Ectopic expression of neurogenin 3 in neonatal pig pancreatic precursor cells induces (trans)differentiation to functional alpha cells

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ARTICLE

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Ectopic expression of neurogenin 3 in neonatal pig pancreatic precursor cells induces (trans)differentiation to functional alpha cells

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Abstract *Aims/hypothesis:* Neurogenin 3 (NEUROG3), a basic helix-loop-helix transcription factor that is needed for endocrine cell development in the embryonic pancreas, has been shown to induce transdifferentiation of duct cells from adult pancreas towards a neuro-endocrine phenotype. Our study explored the endocrine transdifferentiation potential of NEUROG3 in neonatal pancreatic precursor cells. *Materials and methods:* A replication-deficient adenovirus expressing *Neurog3* and green fluorescent protein (*GFP*) (Ad-NEUROG3) was used to infect neonatal pig pancreatic cell preparations enriched for endocrine islet and cytokeratin-positive precursor cells. GFP-positive cells were sorted using flow cytometry on days 3 and 8 after infection and characterised at the transcript and protein level. For in vivo experiments, the total population of Ad-NEUROG3-infected pancreatic cells was transplanted, then later removed for determination of graft hormone content and immunohistochemistry. *Results:* Among the GFP-positive cells, the fraction of precursor cells decreased by more than 85% at day 8 after infection, while the fraction of glucagon-positive cells increased 2.5-fold and the beta cell number remained the same. Transplantation of the Ad-NEUROG3-infected pancreatic cell preparation failed to reverse streptozotocin-induced hyperglycaemia, while non-infected cells and a control cell preparation infected with replication-deficient adenovirus expressing only *GFP* were able to do so. At day 109 after transplantion, kidneys grafted with Ad-NEUROG3-infected pancreatic cells contained significantly decreased insulin and increased glucagon levels. Abundant glucagon-immunopositive cells were seen in Ad-NEUROG3-infected grafts, which were virtually devoid of proliferating insulin-positive cells. *Conclusions/interpretation:* In summary, adenoviral delivery of NEUROG3 to pancreatic precursor cells from neonatal pig pancreas promotes alpha cell differentiation in vitro and in vivo.

Keywords Adenovirus · Alpha cell · Differentiation · Neonatal pig pancreatic cells · Neurogenin 3 · Transplantation

Abbreviations ABC: avidin-biotin complex · Ad-GFP: replication-deficient adenovirus expressing *GFP* · Ad-NEUROG3: replication-deficient adenovirus expressing *Neurog3* and *GFP* · CK7: cytokeratin-7 · DAB: diaminobenzidine · EYFP: enhanced yellow fluorescence protein · GFP: green fluorescent protein · NEUROG3: neurogenin 3 · PCNA: proliferating cell nuclear antigen · TUNEL: Tdt-mediated dUTP nick-end labelling

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Introduction

Type 1 diabetes is an autoimmune disease in which insulin-producing pancreatic beta cells are destroyed. Therefore, lifesaving exogenous insulin must be administered to promote glucose utilisation by the body and maintain normal blood glucose levels. Insulin injections, however, fail to mimic the body's natural release of insulin in response to glucose. The replacement of insulin-producing beta cells by islet transplantation may provide patients with a more physiological therapeutic alternative to insulin injections [1–3]. Yet for islet transplantation to become the current treatment of choice for diabetes, an unlimited supply of islets must first be found. Currently, extensive research is being devoted to the study of xenogeneic islets as a potential source of tissue for transplantation into patients with type 1 diabetes. In particular, neonatal pig pancreatic islet preparations may be preferred over adult pig pancreatic islets, since our group and others have shown that neonatal islets are easier to isolate and maintain

in culture, contain many cytokeratin-7 (CK7)-positive endocrine precursor cells, and are less immunogenic than adult pig islets [4–7]. While pancreatic cells from neonatal pigs are composed of only ~25% beta cells, the differentiation of the CK7-positive precursor population into beta cells is necessary to reverse hyperglycaemia in diabetic mice, which normally takes 6 to 9 weeks [4, 5]. Thus, inducing the differentiation of beta cells from precursors within the neonatal pancreas in vitro prior to transplantation would perhaps lead to enhanced in vivo function after transplantation. This has been demonstrated to be the case when neonatal pancreatic cells are provided an alginate extracellular matrix or cultured with nicotinamide [4, 8].

Another approach to induce beta cell neogenesis is by ectopic expression of embryonic transcription factors such as PDX-1 or NeuroD1, since they are involved in islet cell development [9–11]. In particular, neurogenin 3 (NEUROG3), a member of the basic helix-loop-helix transcription factor family, is expressed in pancreatic epithelial precursor cells prior to endocrine differentiation, which, if absent, results in an absence of the endocrine component of the pancreas [12–15]. Following ectopic expression of NEUROG3 in adult human pancreatic ductal cells, a neuroendocrine phenotype was induced with an increased fraction of insulin-positive cells 10 days after transduction [16]. Similarly, NEUROG3 transfection of retinoic acid-treated mouse ES cells was shown to induce insulin transcription and other endocrine genes [17]. In contrast, in mouse embryos with premature expression of NEUROG3 in most pancreatic precursor cells no beta but rather alpha cells developed [13, 15], suggesting that additional signals are required to deviate the differentiation of precursor cells from alpha cells into beta cells. In addition, Grapin-Botton et al. demonstrated predominant alpha cell development and some delta cell differentiation from chick endoderm following ectopic NEUROG3 expression [18]. A recent report by Dominguez-Bendala et al. demonstrated that direct administration of cultured embryonic pancreatic explants with NEUROG3 protein rather than by genetic manipulation with the *Neurog3* gene can also promote alpha cell differentiation at the expense of other pancreatic cell types [19]. The aim of this study was to induce endocrine cell differentiation in pancreatic cells from neonatal pigs by ectopic adenovirus-mediated expression of NEUROG3.

Materials and methods

Neonatal pig pancreatic cells

Cells were obtained from 1- to 2-day-old Duroc pig pancreases (Swine Research and Technology Center, University of Alberta, Edmonton, AB, Canada). Neonatal pancreatic cell aggregates were isolated and cultured as previously described by Korbutt et al. [4].

Adenoviral transduction

A replication-deficient adenovirus containing the genes for murine *Neurog3* as well as enhanced green fluorescent protein (*GFP*) as a reporter, both driven by separate cytomegalovirus promoters, was used. Cells were infected using a modification of a previously described protocol [16]. Prior to infection, pancreatic cells were collected in a cation- and phenol-red-free Hank's balanced salt solution (Sigma, St Louis, MO, USA) supplemented with 1 mmol/l EGTA and 0.5% BSA [4], with frequent pipetting for 10 min before infection in order to break down the extracellular matrix and enhance access of viral particles within the aggregates. Preliminary experiments showed this treatment to significantly increase infection efficiency over untreated aggregates (data not shown). Cells were then seeded in 60-mm non-tissue-culture-treated plates (Fisher Scientific, Edmonton, AB, Canada) at 10×10^6 cells/5 ml Hams F10 medium supplemented with 10% neonatal pig serum. These cultures were infected at a multiplicity of infection of 50 with either a replication-deficient adenovirus expressing *Neurog3* and *GFP* (Ad-NEUROG3) or a control replication-deficient adenovirus expressing only *GFP* (Ad-GFP) for 4 h at 37°C, and were subsequently washed to remove viral particles and then cultured in Hams F10 plus 10% neonatal pig serum for either 3 or 8 days. A non-infected control group received no virus and was cultured in the same medium as the infected group for a similar time period.

Post-infection in vitro assessment

Prior to infection, intact cellular aggregates were washed with PBS, fixed in 4% paraformaldehyde for 30 min and stored in PBS. Samples were subsequently embedded in a 2% low melting point agarose solution and allowed to harden at 4°C before processing, paraffin embedding and sectioning (5 μm).

At 3 and 8 days after infection, infected and control preparations were assessed for cellular insulin and DNA content as well as for cellular composition by immunocytochemistry. Insulin and DNA content were analysed as previously described [4] and expressed as percent recovery compared with controls. Cell composition was determined by dissociating aggregates into single cell suspensions by gentle agitation in a 37°C water bath in cation- and phenol-red-free Hank's balanced salt solution, supplemented as above and also with trypsin (25 μg/ml; Boehringer Mannheim, Indianapolis, IN, USA) and DNAse (4 μg/ml; Boehringer Mannheim). Cells were then placed on positively charged Histobond microscope slides (Paul Marienfeld, Lauda-Koenigshofen, Germany) and fixed in a water-based Bouin fixative for 12 min before storage at 4°C in 70% ethanol [20].

To assess which cells expressed NEUROG3 after infection, fluorescence-activated cell sorting was used to separate GFP-positive (i.e. NEUROG3-positive) and negative cell populations. Pancreatic single cell suspensions as

described above were obtained on either days 3 or 8 after infection and analysed by flow cytometry (EPICS Elite ESP flow cytometer; Coulter, Hialeah, FL, USA). Presorted and GFP-positive and -negative cells were collected and assessed for cell composition by immunocytochemistry. Immunostaining was visualised using the avidin-biotin complex (ABC) method with peroxidase and diaminobenzidine (DAB) as the chromagen. Endogenous peroxidase was quenched with a 10% H_2O_2 methanol solution. Microwave antigen retrieval for NEUROG3, CK7 and synaptophysin staining was performed in 10 mmol/l citrate buffer (pH 6.0). Blocking was performed with 20% normal goat serum (Fischer) for 15 min. Primary antibody concentrations were as follows: 1:1,000 guinea-pig anti-porcine insulin (Dako Diagnostics Canada, Mississauga, ON, Canada), 1:5,000 guinea-pig anti-glucagon (Linco Research, St Charles, MO, USA), 1:50 rabbit anti-human synaptophysin (Dako), 1:200 mouse anti-human CK7 (Dako) and 1:1,000 rabbit anti-mouse NEUROG3 (Michael German, UC San Francisco, CA, USA). Primary antibody incubations were for 30 min followed by two washes in PBS before addition of secondary antibody. All biotinylated secondary antibodies were obtained from Vector Laboratories (Burlingame, CA, USA) and used at a concentration of 1:200 for 20 min. For insulin and glucagon, anti-guinea-pig IgG was used, while for synaptophysin and NEUROG3, anti-rabbit IgG and for CK7 anti-mouse IgG secondary antibodies were used, respectively. ABC complex (Vector) incubation time was for 40 min and visualisation was with DAB (BioGenex, San Ramon, CA, USA). Single cell counts were performed on a minimum of 500 cells per sample and percentages calculated as a number of positive cells per 500 cells.

RT-PCR

Infected and control samples taken on days 3 and 8 were suspended in Trizol reagent and stored at −80°C for molecular analysis. All samples were subsequently thawed and RNA extracted according to the manufacturer's protocol (Gibco, Burlington, ON, Canada). cDNA was transcribed from 1 μg mRNA with 10 U (200 Uμ/l) Superscript reverse transcriptase in 1×buffer containing 0.01 mol/l dithiothreitol, 0.5 mmol/l dNTPs and 0.02 μg/μl oligo dT15. For each sample, 2 μl of cDNA were used per 25 μl reaction along with 1×PCR buffer, 2 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 1 U Taq polymerase (5 U/ml), and 0.5 mmol/l of each primer pair. For all PCR reactions, 35 cycles were performed, with 30 s denaturation at 94°C, 30 s annealing at 58°C and 30 s extension at 72°C. Final polymerisation was at 72 C for 10 min followed by a 4°C hold. Products were separated on a 2% ethidium bromide-stained agarose gel and images captured with Alpha Digidoc software (Perkin-Elmer, Boston, MA, USA). Primer sequences were as follows: 5′-GCT TCT TCT ACA CGC CCA AG-3′ (forward) and 5′-CCA GCT GGT AGA GGG AAC AG-3′ (reverse) (insulin 102-bp fragment); 5′-CCC AAG ATT TTG TGC AGT GGT T-3′ (forward) and 5′-GCG GCC AAG TTC TTC AAC AAT-3′ (reverse) (glucagon 221 bp fragment); 5′-CCG GAT GAC GCC AAA CTT ACA-3′ (forward) and 5-ACA CCA GTG CTC CCG GGA G-3′ (reverse) (murine NEUROG3 288-bp fragment); and 5′-TGT ATT CCC CTC CAT CGT G-3′ (forward) and 5′-GGA TCT TCA TGA GGT AGT CTG TC-3′ (reverse) (β-actin 500-bp fragment). Negative controls consisted of water (Sigma) instead of experimental cDNA.

Transplantation

Immune-deficient C57BL/6-rag1$^{tm1/mom}$ (B6 *rag1*−/−, H2^{b}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and used as recipients. Mice were rendered diabetic by a single i.p. injection of 175 mg/kg streptozotocin (Sigma) dissolved in cold acetate buffer (pH 4.5) 2 days before transplantation. Diabetes was defined as a minimum of two consecutive blood glucose measurements ≥20 mmol/l. Blood from the tail vein of each recipient was collected once a week to monitor glucose levels using a glucose meter (One Touch Ultra; Lifescan, Milpitas, CA, USA). On the day of transplantation, mice were anaesthetised using halothane and grafts of 2,000 aggregates were transplanted in a single operation beneath the left renal capsule using a micromanipulator syringe as previously described [4]. In addition to non-infected control and Ad-NEUROG3-infected groups, an Ad-GFP-infected control group was included to assess the effect of GFP on endocrine function after transplantion. Transplanted animals were monitored once a week for blood glucose levels. All mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care.

Graft hormone content and morphology

Graft insulin or glucagon content was assessed before and after transplantation. For hormone extraction, grafts were homogenised and then sonicated at 4°C in 2 mmol/l acetic acid (0.25% BSA). After 2 h at 4°C, tissue homogenates were resonicated, centrifuged (8,000 ×*g*, 25 min), then supernatants were collected and the pellets further extracted by sonication in acetic acid, then stored for 1 h at 4°C. The second supernatant was collected after centrifugation, combined with the first supernatant, the total volume was measured, and samples were assayed for insulin or glucagon content. For morphological assessment, harvested grafts were immersed in Z-fix solution (Anatech, Battle Creek, MI, USA) and embedded in paraffin. Sections, 5 μm thick, were immunostained for the presence of insulin-, glucagon- and CK7-positive cells, as described above and counterstained with Harris' haematoxylin for 2 min.

Apoptosis was detected by Tdt-mediated dUTP nick-end labelling (TUNEL) staining (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Sections were counterstained with an anti-insulin antibody and phycoerythrin-conjugated goat anti-guinea-pig IgG secondary antibody (1:200; Jackson Immunoresearch, West Grove, PA, USA). Cell proliferation was assessed using a monoclonal mouse anti-PCNA (proliferating cell nuclear antigen) (1:50, Dako) and Cy3 anti-mouse IgG secondary antibody (1:200; Jackson Immunoresearch).

Incubation times for primary and secondary antibodies were 1 h at room temperature in a humidified chamber.

Statistical analysis

Data are expressed as means±SEM of *n* observations. Statistical significance of differences was determined using one-way ANOVA with SPSS statistical software, version 12.0 for Windows (Chicago, IL, USA). $p<0.05$ was considered to be statistically significant.

Results

In vitro assessment of neonatal pancreatic cells infected with Ad-NEUROG3

Following collagenase digestion of neonatal pancreases and 7 to 9 days of tissue culture, cellular aggregates formed in suspension (Fig. 1). Immunocytochemical staining of aggregates for insulin (Fig. 1a), glucagon (Fig. 1b), CK7 (Fig. 1c) and synaptophysin revealed that 23.5±3.3, 17.6±4.3, 39.7±8.6 and 55.3±4.3% of cells were immunopositive, respectively. After infection with the Ad-NEUROG3 virus, ectopic expression of *Neurog3*, as visualised by GFP fluorescence and *Neurog3* mRNA expression, remained detectable at 3 and 8 days after infection in all preparations examined (Fig. 2). No GFP fluorescence or *Neurog3* mRNA were detected in non-infected controls. In addition to *Neurog3*, mRNAs encoding insulin and glucagon were detected on days 3 and 8 after infection (Fig. 2a). The proportion of cells which produced NEUROG3 was 40.3±1.5 and 35.6±2.9% at days 3 and 8 after infection, respectively (Table 1). The percentage of alpha, beta or CK7-positive cells in the total population of pre-sorted Ad-NEUROG3-infected cells did not differ significantly from non-infected controls on either days 3 or 8 (Table 1). Similarly, in Ad-NEUROG3-infected preparations, the percentage of synaptophysin-positive cells did not differ

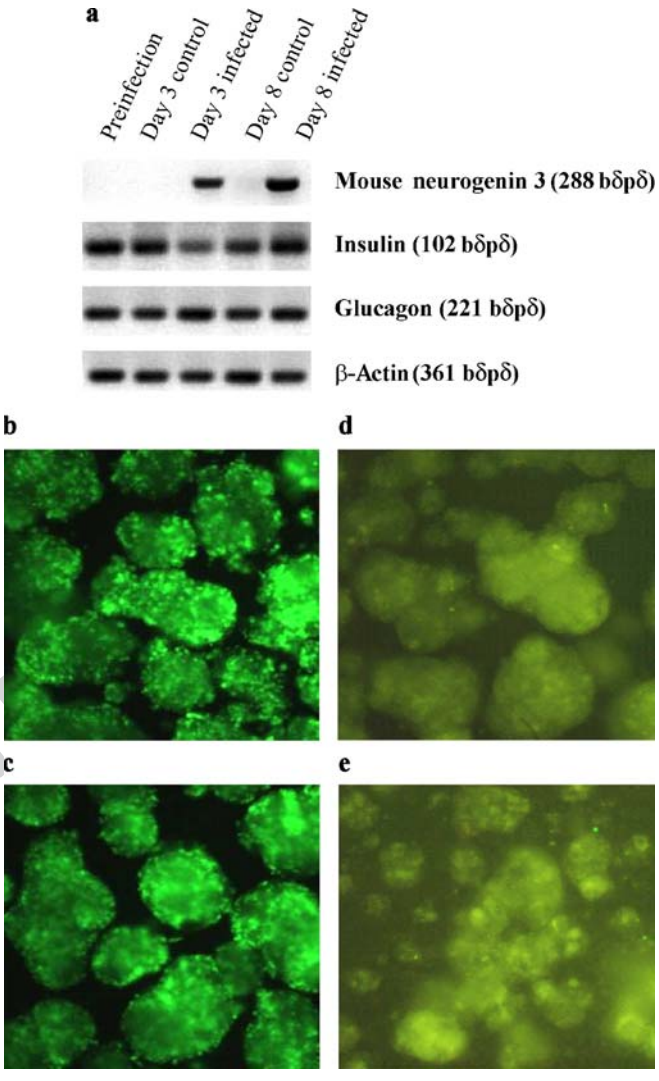

Fig. 2 **a** Representative RT-PCR analysis of non-infected control and Ad-NEUROG3-infected cells after 3 or 8 days of in vitro culture. Samples were assessed for transcription of murine NEUROG3, insulin and glucagon genes along with actin as an internal control (n=3). *Neurog3* mRNA was not detected in non-infected control cells. **b** Expression of the reporter gene, *green* fluorescent protein (*GFP*), in pancreatic preparations at 3 and 8 (**c**) days after infection suggests successful transduction of neonatal pig pancreatic cell cultures with Ad-NEUROG3. Non-infected controls expressed no *GFP* on days 3 (**d**) or 8 (**e**) (×200 magnification)

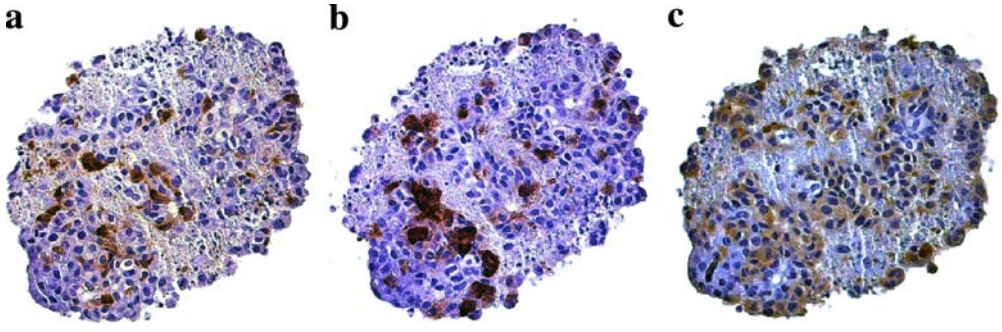

Fig. 1 Immunohistochemical staining of representative cellular aggregates for insulin (**a**), glucagon (**b**) and CK7 (**c**) prior to infection (×400 magnification). The cellular composition of aggregates was determined by dissociating aggregates into single cells and calculating the proportion of insulin-, glucagon- and CK7-positive cells

Table 1 Cellular composition (means±SEM, n=4) of Ad-NEUROG3-infected neonatal pancreatic cells on days 3 and 8 after infection

	Day 3				Day 8			
	Control	Ad-NEUROG3			Control	Ad-NEUROG3		
		Pre-sort	GFP^+	GFP^-		Pre-sort	GFP^+	GFP^-
NEUROG3	0	40.3±1.8	96.9±0.6	2.8±0.7	0	35.6±2.9	96.2±1.3	2.4±1.1
Insulin	17.3±1.6	15.4±1.5	16.9±3.5	14.4±1.0	26.7±2.3	28.2±5.2	22.8±2.3	28.9±3.5
Glucagon	15.1±2.2	18.3±3.4	19.7±1.3	22.6±3.7	27.0±1.1	31.0±1.0	73.8±6.9*	21.4±3.2
CK7	43.2±3.6	41.2±4.5	45.6±3.2	46.7±2.3	34.5±5.6	36.3±2.1	5.2±4.5*	33.8±5.7
Synaptophysin	59.6±9.9	53.5±10.9	nd	nd	58.8±3.1	55.1±7.2	nd	nd

*p<0.05 compared with GFP^- cells on day 8
nd Not determined

compared with non-infected controls on day 3 (53.5±10.9 vs 59.6±9.9%, respectively) or day 8 (55.1±7.2 vs 58.8±3.1%, respectively) after infection (Table 1). The cellular DNA content was assessed on days 3 and 8 after infection and served as an accurate assessment of the number of viable cells [21]. Following infection with Ad-NEUROG3 virus, the cellular DNA content of pancreatic cells remained similar to non-infected controls (100.0%) (Fig. 3). In contrast, the cellular insulin content on day 3 after infection was reduced by 42.7±6.3%, p<0.05 (Fig. 3); however, by day 8, the recoverable cellular insulin content was only decreased by 25.3±4.6%, p<0.05 (Fig. 3).

To assess the phenotype of the Ad-NEUROG3-infected cells, single cell suspensions of GFP-positive and -negative cells were sorted and stained for NEUROG3, insulin, glucagon and CK7. The GFP-positive cell population contained ~96% *Neurog3*-expressing cells, whereas <3% of the cells in the GFP-negative population expressed *Neurog3* (Table 1). On day 3, the percentage of insulin-, glucagon- or CK7-positive cells in the GFP-positive population did not differ significantly from that found in either the pre-sorted condition, GFP-negative population or non-infected controls (Table 1). However, by day 8, the majority of GFP-positive cells were glucagon-positive alpha cells (73.8±6.9%), contrasting with the pre-sorted condition, the GFP-negative population or non-infected controls, which contained 31.0±1.0, 21.4±3.2 and 27.0±1.1% alpha cells, respectively (Table 1). There was no difference in the percentage of beta cells in both GFP-positive and -negative populations, whereas there was a significant decrease in the percentage of CK7-positive cells in the GFP-positive population (5.2±4.5 vs 33.8±5.7%, p<0.05; Table 1).

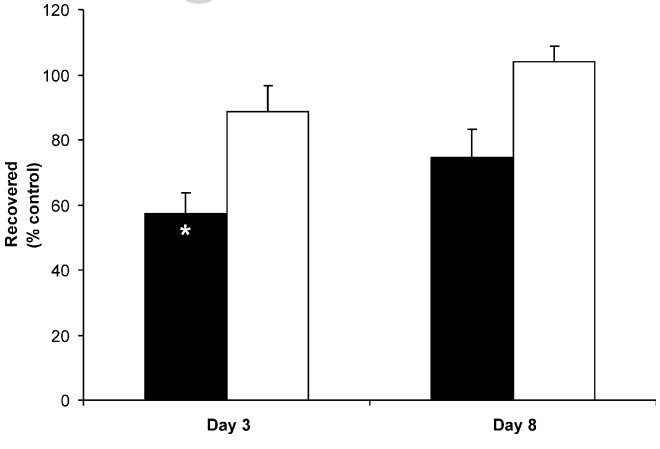

Fig. 3 Cellular insulin (*black bars*) and DNA (*white bars*) contents were assessed in non-infected control and Ad-Neurog3-infected neonatal pig pancreatic cells after 3 and 8 days of culture. Ad-NEUROG3 infection significantly reduced the cellular insulin content by day 3 of culture. Means±SEM (n=4); *p<0.05 vs control

In vivo assessment of transplanted neonatal pancreatic cells infected with Ad-NEUROG3

Twenty-four hours after infection, Ad-NEUROG3, Ad-GFP and non-infected control groups were transplanted into immune-deficient streptozotocin-diabetic mice. Graft characterisation prior to transplantation revealed that all groups of pancreatic preparations contained a similar cellular insulin content and beta cell number (Table 2). Mice receiving control (n=5) or Ad-GFP-infected grafts (n=4) achieved normoglycaemia by day 70 after transplantation (blood glucose value ≤10 mmol/l) whereas recipients of Ad-NEUROG3-infected cells (n=6) did not achieve euglycaemia (blood glucose levels ≥20 mmol/l) throughout the 109-day follow-up period (Fig. 4a). In recipients of both non-infected control and Ad-GFP-infected cells, removal of the graft-bearing kidney after normoglycaemia resulted in a rapid return to hyperglycaemia, indicating that the grafts were responsible for the normoglycaemic state (Fig. 4a).

Grafts retrieved from non-infected control and Ad-GFP groups of animals contained similar levels of cellular insulin and glucagon (Fig. 4b). The graft insulin content of non-infected controls increased 23.7±3.2-fold (from 4.7±0.5 to 110.3±13.7 μg) after transplantion and Ad-GFP-infected grafts increased 31.1±1.6-fold (from 4.1±0.9 to 140.6±13.5 μg). Ad-NEUROG3-infected grafts, on the

Table 2 Composition of grafts prior to transplantation (means±SEM, n=4)

	Control	Ad-NEUROG3	Ad-GFP
Beta cells ($\times 10^6$)	0.6±0.5	0.6±0.3	0.6±0.6
Insulin content (μg)	4.7±0.5	4.2±0.6	4.1±0.9

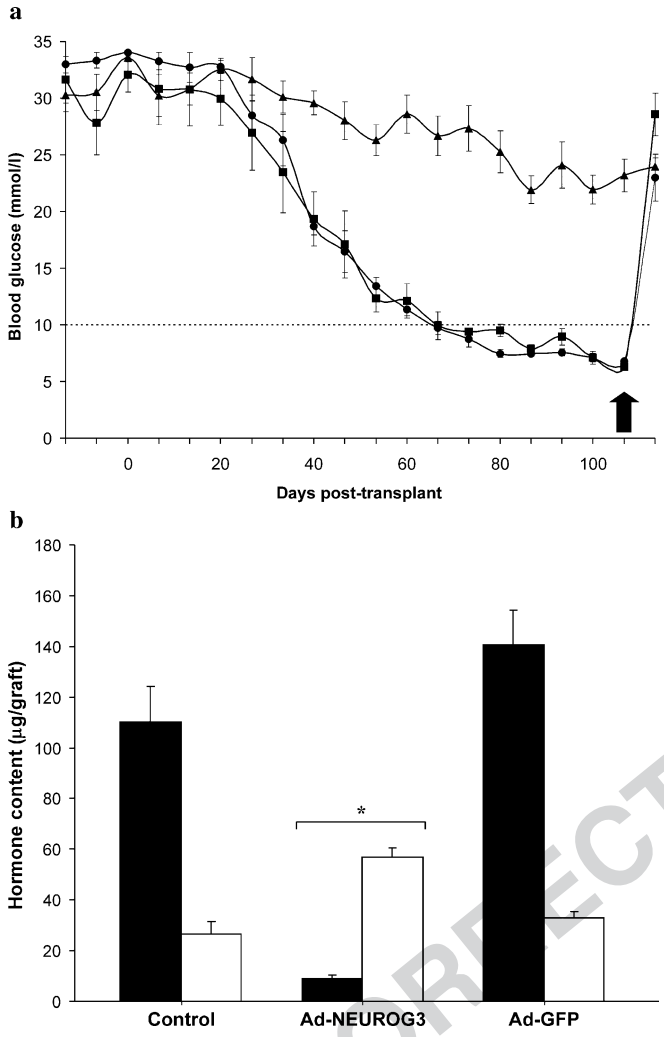

Fig. 4 **a** Blood glucose values of mice following transplantation of control (●; n=5), Ad-GFP- (■; n=4) or Ad-NEUROG3- (▲; n=6) infected grafts. Transplantation was performed 24 h after adenovirus infection and grafts were harvested (*arrow*) for histological and hormone content analysis 109 days after transplantion. Recipients of Ad-NEUROG3 grafts failed to achieve normoglycaemia in the time that recipients of non-infected and Ad-GFP control grafts did. Values are means±SEM. **b** Cellular insulin (*black bars*) and glucagon (*white bars*) contents of graft-bearing kidneys harvested from animals 109 days after transplantion. The insulin content of Ad-NEUROG3 grafts was significantly reduced compared with non-infected and Ad-GFP control grafts. Ad-NEUROG3 graft glucagon content was greater than controls. n=3; *p<0.05 vs control and Ad-GFP

other hand, increased only 2.2±0.5-fold (from 4.2±0.6 to 9.1±1.3 µg), p<0.05 (Fig. 4b). When the glucagon content of the grafts was compared, Ad-NEUROG3-infected grafts contained significantly more glucagon (57.0±3.4 µg) when compared with non-infected control (25.6±4.9 µg) and Ad-GFP-infected grafts (33.1±2.5 µg), p<0.05 (Fig. 4b). Histological examination of the Ad-NEUROG3-infected grafts taken from mice on day 109 after transplantion revealed glucagon-positive alpha-cell-rich areas (Fig. 5h) but very few faint insulin-positive cells (Fig. 5g). In contrast, non-infected control and Ad-GFP-infected grafts consisted predominantly of well-granulated insulin-positive cells (Fig. 5a,d) with few glucagon-positive cells (Fig. 5b,e). Similar staining patterns of CK7-positive cells were seen in all groups of transplanted grafts (Fig. 5c,f,i). Randomly scattered NEUROG3-positive cells were detected in Ad-NEUROG3-infected grafts up until 2 weeks after transplantion, after which none were detected (data not shown). No marked differences in morphology were observed between the non-infected control and Ad-GFP groups.

Assessment of apoptotic cells in Ad-NEUROG3-infected grafts 2 weeks following transplantation was performed using the TUNEL assay (Fig. 6a,b). In both non-infected control and Ad-NEUROG3-infected grafts, very few apoptotic beta cells were detected. In contrast, the number of proliferating beta cells was markedly reduced in Ad-NEUROG3-infected grafts compared with non-infected control grafts at 2 weeks after transplantion (Fig. 6c,d).

Discussion

This study shows that ectopic expression of *Neurog3* in neonatal pig pancreatic precursor cells induces (trans) differentiation to functional alpha cells. In vitro assessment of pancreatic cell composition after Ad-NEUROG3 infection showed no effect by day 3, but by day 8 there was a marked increase in the proportion of alpha cells in the NEUROG3-positive population. In addition, the increase in the alpha cell fraction was associated with a significant decrease in the proportion of CK7-positive cells, while the fraction of beta cells remained unchanged. These data suggest that CK7-positive precursor cells expressing ectopic murine NEUROG3 differentiate into alpha cells. The observation that Ad-NEUROG3 induces alpha cell differentiation by day 8 after infection and not day 3 suggests that sufficient time is necessary for the gene-expression events controlling alpha cell differentiation to occur. Whether the porcine *Neurog3* gene was ever expressed in pancreatic cells remains undetermined, since the murine *Neurog3* primers or antibody used in this study did not detect *Neurog3* mRNA or protein, respectively, in non-infected control cells. Similarly, when fetal porcine pancreatic cells were examined for *Neurog3* mRNA using the same murine *Neurog3* primers used in this study, none was detected (H. Heimberg unpublished observation). Adenoviral infection of pancreatic cells had no significant effect on cellular DNA content recovery and thus the infection procedure does not appear to cause significant cell death. This supports the assumption that the decrease in the proportion of CK7-positive cells in the NEUROG3-positive population in vitro is not due to selective cell death, but rather the differentiation of these precursor cells into alpha cells.

NEUROG3 mRNA and protein are normally not detected in differentiated insulin- and glucagon-producing cells [13, 15]. However, in this study, ectopic murine NEUROG3 protein was produced in hormone-producing alpha and beta cells. In a study by Mellitzer et al. [22],

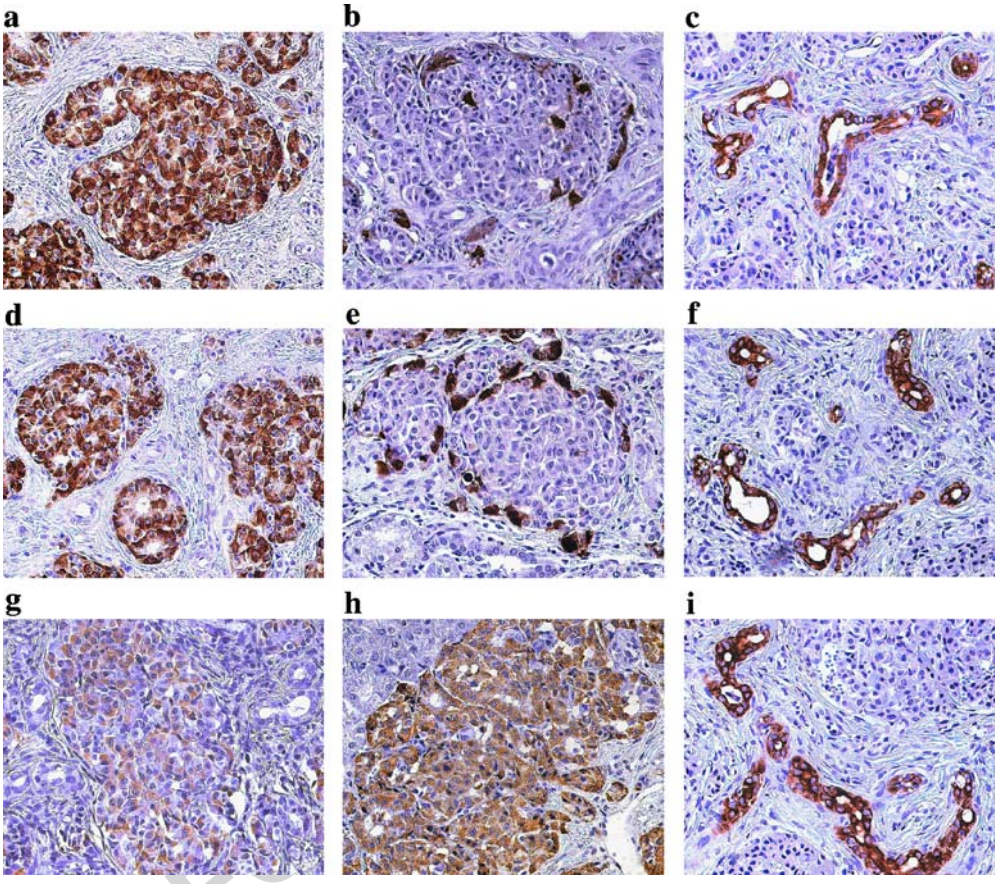

Fig. 5 Immunohistochemical staining of non-infected control (**a**–**c**), Ad-GFP- (**d**–**f**) and Ad-NEUROG3- (**g**–**i**) infected grafts for insulin (**a**, **d**, **g**), glucagon (**b**, **e**, **h**) and CK7 (**c**, **f**, **i**). Abundant glucagon-immunopositive cells were seen in Ad-NEUROG3-infected grafts, which were almost devoid of insulin-positive cells. Grafts were harvested 109 days after transplantion, fixed and embedded in paraffin. Immunopositive cells were identified using ABC/DAB and counterstained with Harris' haematoxylin (×400 magnification)

transgenic mice were developed in which *Neurog3*-expressing cells of the developing pancreas were labelled with enhanced yellow fluorescence protein (EYFP). The experiments showed that some islet progenitors expressing *Neurog3* (EYFP-positive) also co-stained for insulin or glucagon. The detection of ectopic murine NEUROG3 and not porcine NEUROG3 suggests forced expression of *Neurog3* in neonatal pig pancreatic cells does not necessarily affect hormone production, at least in the short-term. Perhaps if the endogenous porcine *Neurog3* gene were activated, hormone expression would eventually be extinguished. This has been reported to be the case at least in adult human beta cells forced to proliferate in vitro [23]. Expression of *Neurog3* was reported in dedifferentiated in-vitro-expanded human beta cells along with an accompanying loss of expression of genes characteristic of

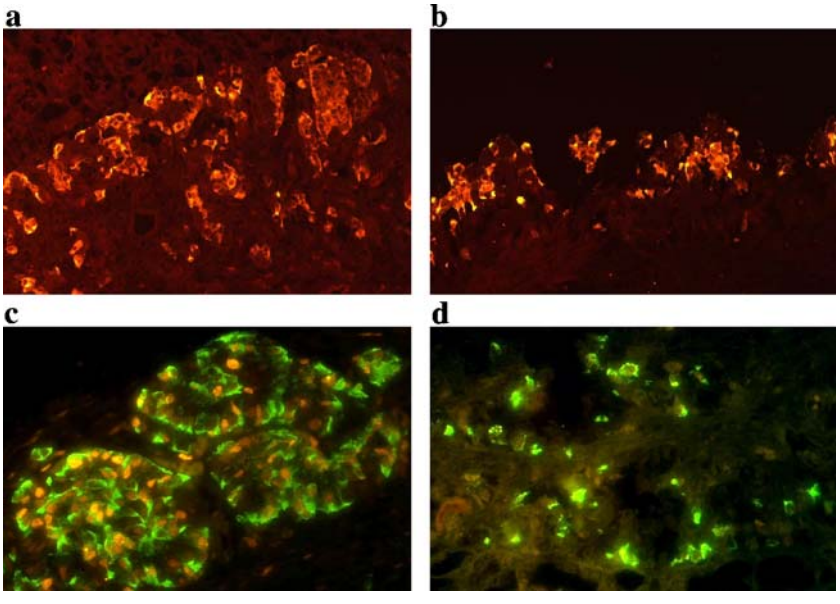

Fig. 6 In vivo assessment of cell death (**a, b**) and proliferation (**c**, **d**) in Ad-NEUROG3-infected grafts. **a** Representative non-infected control and (**b**) Ad-NEUROG3-infected grafts showing very few apoptotic beta cells (insulin staining, *red*; TUNEL labelling with FITC, *green*) at 2 weeks after transplantion. **c** Numerous proliferating beta cells (insulin staining, *green*; PCNA staining, *orange*) were seen in non-infected control grafts, whereas Ad-NEUROG3-infected grafts (**d**) contained very few proliferating cells (×400 magnification)

beta cell differentiation. Similarly, in an adult mouse model of beta cell regeneration, intra-islet endocrine progenitor cells transiently expressed *Neurog3* mRNA, implicating reactivation of endogenous *Neurog3* during regeneration of beta cells [24]. However, a recent report by Lee et al. found beta cell regeneration after partial pancreatectomy does not involve *Neurog3* gene reactivation [25]. The subsequent loss of insulin production in transplanted Ad-NEUROG3-infected grafts may be due to a lack of beta cell differentiation because of forced NEUROG3 production. However, unlike beta cells, alpha cells appear unaffected by the presence of NEUROG3 and thus were able to maintain glucagon production even in vivo after transplantation. However, until we are able to detect endogenous porcine *Neurog3* gene expression we cannot be certain.

The induction of glucagon-positive alpha cell differentiation in neonatal pig pancreatic cells is not surprising since others have reported similar findings resulting from overexpression of *Neurog3* in pancreatic precursor cells [16, 17, 26]. The developmental age, and hence plasticity, of the infected tissue appears to affect the differentiation potential, as it would appear neonatal precursors behave similarly to precursor cells of embryonic origin in their ability to become alpha cells following *Neurog3* overexpression.

Genetic engineering of pancreatic islets may enhance metabolic function or reduce graft immunogenicity after transplantation. In this study, the metabolic function of Ad-NEUROG3-infected pancreatic cells was assessed in an in vivo transplantation model. Unlike non-infected control and Ad-GFP-infected cells, Ad-NEUROG3-infected cells failed to reverse streptozotocin-induced diabetes when transplanted into immune-deficient mice. Assessment of the proportion of beta cells and insulin content prior to transplantation revealed a similar beta cell mass and insulin content was transplanted in all groups. The use of an Ad-GFP control ruled out the possibility that GFP expression in pancreatic cells may interfere with endocrine function. Consistent with our data, others have reported Ad-GFP expression in transplanted pancreatic islets does not affect in vivo function [27].

The failure of Ad-NEUROG3-infected grafts to reverse hyperglycaemia in animals was due to an inadequate beta cell mass in grafts, as determined by significantly reduced insulin content. Prior to infection with Ad-NEUROG3, the insulin content was similar to that found in non-infected and Ad-GFP control groups; unfortunately, however, glucagon content was not assessed. Despite only a 2.2-fold increase in insulin content, significantly increased glucagon content was observed in Ad-NEUROG3-infected grafts after transplantion compared with controls. The increased alpha cell mass suggests a biased differentiation of precursor cells in neonatal pig pancreatic cells towards an alpha cell phenotype, which was made possible by ectopic *Neurog3* overexpression. Exposure of infected precursor cells to the hyperglycaemic environment of the recipient after transplantation may also have contributed to the increased alpha to beta cell mass, which is also seen in diabetes [28]. To make full use of the potential of pancreatic precursor cells and achieve optimal metabolic function in recipients of differentiated islet precursor cells, other islet cell types will ultimately be required.

What is the mechanism by which NEUROG3 promotes transdifferentiation? Previous studies have indicated that NEUROG3 activates islet transcription factors such as Nkx2.2, Pax4 and IA1 in addition to endodermal transcription factors like Foxa2 and HNF1α, while repressing its own promoter [29–32]. In this study, interactions between these transcription factors in vitro and in vivo resulted in the rapid transdifferentiation of neonatal pig pancreatic cells into alpha cells. Nonetheless, insulin content was significantly reduced in vitro by day 3 after infection, but was partially restored by day 8. Culture supplements affecting beta cell proliferation and differentiation such as nicotinamide, 3-isobutyl-1-methyl-xanthine and pig serum may have contributed to this recovery in vitro, but because they are not present in the murine post-transplantion environment in vivo, the insulin content (i.e. beta cell mass), was not successfully restored to meet the critical threshold level of insulin secretion required to reverse hyperglycaemia in diabetic mice [4]. Evidence to suggest beta cell proliferation was markedly impaired in Ad-NEUROG3-infected grafts was apparent in 2-week-old Ad-NEUROG3-infected grafts, which contained significantly reduced PCNA-positive beta cells. Beta cell death does not appear to be a major contributing factor to the reduced beta cell mass seen in Ad-NEUROG3-infected grafts, since no significant changes in measurable cellular DNA content in vitro and apoptotic beta cells in vivo were found compared with non-infected controls.

Co-expression of *Neurog3* with other downstream transcription factors may be necessary to induce differentiation of all endocrine islet cell types. By co-expressing multiple transcription factors together or in sequence in neonatal pig pancreatic cells, islet cell differentiation may be repeated, as occurs in situ during development, thus resulting in an increased beta cell mass. A better understanding of the mechanisms involved in the differentiation of islet endocrine cells is likely to be essential in directing the formation of such cells from precursor cells for use in cell-therapy treatment of diabetes mellitus.

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