day 8 following the first dose of streptozotocin).

Rats were weighed three times per week between 0900 and 1000 h.

Hydrodynamic gene delivery to the rat liver via the isolated inferior vena cava segment

This was performed under isoflurane general anaesthesia, using a Zeiss operating microscope for operator and assistant, as previously described (Sawyer *et al.* 2008). Briefly, a Harvard programmable syringe pump (model PHD 2000, Harvard Apparatus, Holliston, MA, USA) was used for delivery of the DNA. The inferior vena cava (IVC) segment into which the hepatic veins drain was isolated between 4–0 braided silk ties, one above and one below the liver. A 21-gauge needle was inserted into the IVC segment and the pump was activated. A volume corresponding to 2% of the body weight (i.e. 4 ml for a 200 g rat) was delivered at 100 ml/min. After 1 min, the suprahepatic and then the infrahepatic ties were removed.

Expression plasmids

Details of each plasmid are given in the Results section. Plasmids were prepared as lipopolysaccharide-free gigapreps (Qiagen), stored in aliquots at 1 mg/ml in water at -35°C and diluted to the appropriate concentration in 0.15 M NaCl just before use. For the initial studies evaluating the level and longevity of rat *Pdx1* expression, pEPI(CMV)-*pdx1* was used at 50 $\mu\text{g}/\text{ml}$ and the other pEPI plasmids at equimolar concentrations: pEPI(HCR + A1AT)-*pdx1* (55 $\mu\text{g}/\text{ml}$), pEPI(UCOE + HCR + A1AT)-*pdx1* (111 $\mu\text{g}/\text{ml}$) and pEPI(10 kb A1AT)-*pdx1* (156 $\mu\text{g}/\text{ml}$). CpG-depleted plasmid (pCpG)-*pdx1* was used at 50 $\mu\text{g}/\text{ml}$, which is a ~ 1.5 -fold molar excess over the pEPI constructs. For the combination studies with the pCpG plasmid, pCpG-*pdx1*, pCpG-*ngn3* and pCpG-*mafA* were used at 50, 48 and 53 $\mu\text{g}/\text{ml}$, which are equimolar concentrations. For the combination studies with the pEPI plasmid, pEPI(CMV)-*pdx1* was used at 66 $\mu\text{g}/\text{ml}$ and the pEPI(CMV)-*ngn3* and pEPI(CMV)-*mafA* at the equimolar concentrations of 64 and 68 $\mu\text{g}/\text{ml}$ respectively.

Harvesting of liver

Under isoflurane general anaesthesia, the abdomen was opened and the rat exsanguinated via a needle in the abdominal aorta. The liver was immediately removed and samples were taken for various analyses. For each assay, the liver sample was taken from the same anatomical site.

Preparation of total RNA from frozen liver samples

Liver samples (100–150 mg) were snap frozen in liquid nitrogen and stored at -80°C . The frozen sample was placed into 2 ml ice-cold TRIzol (Invitrogen) and homogenised with a ground glass homogeniser. Total RNA was extracted using chloroform/isoamylalcohol precipitated with isopropanol, washed in ethanol, dissolved in RNase/DNase-free water and stored in aliquots at -80°C .

Preparation of cDNA

Total RNA (10 μg) was subjected to two rounds of DNase treatment (Applied Biosystems) to remove any contaminating chromosomal or plasmid DNA. DNA-free RNA (2 μg) was converted to cDNA using random hexamers with a high-capacity cDNA RT kit (Applied Biosystems) and diluted to 100 μl in RNase/DNase-free water. This was stored at -35°C .

TaqMan quantitative PCR

TaqMan PCRs were performed in duplicate, including RT minus controls, using a 7900 HT Fast Real-Time PCR system (Applied Biosystems). The fluorescent marker

was 6-carboxyfluorescein (FAM) at the 5' end of probes with a non-fluorescent quencher (NFQ) at the 3' end. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were used as internal controls to normalise RNA levels, and the data were analysed using relative quantification.

To compare levels of *Pdx1* expression from different expression plasmids, primers and probes were based on the coding region of *Pdx1* (forward primer, 5'-CTCCCTTT-CCCGTGGATGAA-3', reverse primer, 5'-CGGTTCTG-CTGCGTATGC-3'; fluorescent probe, 5' (FAM)-CCT-GCCCACTGGCTTT-(NFQ) 3'). To evaluate rat insulin-1, insulin-2 and GAPDH, primers and fluorescent probes were provided by Applied Biosystems.

SYBR Green PCR

Power SYBR Green PCR master mix (Applied Biosystems, code: 4367659) was used. Primers were based on the 3' UTR of rat prohormone convertase 1/3: forward primer, 5'-CCTTCTCTTAATATGCCAAC-3' and reverse primer, 5'-GCTACTAATCTCACTCAAAGC-3' gave 143 bp PCR product. A dissociation curve was run to ensure that a single PCR product was obtained.

Semi-quantitative PCR

Semi-quantitative PCRs were performed using Amplitag GoldDNA polymerase (Applied Biosystems) in an MJ Research Tetrad PTC-225 thermocycler (GMI, Inc., MN, USA). cDNA (2 μl ; corresponding to 40 ng cellular RNA) was used as template, and reaction products were visualised using ethidium bromide on 1.5% agarose gels. GAPDH was used as an internal control. Pancreatic endocrine markers (glucagon, insulin-1, insulin-2, somatostatin and pancreatic polypeptide), pancreatic exocrine markers (elastase and trypsin), pancreatic β cell markers (Kir6.2 and the L type, voltage-gated calcium channel) and pancreatic transcription factors (*Pdx1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1*) were evaluated. The primers are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. Normal liver, normal pancreas and the INS-1 rat insulinoma cell line were used as controls.

DNA expression constructs

DNA polymerase from high-fidelity PCR selection kit (Invitrogen) was used to generate coding sequences and functional elements. These were cloned into the TOPO-XL-PCR transfer vector (Invitrogen) and sequenced (GATC-Biotech Ltd., Germany) to confirm accuracy of the PCR, using NCBI (GenBank) as the reference sequence. When DNA was subcloned into expression constructs, the ligation sites were sequenced to confirm fidelity of the ligation.

Immunohistology

Rabbit antisera to human PDX1, mouse NGN3 and human insulin (codes H-140: SC-25403; D-15-R:SC-13794-R and H-86:SC-9168 respectively; Santa Cruz Biotechnology, Inc.) and rabbit antisera to mouse MAFA (code A300-611A; Bethyl Laboratories, Inc., Montgomery, TX, USA) were used. These antisera cross-react strongly with the rat homologues. A mouse IgG1 monoclonal antibody to rat C peptide not reactive to rat proinsulin (code NBP1-05433, Novus Biologicals, Cambridge, UK) was used. Normal rabbit IgG or the F15-42-1 mouse IgG1 monoclonal antibody to human Thy-1 (McKenzie & Fabre 1981) was used as the negative control. Frozen sections (8 µm thickness) were placed on polylysine-coated slides (Merck) and air-dried overnight. The sections were fixed in acetone and stained using the immunoperoxidase technique with peroxidase-labelled goat anti-rabbit immunoglobulin or rabbit anti-mouse immunoglobulin as appropriate (Dako), and counterstained with Harris' hematoxylin using standard techniques.

Results

Expression constructs

Four expression constructs were based on the pEPI plasmid, the key functional elements of which are a CMV promoter and a ~2 kb scaffold/matrix attachment region (S/MAR) from the human β interferon gene (Baiker *et al.* 2000). The S/MAR confers several properties, including the capacity for episomal replication (Jenke *et al.* 2004). pEPI was modified by excising the gene for the neomycin/kanamycin fusion protein and replacing it with a kanamycin resistance gene subcloned from the TOPO plasmid (Invitrogen). This avoids *in vivo* expression of the neomycin resistance gene, which encodes a potentially immunogenic bacterial protein. This plasmid was designated pEPI(neo⁻).

Detailed steps for constructing the DNA expression plasmids are given in [Supplementary Materials and Methods](#), see section on [supplementary data](#) given at the end of this article. Briefly, rat *Pdx1* cDNA was obtained by PCR and replaced the gene for green fluorescent protein (GFP) in pEPI(neo⁻). This plasmid was designated pEPI(CMV)-*pdx1* (6927 bp) ([Fig. 1A](#)). pEPI(CMV)-*ngn3* (6720 bp) and pEPI(CMV)-*mafa* (7160 bp) were similarly constructed.

Argyros *et al.* (2008) have reported that luciferase gene expression in mouse liver is prolonged by replacing the CMV promoter of pEPI with a short alpha1-antitrypsin promoter and a liver-specific enhancer. We obtained the ApoE enhancer/alpha1-anti-trypsin promoter by PCR from the PBS-HCRHPI-A plasmid (Miao *et al.* 2000) and replaced the CMV promoter in pEPI(CMV)-*pdx1* with this promoter to produce pEPI(HCR + A1AT)-*pdx1* (7514 bp) ([Fig. 1B](#)).

The ubiquitous chromatin-opening element (UCOE; Antoniou *et al.* 2003) prevents methylation of integrated plasmids (Lindahl & Antoniou 2007) but has not previously been evaluated for episomal gene expression *in vivo*. A UCOE was cloned into pEPI(HCR + A1AT)-*pdx1* adjacent to the ApoE enhancer to produce pEPI(UCOE + HCR + A1AT)-*pdx1* (11 756 bp) ([Fig. 1C](#)).

A ~10 kb length of mammalian genome sequence is likely to contain an origin of replication (Gilbert 2004, Hibbitt *et al.* 2009) and also is less likely to undergo hypermethylation and gene silencing. For these reasons, 10 kb of the human alpha1-antitrypsin promoter was obtained by PCR and replaced the CMV promoter in pEPI(CMV)-*pdx1* to produce pEPI(10 kb A1AT)-*pdx1* (16 414 bp) ([Fig. 1D](#)).

CpG depletion of plasmids is that are known to prolong gene expression (Yew *et al.* 2002) *Pdx1*, *Nggn3* and *MafA* were cloned into the pCpG plasmid (InvivoGen, Toulouse, France), which is completely devoid of CpG dinucleotides, to give pCpG-*pdx1* (4477 bp) ([Fig. 1E](#)), pCpG-*ngn3* (4270 bp) and pCpG-*mafa* (4710 bp).

Quantitative kinetics of *Pdx1* expression

The use of a rat protein for *in vivo* gene expression studies on rats removes the possibility of immune responses to the commonly used immunogenic reporter genes (Limberis *et al.* 2009, Wang *et al.* 2009).

For the pEPI constructs, PCR primers and fluorescent probe for TaqMan PCR were based in the plasmid-derived 5' UTR and spanned the exon-exon junction ([Supplementary Fig. 1A and B](#), see section on [supplementary data](#) given at the end of this article). For each plasmid, levels of *Pdx1* expression are given relative to day 1 ([Fig. 2A, B, C, D and E](#)). With pEPI(CMV)-*pdx1*, *Pdx1* levels fell ~150-fold by day 3 ([Fig. 2A](#)). Replacement of the CMV promoter with the ApoE locus control region plus short alpha1-antitrypsin promoter ([Fig. 2B](#)) or the 10 kb genomic alpha1-antitrypsin promoter ([Fig. 2C](#)) gave more sustained levels of *Pdx1* expression, with *Pdx1* levels at ~20% of day 1 levels at day 14. Adding a UCOE element to pEPI(HCR + A1AT)-*pdx1* did not alter the pattern of expression, except that the level of expression between days 14 and 28 appeared to be sustained ([Fig. 2D](#)).

The pCpG ([Fig. 2E](#)) behaved similarly to the pEPI plasmids without the viral promoter. The PCR primers and fluorescent probe for TaqMan PCR for pCpG-*pdx1* were from the coding region of *Pdx1* (see Materials and Methods section) and therefore would not distinguish plasmid-derived *Pdx1* from endogenous *Pdx1*. However, using primers and probe specific for the 3' UTR of endogenous *pdx1* ([Supplementary Fig. 1C](#)), no endogenous *Pdx1* was detected by TaqMan PCR (data not shown).

A comparison of the levels of *Pdx1* expression with the five constructs is given in [Fig. 2F](#). For this figure, all samples in [Fig. 2A, B, C, D and E](#) were analysed on the same plate using primers and fluorescence probe based on the coding region of *Pdx1* (see Materials and Methods section). Levels are given

Figure 1 Expression plasmids for rat *Pdx1*. (A) The pEPI plasmid with a ~ 2 kb S/MAR from the human β interferon gene (Baiker *et al.* 2000, Sawyer *et al.* 2008) was modified by replacing the composite neomycin/kanamycin resistance gene with a kanamycin resistance gene under a bacterial promoter. The gene for GFP was replaced with rat *pdx1* to give pEPI(CMV)-*pdx1*. I indicates an intron in the 5' UTR. pAn indicates a polyadenylation signal. Note that the S/MAR is within the *Pdx1* transcription unit. (B) The CMV promoter of pEPI(CMV)-*pdx1* was replaced with the alpha1-antitrypsin promoter/ApoE locus control region of PBS-HCRHPI (Argyros *et al.* 2008) to give pEPI(HCR+A1AT)-*pdx1*. (C) The HNRPA2B1/CBX3 UCOE (Miao *et al.* 2000) was cloned into the pEPI(HCR+A1AT)-*pdx1* 3' to the enhancer to produce pEPI(UCOE+HCR+A1AT)-*pdx1*. (D) The CMV promoter of pEPI(CMV)-*pdx1* was replaced with a ~ 10 kb genomic promoter of human alpha1-antitrypsin to produce pEPI(10 kb A1AT)-*pdx1*. (E) Rat *Pdx1* was cloned into pCpG, an expression plasmid completely devoid of CpG dinucleotides and with an EF-1 promoter (hEF1p; InvivoGen, Inc.) to produce pCpG-*pdx1*. I, pAn and MAR as earlier. pAn indicates a polyadenylation signal. Note that the S/MAR is not within the *Pdx1* transcription unit.

relative to pEPI(CMV)-*pdx1* at day 1. pEPI(CMV)-*pdx1* and pCpG-*pdx1* had the highest levels of expression at day 1, with little difference between them. Removal of the CMV promoter from pEPI resulted in markedly lower levels of gene expression at day 1 ($P=0.003$, unpaired Student's *t*-test for

comparisons of pEPI(CMV) with each of the other three pEPI plasmids). Thus, although the three pEPI plasmids without a CMV promoter gave a more sustained level of *Pdx1* expression (Fig. 2B, C, D and E), the actual level of expression was much lower (Fig. 2F). The pCpG plasmid by contrast

Figure 2 Quantitative kinetics of *Pdx1* expression from different plasmids. The five expression plasmids were delivered to the livers of normal DA rats using the IVC segment hydrodynamic approach, with volumes corresponding to 2% of body weight at 100 ml/min. pEPI(CMV)-*pdx1* was used at 50 μ g/ml in 0.15 M NaCl (A) and the other pEPI plasmids in the equimolar concentrations of 55, 156 and 111 μ g/ml for (B) to (D) respectively. pCpG-*pdx1* was used at 50 μ g/ml (E), which is a ~ 1.5 molar excess over the pEPI plasmids. Groups of three (pEPI plasmids) or four (pCpG plasmid) rats were killed at days 1, 3, 14 and 28 after gene delivery, and the livers examined by TaqMan PCR for *Pdx1* expression, using primers and probe from the *Pdx1* coding sequence (see Materials and Methods section). Each sample was assayed in duplicate. Values are the mean $\pm$ 1 s.d. calculated relative to day 1. In (F), all 64 rats shown in (A, B, C, D and E) were evaluated simultaneously, and values are given relative to day 1 for pEPI(CMV)-*pdx1*. In all rats, RT minus PCR was negative.

gave a relatively high level of *Pdx1* expression sustained for several days.

TaqMan PCR for endogenous *Pdx1* was performed in all 64 rats shown in Fig. 2F, but none was detected.

Visualisation of transgene expression

As expected from studies with reporter genes (Sawyer *et al.* 2008, 2009), *Pdx1* expression was seen in isolated scattered cells (Fig. 3).

The use of *Pdx1* alone Using adenoviral vectors, *Pdx1* alone (Ferber *et al.* 2000, Kojima *et al.* 2003) and *Ngn3* with

Figure 3 Visualisation of transgene expression in liver after hydrodynamic delivery. Frozen sections of liver taken 1 day after delivery of pEPI(CMV)-pdx1 to normal DA rats were stained using the immunoperoxidase technique and rabbit antibodies to *Pdx1* (A) or normal rabbit Ig (B). Some of the positive cells are marked with an arrow. Very similar results were obtained with livers from rats given the pEPI(CMV)-ngn3 or pEPI(CMV)-mafA plasmids (data not shown). None of the antibodies stained normal liver (data not shown). A $\times 100$; B $\times 200$.

the growth factor β -cellulin (Yechoor *et al.* 2009) have resulted in liver to pancreatic β cell transdifferentiation. As an initial screen for differentiation towards pancreatic β cells, all 64 rats shown in Fig. 2 were screened by TaqMan PCR for rat insulin-1, rat insulin-2 and endogenous rat *Pdx1*. In no case was any expression noted (data not shown).

The use of *MafA* alone As *MafA* appears late in pancreatic β -cell differentiation (Habener *et al.* 2005), we evaluated pCpG-*mafA* alone. Groups of three DA rats were given pCpG-*mafA*, and livers were harvested at days 1, 3, 7, 14 and 28. In none of the 15 rats was insulin-1, insulin-2 or endogenous *Pdx1* seen by TaqMan PCR (data not shown).

The use of multiple transcription factors The pEPI(CMV) and pCpG plasmids encoding *Ngn3* and *MafA* gave excellent transgene expression at day 1 following hydrodynamic delivery of single constructs to the liver, as shown by immunohistochemistry (as in Fig. 3) and semi-quantitative PCR (data not shown). When *Pdx1*, *Ngn3* and *MafA* were given together, either in the pEPI(CMV) plasmid (Fig. 4A) or pCpG plasmid (Fig. 4B and C), insulin-2 expression was readily seen in the transfected livers. With pEPI(CMV), insulin-2 was not detectable at day 1, peaked over days 3, 7 and 14 and returned to background levels by day 28 (Fig. 4A). Insulin-2 levels were ~ 50 -fold higher with pCpG at days 3 and 7, with substantial insulin-2 expression at day 1 (Fig. 4B). Results for individual rats receiving pCpG are given in Fig. 4C. Given the ~ 10 - to 20-fold larger size of liver in relation to pancreas, the insulin mRNA content of the livers at days 3 and 7 was approximately equal to that of pancreas. However, insulin-2 levels were declining at days 14 and 28 relative to days 3 and 7. This suggests that, if the transdifferentiation process had been initiated, it had not reached the stage of irreversibility.

PDX1 and MAFA both bind to the insulin gene promoter and are involved in activation of transcription of the insulin gene (Aramata *et al.* 2005). However, neither factor alone induced insulin expression (see above). The insulin gene expression seen in Fig. 4 therefore probably represents the result of a more complex transdifferentiation process. To investigate this further, the presence of other pancreatic β cell proteins was evaluated. The results in Fig. 5 demonstrate that the prohormone convertase 1/3, essential for processing of inactive proinsulin to insulin, was transiently elevated on days 3 and 7 after the multiple factors in the pCpG plasmid but not the pEPI plasmid. This is consistent with gene delivery initiating a process of transdifferentiation which, however, reversed in the second week.

In none of the 35 rats shown in Fig. 4 was endogenous *Pdx1* detected by TaqMan PCR. Very low levels of insulin-1 were seen at day 3 in the group receiving the pCpG plasmid (data not shown).

Using transgene-specific PCR primers, the expression of *Pdx1*, *Ngn3* and *MafA* was evaluated in the livers of rats given pCpG-*pdx1*, pCpG-*ngn3* and pCpG-*mafA* together

Figure 4 Quantitative kinetics of insulin expression in liver after administration of multiple transcription factors. pEPI(CMV)-pdx1, pEPI(CMV)-ngn3 and pEPI(CMV)-mafA (A) or pCpG-pdx1, pCpG-ngn3 and pCpG-mafA (B) were delivered to normal DA rats using the IVC segment hydrodynamic approach. Livers were harvested at days 1, 3, 7, 14 and 28, with three or four rats per group. TaqMan PCR for insulin-2 expression in all 35 rats is shown, relative to insulin-2 expression in normal pancreas. Normal liver (N) was included as a control. Each rat was assayed in duplicate, and the results are given as the mean $\pm$ s.d. for each group of four rats. In (C), results for individual rats of the pCpG group are given. The Mann-Whitney *U* test was used for the statistical comparisons.

Figure 5 Quantitative kinetics of prohormone convertase 1/3 expression after administration of multiple transcription factors. pEPI(CMV)-pdx1, pEPI(CMV)-ngn3 and pEPI(CMV)-mafA (A) or pCpG-pdx1, pCpG-ngn3 and pCpG-mafA (B) were delivered to normal DA rats using the IVC segment hydrodynamic approach. Livers were harvested at days 1, 3, 7, 14 and 28, with three or four rats per group. These are the same rats as evaluated in Fig. 4. SYBR Green quantitative PCR for expression of prohormone convertase 1/3 is shown relative to expression in normal liver (N). Each rat was assayed in duplicate, and the results are given as the mean $\pm$ s.d. for each group of four rats. The Mann-Whitney *U* test was used for statistical comparisons.

(Fig. 6). All three transcription factors were expressed together in the livers of all rats in the first week after gene delivery. Thereafter levels declined but were detectable in most rats at day 28.

Localisation of insulin protein in liver

Strong insulin expression was seen in scattered cells with hepatocyte morphology in rats given the combination of pCpG-pdx1, pCpG-ngn3 and pCpG-mafA (Fig. 7B). The insulin-positive cells were more readily observable at days 3 and 7 than at days 14 and 28 after gene delivery. However, although the C-peptide was easily detectable in pancreatic islets (Fig. 7D), it could not be detected in

Figure 6 Transgene expression in liver after administration of multiple constructs. Livers from a normal rat (N) and from all rats given pCpG-pdx1, pCpG-ngn3 and pCpG-mafA together (Fig. 4B and C) were evaluated by semi-quantitative PCR for transgenic *Pdx1*, *Ngn3* and *MafA* expression using PCR primers specific for the transgenic products. The reverse primer for the pCpG-pdx1 and pCpG-ngn3 was from the 3' UTR of plasmid origin: 5'-GCAATAGCATCACAATTCACA-3'. The forward primers were from the coding sequence of the expressed gene: 5'-CATTGCCACCGC-CACCACT-3' for pCpG-pdx1 and 5'-TCCCAAGCTGGTAGCCT-GAGC-3' for pCpG-ngn3 respectively. The forward primer for pCpG-mafA was from 5' UTR of plasmid origin: 5'-TCTCC-CTCCTGTGAGTTTGGTTG-3' and the reverse primer from the coding sequence of mafA: 5'-GTCGAGGATAGCGAGCCGGT-3'. Samples for days 1, 3 and 7 were run on one gel, and for days 14 and 28 and normal liver on a different gel.

the livers of rats with insulin expressing cells as in Fig. 7B. It should be borne in mind that immunohistology is not a sensitive technique for detecting target molecules.

Semi-quantitative evaluation of markers for pancreas differentiation

Transient expression of insulin-2 in rats given the pEPI(CMV) construct (Fig. 8A) is consistent with the TaqMan quantitative assays for this group (Fig. 4A). The other pancreatic hormones (glucagon, somatostatin and pancreatic polypeptide) could not be detected. The L-type voltage-gated Ca⁺⁺ channel of pancreatic β cells was detectable from day 3, most strongly at day 7. Interestingly, the pancreas exocrine markers (trypsin and elastase) were readily detected in some rats, suggesting transdifferentiation towards exocrine as well as endocrine pancreas. The pancreas transcription factors (*Pdx1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1*) were not detected.

Insulin-2 was easily detected in all rats given the pCpG construct (Fig. 8B). The other pancreatic islet hormones were not detectable, nor were pancreatic transcription factors. However, the pancreatic β-cell marker Kir6.2 (a component of the ATP-sensitive K⁺ channel of pancreatic β cells) and the Ca⁺⁺ channel were expressed from day 3. The pancreas exocrine markers (trypsin and elastase) were also seen in most livers.

Studies on hyperglycaemic rats

To see whether the insulin detected in the transfected livers was functional and whether the addition of hyperglycaemia to the delivery of the transcription factors might enhance the transdifferentiation effect, we delivered *Pdx1*, *Ngn3* and *MafA* together in the pCpG plasmid to six hyperglycaemic DA rats.

pEPI(CMV) encoding an irrelevant rat protein (lactase; at 86 μg/ml) was given to five hyperglycaemic control DA rats.

There was no difference between the experimental and control groups with regard to non-fasting blood glucose (Fig. 9A). However, fasting blood glucose was lower in the experimental group on days 1, 3 and 7 after gene delivery (Fig. 9B; *P*≤0.05 for each of these days, Fisher's exact test). This is consistent with the high levels of insulin-2 mRNA (Fig. 4B) and the simultaneous presence of the prohormone convertase 1/3 (Fig. 5B) in the livers of these rats over this period. The average weight loss from the time of gene delivery to day 25 was 12.8% in the control group, but only 1.1% in the experimental group (Fig. 9C; *P*≤0.05, Fisher's exact test). Body weights at days 14, 18, 21 and 25 were higher in the experimental group (*P*≤0.04 for each time-point, unpaired *t*-test). This is consistent with the low but definite expression of insulin-2 mRNA over the 25 days of the study (Figs 4B and 8B).

Discussion

We have quantitated rat *Pdx1* expression in liver following the hydrodynamic delivery of five distinct DNA expression plasmids to normal rats. This was to determine the level and longevity of gene expression with each construct and to see whether *Pdx1* expression alone could initiate transdifferentiation in normoglycaemic rats. Each plasmid gave markedly different patterns of expression. However, in none of the 64 rats examined by quantitative PCR over days 1, 3, 7, 14 and 28 after delivery of *Pdx1* alone was there any insulin

Figure 7 Immunohistological localisation of insulin in rat liver. Frozen sections of normal rat pancreas (A and D) or of liver from a normoglycaemic rat 3 days after delivery of pCpG-pdx1, pCpG-ngn3 and pCpG-mafA via the IVC segment hydrodynamic technique (B and C) are shown. Sections were stained with rabbit antibodies to insulin (A and B), normal rabbit IgG (C) or a mouse monoclonal antibody to rat C-peptide (D) using the indirect immunoperoxidase technique. The insulin-positive cells in liver are marked with an arrow. A and C, ×200; B, ×400.

Figure 8 Semi-quantitative PCR of markers of pancreas differentiation. The rats given pEPI(CMV)-pdx1, pEPI(CMV)-ngn3 and pEPI(CMV)-mafa (A) or pCpG-pdx1, pCpG-ngn3 and pCpG-mafa (B) were evaluated by semi-quantitative PCR for markers of pancreas differentiation. The primers used for each PCR are given in Supplementary Table 1. Note that the primers for *Pdx1* were specific for endogenous *Pdx1* of cellular origin. The negative control was normal liver (N). Positive controls were the rat insulinoma cell line INS-1 (I) and normal rat pancreas (P). GAPDH was used as the internal control. P. Polypeptide is pancreatic polypeptide. Kir6.2 is a component of the ATP-sensitive K^+ channel in pancreatic β cells. Ca channel is the L-type voltage-gated Ca^{++} channel of pancreatic β cells. In (A), samples for days 1, 3, 7, 14 and 28 were run on one gel, and for the controls on a different gel. One of the samples for day 28 gave an unusual picture, which was not reproducible on mRNA taken from another part of the liver, and was therefore omitted. In (B), samples for days 1, 3 and 7 were run on one gel, and the samples for days 14 and 28 and controls on a different gel.

expression. Chen *et al.* (2009) have reported that the hydrodynamic delivery of *Pdx1* alone in a commercially available plasmid (pCDNA 3.1, Invitrogen) to hyperglycaemic mice resulted in insulin-positive cells by immunohistology in the liver at week 1 but not week 2 after gene delivery. By contrast, Wang *et al.* (2007) did not obtain insulin expression in liver by hydrodynamic delivery of *Pdx1* alone in hyperglycaemic mice, using a plasmid containing an alpha1-antitrypsin promoter and liver-specific enhancer (Argyros *et al.* 2008). It is difficult to compare these studies, other than to note the different results with the different plasmids in hyperglycaemic mice. In our studies with *Pdx1* alone, rats rather than mice were used, and the rats were

normoglycaemic in order to avoid the confounding effects of insulin expression in liver as a consequence of hyperglycaemia *per se* (Kojima *et al.* 2004).

In contrast to the results with *Pdx1* alone, we were able to obtain strong insulin expression using the hydrodynamic

Figure 9 Studies on streptozotocin-treated hyperglycaemic rats. Hyperglycaemic DA rats (non-fasting blood glucose ≥ 20 mM) received *Pdx1*, *Ng3* and *MafA* in pCpG plasmids (experimental group $n=6$) or pEPI(CMV) encoding rat lactase (control group $n=5$). The mean $\pm$ s.d. of non-fasting blood glucose (A), fasting blood glucose (B) and body weight (C) on the days indicated are shown. Weight loss from the time of gene delivery to day 25 was less in the experimental group (1.1% in comparison with 12.8%; $P < 0.05$, Fisher's exact test). Star indicates $P \leq 0.05$ for comparisons between experimental and control groups.

approach in normoglycaemic rats, with a combination of three transcription factors, *Pdx1*, *Ngn3* and *MafA*. Two plasmids were evaluated for the delivery of multiple genes. Both plasmids provided comparably high levels of gene expression in liver 1 day after gene delivery, but pCpG gave more prolonged expression than pEPI(CMV). Both plasmids stimulated insulin expression in liver, but pCpG gave insulin levels ~75-fold higher than pEPI(CMV). The insulin content of the liver at days 3 and 7 was approximately equivalent to that of a pancreas. However, levels decreased substantially over days 14–28. The levels of prohormone convertase 1/3 were also elevated at days 3 and 7, but not at later times, with the pCpG plasmid. These data suggest that, if a transdifferentiation process had been initiated in the normoglycaemic rats, it was reversible.

It is known that hyperglycaemia promotes transdifferentiation to pancreatic β cells (Vanderford *et al.* 2007). All previous published work in this area has been on hyperglycaemic animals. Our studies on hyperglycaemic rats demonstrated that at least some of the insulin produced in the liver was functionally active and therefore that it had undergone appropriate processing and release from the cell. However, lowering of the fasting blood glucose was seen only in the first week after gene delivery, suggesting that any transdifferentiation was reversible, even in the presence of hyperglycaemia.

It is uncertain whether the delivery of *Pdx1*, *Ngn3* and *MafA* to the rat liver initiated a process of transdifferentiation, or simply directly stimulated insulin expression. The fact that multiple transcription factors were required, and that the complex process of secreting mature insulin was achieved, would suggest transdifferentiation. However, the process was reversible and inferior to the results obtained using adenoviruses to deliver the same transcription factors to mouse pancreas. It is possible that the exocrine pancreas cells, being closer embryologically to pancreatic β cells, are a more favourable target than liver cells for transdifferentiation. However, it is also possible that the level and, in particular, the longevity of gene expression, even from our best plasmid (pCpG), were insufficient to drive the liver cell into irreversible transdifferentiation.

Hydrodynamic gene delivery to the liver is a relatively inefficient procedure in the rat, giving of the order of 2 or 3% transfected cells (Fig. 3). This is potentially a problem in two respects. First, the number of liver cells potentially able to transdifferentiate into pancreatic β cells is low. However, given the large size of the liver in comparison with pancreatic islets, even a low percentage of transdifferentiated liver cells is likely to be sufficient. In our study, even though we achieved only a scattering of insulin-positive cells (Fig. 7), the amount of insulin in the livers was equivalent to a pancreas. The second potential problem concerns the necessity for co-expression of all three constructs in the required target cell. Given the large number of plasmids in liver cells following hydrodynamic gene delivery – measured at ~20 000 copies/cell in the mouse (Argyros *et al.* 2008) – it

is unlikely that expression of single plasmids in individual transfected cells is a problem. This has formally been proven by the simultaneous hydrodynamic delivery in mice of two distinct plasmids, one encoding a GFP and the other a red fluorescent protein. In all cases, liver cells expressed both proteins (Richard Harbottle, Simon Waddington and Charles Coutelle, Imperial College London; personal communication of work performed in 2002). The precise nature of the liver cells that undergo transdifferentiation to pancreatic β cells is also uncertain. If it is a relatively rare cell type, such as the oval cell, the relative susceptibility of this cell to hydrodynamic gene delivery will be crucial. However that may be, in our studies, the insulin-expressing cells frequently had a hepatocyte morphology, and the amount of insulin produced by the liver was equivalent to a pancreas. The fundamental problem highlighted by our study is unlikely to be inefficiency in the number of cells undergoing transdifferentiation, but the fact that the transdifferentiation process was not irreversible.

Semi-quantitative PCR following administration of adenoviral vectors showed strong expression of the transcription factors at 10 days after adenovirus delivery and weak expression at day 30 (Zhou *et al.* 2008). Thus, strong expression of the transcription factors for several weeks seems sufficient. From a practical point of view, the marked difference in efficacy between pCpG and pEPI(CMV) in our studies is important to note. For the future, it is possible that expression plasmids that provide more sustained gene expression than pCpG will be superior for irreversible transdifferentiation.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0033>.

Declaration of interest

J-P R is an employee of InvivoGen, Inc., a company involved among other things in the development of DNA expression constructs for experimental and clinical application. There is otherwise no potential conflict of interest among the authors.

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