Identification and action of N-myc downstream regulated gene 4 A2 in rat pancreas

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Abstract

Pancreatic islets and acinar tissue develop from duct epithelium and share expression of several transcription factors and other molecular markers also involved with the development of neural tissues. We examined rat pancreatic tissue from fetal life until adulthood for the expression of N-myc downstream regulated gene 4 (*NdrG4*), a gene shown to be expressed during neuronal cell differentiation. Isolated pancreatic ducts from neonatal rats were maintained in culture and gave rise to clusters of cells expressing nestin (NES) and PDX-1, which subsequently contained immunoreactive glucagon. Using reverse transcription PCR (RT-PCR), we identified mRNA expression and immunoreactive protein presence for NDRG4 in cultured duct-derived cells, and brain of neonatal rats. By PCR cloning of the ductal cell-derived DNA the molecular form of NDRG4 expressed in

pancreatic ducts and ARIP rat pancreatic cells was identified as NDRG4A2, and its presence in intact pancreas of fetal and neonatal rats was demonstrated by immunohistochemistry. Incubation of ARIP cells with glucagon-like polypeptide-1 (GLP-1), increased the expression of NDRG4A2 and PDX-1, while decreasing DNA synthesis and promoting the appearance of glucagon-positive cells. This inhibitory effect of GLP-1 on DNA synthesis and the stimulatory effect on endocrine differentiation were reversed when the translation of NDRG4A2 was prevented using siRNA. These findings indicate that NDRG4A2 is expressed in pancreatic duct cells under GLP-1 control and may be related to a reduction in proliferation and the onset of the pancreas cell differentiation. *Journal of Endocrinology* (2009) **201**, 15–25

Introduction

Both islet endocrine cells and acinar tissue develop from pancreatic epithelium cells during the fetal and neonatal development of the rat, and in the human fetus (Cerf 2006, Murtaugh 2007). The initial development of both lineages depends on the expression of key transcription factors such as PDX-1 and PTF1 α within the ductal cells (Murtaugh 2007). PDX-1 is also required in the mature β -cell where it trans-activates the insulin and GLUT2 gene promoters. Other transcription factors including neurogenin3, NEUROG 3, (previously known as NEUROD) BETA2, PAX-4 and -6, and NKX2.2 that are necessary to complete the differentiation of individual endocrine cell lineages, and many of these have a determining role in the differentiation of both neural cells and pancreatic cell types (Habener *et al.* 2005). Pancreatic ductal cells can be manipulated *in vitro* giving rise to pseudo-islet structures with multiple endocrine cell types (Ramiya *et al.* 2000, Soria 2001, Bonner-Weir *et al.* 2004). Typically, this trans-differentiation process involves the expression of PDX-1. However, there is a paucity of specific markers of pancreatic progenitor cells prior to their expression of endocrine hormones. One such marker was thought to be the

intermediate filament protein, the nestin that is also expressed in developing neural tissues (Lendhal *et al.* 1990). While nestin positive cells were described in isolated adult human islets and identified as a sub-fraction expressing the ATP-binding cassette transporter (ABCG2; Lechner *et al.* 2002), the corresponding cells *in vivo* are usually mesenchymal in nature and have been linked to the development of new endothelial cells within the supporting vasculature (Treutelaar *et al.* 2003). This does not preclude nestin expression also being associated within the lineage development of some pancreatic endocrine or acinar precursor cells (Delacour *et al.* 2004, Joannette *et al.* 2004).

We therefore hypothesized that there might be other genes co-expressed in developing pancreas and brain that might be involved in pancreatic cell differentiation from ductal cells. One such candidate is the N-myc downstream regulated gene 4 (*NdrG4*)/*Bdm1* family. These genes are expressed throughout regions of the brain in the neonatal rat and are required for neurite outgrowth (Yamauchi *et al.* 1999, Ohki *et al.* 2002). A mutant *NdrG1* gene is the cause of hereditary motor and sensory neuropathy-Lom, marked by progressive axonal loss on the peripheral nervous system. All *NdrG* proteins contain an α/β hydrolase structure common to hydrolytic

enzymes (Shaw *et al.* 2002). Here, we describe the identification of NDRG4A2 in rat pancreatic duct cells, and show a possible association to pathways of endocrine cell differentiation.

Materials and Methods

Pregnant Wistar rats were purchased from Charles River Ltd, St Constant, QC, Canada. Animals were maintained under standard conditions and had access to rodent chow and water *ad libitum*. All procedures in this study had the prior approval of the animal care committee of the University of Western Ontario and were performed in accordance with the guidelines of the Canadian Council for Animal Care. Collagenase, fetal bovine serum, 100 bp and 123 bp DNA ladders, Taq DNA polymerase, and Superscript II RNase H reverse transcriptase, siRNA, DMEM, Ham's F-12 and F12K medium were each purchased from GIBCO BRL. RQ1 DNase was purchased from Promega and QIA Quick Gel Extraction Kit from QIAGEN Inc. Trizol reagent, Taq DNA polymerase, oligo dT primers and reverse transcriptase were obtained from Invitrogen Inc. Mouse anti-nestin monoclonal antibody was purchased from Chemicon International Inc., Temecula, CA, USA while rabbit anti-human NDRG-4 was kindly provided by Dr K Kokame, National Cardiovascular Center Research Institute, Osaka, Japan. Monoclonal antibody against human NDRG4 was obtained from the Abnova Corporation, Taipei, Taiwan together with recombinant NDRG4 peptide for antibody blocking studies. Rabbit-anti-porcine glucagon was a gift from Dr Ungar, Dallas, TX, USA and guinea pig anti-insulin was kindly provided by Dr T J McDonald, University of Western Ontario, London, ON, Canada. Mouse anti-human cytokeratin 20 and monoclonal antibody against endothelial cell CD31/PECAM-1 were obtained from Dako Corporation, Santa Barbara, CA, USA, and rabbit anti-human α amylase from Sigma Chemical Co. Rabbit anti-rat PDX-1 was provided by Dr C Wright, Vanderbilt University. Glucagon-like polypeptide 1 (GLP-1) and all other reagents were purchased from Sigma.

Isolation and culture of pancreatic duct cells

Neonatal rats were killed by decapitation on postnatal day 4 and the pancreas, brain (cerebral cortex), and duodenum removed into Hank's buffered salts solution (HBSS) or frozen under liquid nitrogen for RNA isolation. Pancreatic ducts were isolated by a modification of the methods described originally by Remiya *et al.* (2000). Briefly, pancreata were dissected and pooled from 8–10 animals and digested with collagenase (2.8 mg/ml) in HBSS (5 ml). Tissue digestion was performed for ~10 min at 38 °C in a shaking water bath (200 cycles/min). The dissection was terminated by the addition of 10 ml ice-cold HBSS, the contents dispersed by pipette, and the tissue collected by centrifugation at 1000 g

for 10 min. Two further washes with ice-cold HBSS were performed. Elongated ductal structures were hand-picked under a dissecting microscope and cultured in Ham's F-12/DMEM (50/50) with 2% fetal bovine serum supplemented with 10 000 U/ml penicillin and 50 µg/ml streptomycin in tissue-culture grade plastic dishes (Falcon, Lincoln Park, NJ, USA) at 37 °C in a humidified atmosphere of 95% O₂/5% CO₂. The yield of ducts was ~150 per 10 animals. Ductal structures were allowed to attach to the culture dishes and medium was changed every 4 days. After 2 weeks culture, monolayers of cells had formed from the ducts and spherical clusters of cells budded from these, became detached and floated within the culture medium. These were collected and sub-cultured to be further analyzed for RNA or protein presence. Immunocytochemical staining with antiserum against cytokeratin 20 demonstrated that over 90% of the original monolayers were immunopositive, while no staining was seen for α amylase or for CD31 as a marker for endothelial cells deriving from capillaries. Less than 1% of cells in the original monolayers were immunopositive for insulin or glucagon. The positive staining for cytokeratin, coupled with the absence of endocrine hormone-positive cells, acinar or endothelial cells suggests that the cultures consisted predominantly of ductally-derived epithelial cells.

RNA extraction and reverse transcription PCR (RT-PCR)

To detect the expression of specific mRNAs within duct-derived or ARIP cell cultures, RT-PCR was used as previously described (Wang *et al.* 2000). Briefly, total RNA was extracted from duct-derived epithelial cells, newly isolated ducts, neonatal rat brain or duodenum using Trizol Reagent (Invitrogen). Purified RNA (100 µg) was extracted using the RNeasy Mini Kit (Qiagen) to remove any contamination of genomic DNA, and the integrity was verified by the separation using gel electrophoresis and visualization by ethidium bromide staining. The amount of RNA was estimated by absorbance at 260 nm, and samples were stored at –80 °C. Three µg total RNA was reverse transcribed with 300 U of SuperScript II RNase H[–] reverse transcriptase (Invitrogen) using 0.5 µg oligo (dT)_{12–18} primers (Invitrogen) according to manufacturers instructions. The primers utilized are shown in Table 1, and included those designed to amplify a region of the open reading frame of rat *NdrG4* (sequence 181–480). After a denaturation step of 3 min at 95 °C, 0.5 µl Taq DNA polymerase (Invitrogen) was added at 80 °C to perform a hot-start. Following seven cycles of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing and 1 min at 72 °C for extension, 2 µl β -actin primers (50 µM) were added to complete 35 more cycles and extension for the last 10 min at 72 °C. To establish a linear range of amplification, several different cycle numbers of PCR (20, 25, 30, 35, 40, 42, 45) were run. Negative controls without reverse transcriptase (–RT), and without cDNA (–PCR) were run, and no genomic DNA contamination was found. The β -actin primers covered exon/intron boundaries,

Table 1 Primer sets used for the identification of mRNAs encoding N-myc downstream regulated gene 4 (*NdrG4*), *Pdx-1*, nestin, *N-myc*, insulin, glucagon, and actin in pancreatic ductal cell and ARIP cell cultures

<i>NdrG4</i> antisense:	TCA, ATG, TTC, ATC, AAC, ACC, AGC
Sense:	TCT, CAA, TCA, CAA, GCT, GTG, CTT
<i>Pdx-1</i> antisense:	CGC, TGA, GAG, TCC, GTG, AGC, TGC
Sense:	TGC, AAA, CTG, GGG, TGG, CGG, CGG
Nestin antisense:	TGG, TGA, GGG, TTG, AGG, TTT, GT
Sense:	AGG, CTT, CTC, TTG, GCT, TTC, TGG
<i>N-myc</i> antisense:	CGA, TGT, GGT, CAC, GGT, AGA, GA
Sense:	AAG, TCC, GAG, CGT, GTT, CGA, TCT
Insulin antisense:	TGC, CCA, GGC, TTT, TGT, CAA, ACA, GCA, CCT, T
Sense:	CTC, CAG, TGC, CAA, GGT, CTG, AA
Glucagon antisense:	GTG, GCT, GGA, TTG, TTT, GTA, ATG, CTG
Sense:	CGG, TTC, CTC, TTG, GTG, TTC, ATC, AAC
Actin antisense:	GCC, CCT, CTG, AAC, CCT, AAG
Sense:	CAT, CAC, AAT, GCC, AGT, GGT, A

and no signal at 970 bp of genomic DNA contamination was found (data not shown) compared with the expected product size of 660 bp. The amplified PCR products were visualized on 2% agarose gels stained with ethidium bromide and analyzed using a gel imaging system (AlphaEaseFC-FluorChem 8800 software, Alpha Innotech Corp., San Leandro, CA, USA). For the identification and cloning of *NdrG4*, individual bands were removed from the gels, purified by QIA Quick Gel Extraction Kits, and the contained cDNA fragments extracted and re-amplified by PCR using the same primers. The DNA was sequenced within the University of Western Ontario core facility and the sequences identified through the NIH Gene Bank.

Cloning of *NdrG4A2*

PCR cloning was used to identify the entire cDNA sequence of the gene *NdrG4A2* gene expressed within pancreatic duct-derived epithelial cells and in the rat ARIP pancreatic duct cell line. RNA was extracted from cells and digested by DNAase as described above. RT-PCR was performed as described above and the individual bands were excised and sequenced. Identified sequences were compared with those published for *Bdm1* and *NdrG4A2*. To identify the 3' end and 5' end of *NdrG4A2* mRNA the RACE method was used as described by Frohman *et al.* (1988). Purified RNA (200 ng) was used as the template in a reverse transcription reaction, using the anchor primer 5' ACG, TAA, TAC, GAC, TCA, CTA, TAG, GGC, GAA, TTG, GGT, CGA, C-d (T)₁₈A-3' to identify the 3' end of the *NdrG4B* cDNA. Reactions without reverse transcriptase were used as the negative control. The *NdrG4A2* gene-specific primer (CGG, GTC, CTT, CTG, TAT, GTG, GTT, TCC) and the anchor primer were used in PCR and the products separated on 2% agarose gel and subjected to sequence analysis. To identify the 5' end mRNA of *NdrG4A2*, the purified RNA and *NdrG4A2* gene-specific primer (TCA, ATG, TTC, ATC, AAC, ACC, AGC) was used in the reverse transcription. cDNA (5 µl), 5× TdT

buffer (2 µl), 15 mM CoCl₂, (1 µl) 1 mM dATP (1 µl), and terminal transferrase (1 µl, 25 U) were mixed and incubated at 37 °C for 30 min. The reaction was purified on a QIA column and the eluate (30 µl) mixed with 10 Tag buffer (5 µl), 2.5 mM dNTP (3 µl), 20 µM (dT)₁₇ adaptor (GAC, TCG, AGT, CGA, CAT, T₁₇; 1 µl), and water (11 µl), and incubated at 95 °C for 5 min; 72 °C for 30 s while adding 2 U of Tag polymerase; 40 °C for 4 min; and at 72 °C for 40 min. The product was subjected to PCR using the *NdrG4A2* specific primer (CAT, GGC, ACA, CCA, CGA, AGT, GTT, TG) and the adaptor primer (GAC, TCG, AGT, CGA, CAT, CG) and the cDNA fragment separated and sequenced.

Immunohistochemistry

Cell clusters derived from ductal cultures were collected and washed three times with PBS. The cells were spread onto glass cover slips by centrifugation for 5 min at 500 g and air-dried at 23 °C before fixation with 70% (v/v) ethanol. Immunohistochemistry was performed on cells and on paraffin sections (5 µm) of rat pancreas (postnatal days 8, 21, and 130) or cerebral cortex (day 8) using antibodies against nestin (1:200 dilution in PBS), insulin (1:15 dilution), glucagon (1:100 dilution), cytokeratin 20 (1:50 dilution), α amylase (1:2000 dilution), PDX-1 (1:2500 dilution), CD31/PECAM-1 (1:50 dilution) or human NDRG4 (1:100 dilution) by the avidin-biotin peroxidase method as previously described (Joanette *et al.* 2004). Biotinylated horse anti-mouse, goat anti-rabbit, and goat anti-guinea pig (1:100 dilution, Vector Laboratories, Burlingame, CA, USA) were used as secondary antibodies. After washing in 50 mM Tris-HCl (pH 7.5), peptide immunoreactivity was localized by incubation in fresh 1.89 mM diaminobenzidine with 0.03% (v/v) hydrogen peroxide for 2 min and the reaction was quenched in excess 50 mM Tris-HCl (pH 7.5). Cells or tissue sections were counter-stained with Carazzi's hematoxylin, dehydrated and mounted under glass cover slips. Reactions in the absence of either primary or secondary antibody or the

use of non-immune serum, served as negative controls. As an additional control of specificity the monoclonal antibody against NDRG4 was pre-absorbed overnight at 4 °C with 100 µM NDRG4 peptide, any precipitate removed by centrifugation, and the supernatant used in the place of the primary antibody. Immunohistochemistry for NDRG4 was also performed on sections of liver and duodenum from 8 day-old animals for comparison with pancreas.

Western blot analysis

Proteins were extracted from ARIP cell cultures using 1 ml lysis buffer (20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 20 µM leupeptin, 1 mM benzamidine, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 µM microcystin) for $\sim 2 \times 10^5$ cells at 4 °C for 30 min. Detergent-insoluble material was precipitated by centrifugation at 12 000 g for 10 min at 4 °C and lipid layers were removed. Whole protein concentrations from the supernatant were determined by the Micro BCA protein assay (Pierce, Rockford, IL, USA) using BSA as standard protein, as per manufacturer directions. Protein samples were diluted in the SDS sample buffer, and the mixture was boiled for 5 min. Proteins (20 µg) were separated on an 8% SDS/PAGE and then transferred to nitrocellulose membranes. After blocking the membranes for 60 min at room temperature in Tris buffered saline containing 0.05% Tween 20 (TTBS) and 5% non-fat dry milk or 5% BSA, they were probed overnight at 4 °C with human NdrG4 antibody (1:100 dilution). Membranes were washed in TTBS and the proteins detected by enhanced chemiluminescence (Pierce) with HRP-labeled anti-mouse secondary antibody.

Regulation of *NdrG4A2* in ARIP cells

Rat ARIP pancreatic duct cells were maintained in F12 medium containing 7 mM glucose and 10% FCS. Cells were plated in culture dishes (1.7×10^4 per well) and maintained until 70% confluent. They were subsequently cultured in serum-free medium containing 12 mM glucose and increasing concentrations of GLP-1 for 48 h. [³H]-thymidine (2 µCi/ml) was added to some cultures and the isotope incorporated into DNA measured as previously described (Wang *et al.* 2000). RNA was extracted from ARIP cells and analyzed by RT-PCR as described above. To examine the effect of the *NdrG4A2* gene on the regulation of DNA synthesis and the expression of *Pdx-1* in ARIP cells, RNA interference was used as described by Gitlin *et al.* (2002). Briefly, the specific sequence (CCA, AGT, TTG, CAC, TCA, TCT, T) targeted on the open reading frame of *NdrG4A2* gene were chosen and siRNA was synthesized by Invitrogen, together with a non-specific control siRNA of equivalent size designed to have no significant sequence similarity to known mouse, rat or human transcript

sequences. *NdrG4A2* siRNA or control RNA (40 pmoles/ml) was pre-incubated with serum-free medium for 20 min at 23 °C before addition to cell cultures and incubation for 4 h at 37 °C. Transfection efficiency was estimated to be in excess of 80% using a control transfection with Ambion *Silencer* Cycle 3 GFP siRNA (Applied Biosystems, Foster City, CA, USA) and visualization of GFP expression in cells after 24 h by fluorescence microscopy. The culture medium was replaced by fresh medium with the addition of GLP-1 (0–20 nM) and [³H] thymidine (2 µCi/ml) for 48 h at 37 °C. Thymidine incorporation was quantified and RNA extracted and analyzed by RT-PCR as described above. To determine the involvement of *NdrG4A2* in ARIP cell differentiation, cells were incubated in F12 medium containing 7 mM glucose and 1% FCS for 14 days in the presence of GLP-1 (20 nM), with or without *NdrG4A2* siRNA. Cells were then fixed and subjected to immunocytochemistry for glucagon or insulin as described above.

Statistical analysis

Experiments with pancreatic ductal cells, or with the ARIP cell line were performed with three replicate culture plates per variable, and each experiment repeated between three and five times. Data were analyzed by multivariate ANOVA. Immunohistochemistry was performed on tissues derived between three and five separate animals and representative views are shown. To calculate the percent of primary ductal cells immunopositive for both nestin and PDX-1 following dual immunohistochemistry, all cells demonstrating cytoplasmic staining for nestin within a single culture dish were quantified using a Carl Zeiss transmitted light microscope at a magnification of $\times 40$. The percent of immunopositive cells that also demonstrated nuclear immunolocalization of PDX-1 was then calculated. The number of ARIP cells that demonstrated cytoplasmic immunoreactivity for glucagon following incubation in the presence of GLP-1 was quantified relative to the total number of cells per culture flask. Microscopic analyses for cell number were performed with Northern Eclipse (version 6.0) morphometric analysis software (Empix Imaging, Mississauga, ON, Canada).

Results

Isolation and culture of pancreatic ductal cells

Isolated rat pancreatic ducts placed in culture initially formed elongated structures that resembled ducts but had no detectable lumen (Fig. 1A). After culture for two weeks, monolayers of cells had grown out from these and the original ductal structures were no longer discernible. Spherical cell clusters were seen to bud out from the monolayers after three weeks of culture and often detached into the medium. Such clusters, but not the original monolayers were immunopositive for the presence of nestin,

Figure 1 Histological appearance of pancreatic ducts 24 h after isolation and plating onto tissue culture dishes (A), and the appearance of spherical cell clusters derived from the subsequent monolayers of cells and sub-cultured (B–D). Cell clusters demonstrated immunoreactivity for glucagon (B), nestin (C), and NDRG4 (D) (arrows). Many cells immunoreactive for nestin showed nuclear co-staining for PDX-1 (dark blue) (C). Magnification bar A – 50 μ m, B, C and D – 20 μ m. The presence of mRNA for nestin (E) or PDX-1 (F) within duct-derived cell clusters was determined by RT-PCR analysis (lanes B and C). The positive control for nestin consisted of RNA from neonatal rat brain, and for PDX-1, newly isolated pancreatic ducts (lanes D and E). In both panels, reactions without added template (lane A) or without reverse transcriptase (lanes C and E) were used as controls. The molecular markers (M, bp) are shown on the left.

a possible marker of neural and pancreatic cell precursor development (Fig. 1C). Approximately 30% of nestin-positive cells also demonstrated nuclear staining for PDX-1 (Fig. 1C). Clusters were collected, enzymatically dispersed, and re-plated and after 3 weeks groups of cells demonstrated immunoreactivity for glucagon (Fig. 1B), but not insulin, α amylase or CD31. Messenger RNAs for nestin and for *Pdx-1* were expressed within these cultures when analyzed by RT-PCR (Fig. 1E and F).

Identification and cloning of *NdrG4A2* gene in pancreatic duct cells and brain

We sought to identify NDRG4 expression within pancreatic duct-derived cell clusters and identified a positive signal using RT-PCR for RNA isolated from both ductal cells and neonatal rat brain. The sequence of the *Bdm1/NdrG4* mRNA expressed in brain or ductal cells was determined by PCR cloning using different primer pairs (Fig. 2A), while the 3' and 5' ends of *NdrG4A2* mRNA expressed in the duct cells were identified by RACE. The identified sequences of mRNA expressed in duct-derived cells were aligned with the sequences of *Bdm1* (GI 4105411) and *NdrG4A2* (GI 37788064) published in Gene Bank using the pair-wise blast program. The cDNA sequence detected in duct-derived cell clusters was identical to that of *Bdm1*, except for the deletion of 39 nucleotides from positions 926–965 in the open reading frame (Fig. 2B), and was identified as *NdrG4A2*, an isoform with a transcript starting from exon 5 and lacking exon 18 of the gene (Maeda *et al.* 2004).

Using immunocytochemistry with two separate antibodies, we identified NDRG4 within duct-derived cell clusters (Fig. 1D), and determined that $43 \pm 4\%$ of cells also demonstrated immunoreactivity for PDX-1. Immunohistochemistry was also utilized to show the presence of NDRG4 within the intact pancreas of fetal (gestational day 20; Fig. 3A) and neonatal rats (postnatal day 8; Fig. 3B), predominantly associated with the pancreatic duct epithelium and with acinar cells, but not within islet cells. Pancreas isolated from animals at 21 days postnatal age showed only slight staining for NDRG4 within the ducts (Fig. 3C), while by 130 days age no immunoreactivity was detected (Fig. 3D), suggesting that the presence of NDRG4A2 was absent after weaning. Staining in the pancreatic ducts for *NdrG4* was co-localized with cytokeratin (Fig. 4A and B). Staining for NDRG4 was completely absent in control conditions in which the primary antibody was omitted or pre-absorbed with excess antigen (Fig. 3F). No immunohistochemical localization of NDRG4 was seen in two other neonatal tissues derived from gut endoderm, liver or duodenum (not shown), but positive staining was seen within a sub-population of neurons in the cerebral cortex of the neonatal rat (Fig. 3E).

Expression and regulation of *NdrG4A2* in ARIP cells

The expression and sequence of *NdrG4* expressed in the ARIP rat pancreatic duct cell line was determined by RT-PCR cloning and found to be identical to *NdrG4A2* identified in primary ductal cultures. The effect of RNA interference on blocking translation of *NdrG4A2* was examined by RT-PCR

Figure 2 (A) Primer sequences used for the PCR cloning of *NdrG4A2* expressed in pancreatic ductal cells. According to the published sequence of *NdrG4A2* mRNA, various primer pairs were designed as indicated by letters (A–F) and used in RT-PCR analysis for ductal cells or neonatal rat brain tissue as a positive expression control. The triangles indicate the location of introns and the bold line indicates the location of the open reading frame of the gene. (B) Comparison of cDNA sequences for *NdrG4A2* expressed in the neonatal rat brain and that identified in cultured pancreatic ductal cells. Sequences identified by cDNA cloning and RACE were aligned with the published sequences of the *NdrG4A2* (*Bdm1*) gene. A sequence with 39 bp and 13 amino acids is missing in the open reading frame of *NdrG4A2* expressed in ductal cells.

analysis. *NDRG4A2* expression was increased when the ARIP cells were incubated with 20 nM GLP-1 (Fig. 5). However, after addition of siRNA coding for a specific sequence of *NdrG4A2* the expression of the longer cDNA fragment of *NdrG4A2* (181–681) was abolished and the abundance of the short cDNA fragment of the gene (181–481) was substantially decreased. The expression of *NDRG4A2* was not affected when a non-specific siRNA was used, and actin expression was unaltered. These observations indicate that the effect of the siRNA was specific.

We then examined if changes in *NDRG4A2* expression following exposure of ARIP cells to GLP-1 were associated with the reported induction of differentiation into endocrine or acinar phenotypes (Bulotta *et al.* 2002). Exposure to GLP-1 was associated with a significant reduction in DNA synthesis, as determined by the incorporation of [³H] thymidine (Fig. 6A). The inhibitory effect of GLP-1 on [³H] thymidine incorporation by ARIP cells was rescued when siRNA specific for *NdrG4A2* was applied to cells (Fig. 6A), while the addition of a control non-specific RNA did not significantly affect thymidine incorporation. Cells transfected with control siRNA showed immunoreactivity for *NDRG4*, as did non-transfected cells (Fig. 6B–a,c), which was absent in cells exposed to siRNA specific for *NdrG4A2* (Fig. 6B–b). Furthermore, western blot analysis of ARIP cell proteins revealed a 35 kDa species in non-transfected cells, or those transfected with

control siRNA, which was substantially less abundant after transfection of cells with siRNA for *NdrG4A2* (Fig. 6C).

ARIP cells were incubated in the presence of 20 nM GLP-1 for 14 day with or without siRNA for *NdrG4A2*. In the presence of GLP-1 alone or with control siRNA, foci of glucagon-immunoreactive cells were seen (Fig. 6B–d, f; 16 ± 4% of cells), which were absent in control cultures. The presence of glucagon-immunoreactive cells was significantly reduced in the presence of siRNA for *NdrG4A2* (Fig. 6B–e; 5 ± 2% of cells, *P* < 0.01 versus GLP-1 alone), suggesting that inhibition of translation of *NDRG4A2* prevented GLP-1-induced differentiation of glucagon +ve endocrine cells. No immunoreactive insulin was detected in ARIP cultures after 14 days exposure to GLP-1.

The expression of *NDRG4A2* was inversely related to cell DNA synthesis, being increased in response to GLP-1 (Fig. 7), as was the expression of N-myc, while the expression of the housekeeping gene actin was unchanged. Messenger RNA for PDX-1, an indicator of ductal cell differentiation into endocrine or acinar cell lineages, was also increased in response to GLP-1 (Fig. 7).

Cell clusters derived from primary cultures of neonatal rat pancreatic ducts were also cultured in the presence of GLP-1 (20 nM) for up to 3 weeks. The number of cells found to be immunoreactive for glucagon was slightly increased (control 13 ± 2%, GLP-1 18 ± 2%, *P* < 0.05, *n* = 4 preparations).

Figure 3 Localization by immunohistochemistry of NDRG4 within the pancreas of the fetal (gestational day 20) (A), or neonatal rat (8 days postnatal) (B), at day 21 postnatal (C), or as adults at 130 days postnatal (D). Positive staining was seen within cells of the pancreatic duct epithelium (arrows) and within acinar cells (a), but not within islets (I), up until postnatal day 8. The presence of NDRG4 was much reduced in pancreas at 21 days and was absent at 130 days. Staining was also shown within the cerebral cortex from the same animals as in B at postnatal day 8 (E), being localized to a sub-population of neurons (arrows). When the antibody to NdrG4 was pre-absorbed with excess antigen the ductal and acinar staining seen at day 8 was abolished (F). Magnification bar 100 μ m.

Figure 4 Localization by immunohistochemistry of cytokeratin (A) or NDRG4 (B) within adjacent sections of the pancreas of the fetal rat (gestational day 19). Positive staining was seen for both ligands within cells of the pancreatic duct epithelium (amplified inset). Magnification bar 50 μ m.

Figure 5 The effect of siRNA interference for *NdrG4A2* (sequence location shown) on the expression of mRNAs encoding NDRG4A2 and actin. ARIP pancreatic duct cells were cultured in serum-free medium with (lanes 2–4) or without (lane 1) 20 nM GLP-1 after prior transfection with a specific siRNA for *NdrG4A2* (lane 3) or non-specific RNA (lane 4). The mRNAs were quantified using RT-PCR using separate primers for short and long sequences for *NdrG4A2*. The analysis of actin mRNA in the same preparation was used as the loading control and to exclude possible contamination by DNA. A reaction without adding primers was used as the negative control (C).

Discussion

We have identified the expression of a member of the *NdrG* gene family, *NdrG4A2*, within pancreatic ducts and shown that this could be involved in the regulation of pancreatic cell differentiation from ductal precursors in response to GLP-1. Previous studies found that *Bdm1* was homologous to human *NdrG4* (Yamauchi *et al.* 1999, Nakada *et al.* 2002), one of four known members of the *NdrG* family. *NdrG4* has three isoforms, designated *NdrG4B*, *NdrG4B^{Var}*, and *NdrG4H*. These isoforms have an identical 3'- untranslated region (UTR) sequence, while the nucleotide sequences of the 5'- ends of *NdrG4B* and *NdrG4B^{Var}* are identical but differ from *NdrG4H*. The sequences of *NdrG4B* and *NdrG4B^{Var}* are identical except for a deletion of 39 bp in the open reading frame of *NdrG4B* from bp 927 to bp 965 due to alternative RNA splicing. (Zhou *et al.* 2001, Qu *et al.* 2002). After cloning the NDRG expressed in pancreatic ductal cells, we identified the deletion of 39 bp characteristic of *NdrG4B*, suggesting that this was the rat homolog. A recent study has detailed the genomic structure of rat *NdrG4* (Maeda *et al.* 2004). Six transcripts of the gene have been found and named as NDRG4A1, A2, B1, B2, C1, and C2. The transcripts derived from the 5'-ends of exons 1, 2, and 5 are referred to as NDRGC, NDRG4B, and NDRG4A respectively. Transcripts that contain exon 18 of *NdrG4* are denoted NDRG4A1, NDRG4B1, and NDRG4C1, and those lacking exon 18 are *NdrG4A2*, B2, and C2. NDRG4A2, as found here in rat pancreas, is homologous to NDRG4B (Maeda *et al.* 2004).

We identified NDRG4A2 in duct cells of neonatal rat pancreas, in cells derived from ductal cultures with the

potential to develop along an endocrine lineage, and in the ARIP rat pancreatic cell line which can give rise to both acinar and endocrine cells (Hui *et al.* 2001, Liu *et al.* 2004). Previous studies had found that the expression of NDRG4 was restricted to neuronal tissue, the expression of NDRG4A1 and A2 being mainly found in rat brain shortly after birth at a time of neural plasticity (Nakada *et al.* 2002). NDRG4 was also found to be expressed in heart, but not in other rat tissues examined such as spleen, lung, liver, skeletal muscle, kidney, and testis. It was detected in undifferentiated rat PC12 pheochromocytoma cells but was not detected in eight other non-neural cell lines examined (Yamauchi *et al.* 1999). In human, NDRG4B is expressed in brain, NDRG4H in brain and heart, while NDRG4B^{Var} is only marginally expressed in human tissue (Zhou *et al.* 2001, Qu *et al.* 2002). The expression of NDRG4A2 in rat pancreas indicates yet another gene shared between pancreas and brain tissues that might be involved with tissue maturation, as has been found for neurogenin3, NEUROD, and nestin (Gasa *et al.* 2004). However, the RNA processing of NDRG4 in these two tissues could be different allowing for tissue specific actions.

The expression of NDRG4 in rat brain is linked to neuronal developmental processes, and in P19 murine embryonic carcinoma cells is increased after the induction of neuronal differentiation (Bulotta *et al.* 2002). The transfection of NDRG4 into PC 12 neuronal cells regulated the outgrowth of neurites and the expression of the AP-1 DNA binding protein (Yamauchi *et al.* 1999, Ohki *et al.* 2002). By analogy, the expression of NDRG4 in pancreas could also be related to growth and differentiation. Pancreatic duct epithelium is able

Figure 6 (A) The effect of GLP-1, with or without siRNA inhibition of NDRG4A2 synthesis, on [³H] thymidine incorporation (c.p.m./well) by ARIP pancreatic duct cells, (B) the presence of immunoreactive NDRG4 (a–c and f) or glucagon (d and e), and (C) the presence of NDRG4 protein assessed by western blot analysis following transfection with control siRNA or siRNA for *NdrG4A2*. In (A) cells were pre-incubated without siRNA (1–4), with a non-specific RNA control (5 and 7) or with the addition of siRNA specific for *NdrG4A2* (6). Cells were then incubated in serum free medium without (1 and 5), or with GLP-1 at 5 nM (2), 10 nM (3) or 20 nM (4, 6, and 7) concentrations. The addition of 20 nM GLP-1 significantly inhibited thymidine incorporation (1 versus 4, 5 versus 7, **P* < 0.05; *n* = 3). This inhibitory effect was rescued by transfection with siRNA for *NdrG4A2* (6 versus 7, #*P* < 0.05; *n* = 5). (B) ARIP cells were subjected to immunocytochemistry to visualize NDRG4 following transfection with control siRNA (a), siRNA for *NdrG4A2* (b) or no siRNA (c). Immunoreactive NDRG4 was visible in control cultures (arrows) but was absent in cells exposed to *NDRG4A2* siRNA, confirming an inhibition of protein translation. To determine the effects of *NDRG4A2* on differentiation, ARIP cells were incubated for 14 days in the presence of GLP-1 (20 nM) with control siRNA (d), siRNA for *NdrG4A2* (e) or without transfection (f). Cells were then subjected to immunocytochemistry for glucagon (arrows). Cells transfected with siRNA encoding *NdrG4A2* had a lower frequency of glucagon immunoreactivity. Magnification bar 20 μm. (C) ARIP cells were cultured without transfection, following transfection with control siRNA, or following transfection with siRNA for *NdrG4A2*, protein extracted and a western blot performed for NDRG4 protein. A single species of ~35 kDa was visualized in control cultures but was substantially reduced following transfection with siRNA for *NdrG4A2*.

Figure 7 Expression of mRNAs encoding *Pdx-1*, *N-myc*, *NdrG4a2*, and actin within ARIP pancreatic ductal cells by RT-PCR following treatment with increasing concentrations of GLP-1 in serum-free medium (0% FC). The reaction without added primers was used as the negative control (C). The analysis of actin in the same preparations of mRNA was used as the loading control and to exclude the possibility of contamination with DNA.

to function as a precursor pool for cells that can contribute to both endocrine and exocrine lineages *in vitro*, and through budding of cells from the ducts during embryonic development (Githens 1988, Bonner-Weir & Weir 2005). Mouse pancreatic tissue has been shown to contain multi-potential stem cells at low frequency that are capable of giving rise to all pancreatic endocrine cell lineages, as well as neural cell types (Seaberg *et al.* 2004). It is also likely that β -cells can de-differentiate to a ductal phenotype, and subsequently re-differentiate back to endocrine cell types (Wang & Yashpal 2004). In the neonatal rat pancreas, NDRG4A2 was present within pancreatic duct cells and in the developing acinar tissue, but was absent from endocrine cells within the islets of Langerhans. The localization to acinar tissue was substantially reduced by postnatal day 21, and no presence of NDRG4A2 was detected in adult pancreas. The temporal distribution is consistent with a role of NDRG4A2 in the differentiation of acinar tissue, which develops rapidly in the neonatal rodent, and suggests that the gene is no longer expressed once differentiated endocrine cells are present within the islets.

We found that ductal cultures from the neonatal rat pancreas generated cell clusters that were proliferative and expressed the intermediate filament protein, nestin. Nestin-positive cells seen in the neonatal pancreas are likely to predominantly represent cells contributing to the vascular endothelium (Joanette *et al.* 2004), although there are some reports also associating nestin with cells within the endocrine

lineage (Maria-Engler *et al.* 2004). The duct-derived cell clusters could be sub-cultured for up to 6 months and expressed the early transcription factor marker of endocrine and acinar cell commitment, PDX-1. Similar results were found for islet progenitor cells that budded from cultured ductal monolayers derived from adult NOD mice (Ramiya *et al.* 2000). We found no expression or presence of insulin within the ductal cell cultures, although they did demonstrate immunoreactive glucagon presence after 3 weeks of culture. During embryonic development of the mouse, cells containing glucagon and tyrosine hydroxylase are the first to appear in the pancreatic duct at day e10 (Alpert *et al.* 1988).

The ARIP rat pancreatic cell line is a useful tool since these cells can differentiate into endocrine and acinar lineages. Following exposure to GLP-1 they were reported to give rise to insulin-expressing cells with a reduction in proliferative activity (Hui *et al.* 2001), and have been used as an *in vitro* model to study islet neogenesis (Bulotta *et al.* 2002). We found that the expression of NDRG4A2 and PDX-1 in ARIP cells were increased, while DNA synthesis was inhibited, by incubation with GLP-1. Conversely, prolonged exposure to GLP-1 for 14 days increased the percentage of cells immunoreactive for glucagon, but did not cause the appearance of insulin-positive cells. This suggests that NDRG4 may be up-regulated during commitment to differentiation in the pancreatic duct cells, as happens in neuronal tissues. The use of siRNA to block NDRG4A2 translation resulted in the inhibition of GLP-1 action on DNA synthesis and differentiation, implying that NDRG4 may be a GLP-1-dependent effector gene. N-MYC was also expressed within ARIP cells, and was up-regulated after incubation with GLP-1. N-MYC has been shown to regulate NDRG4 expression in neural tissues, and high levels of N-MYC are expressed during development in the fetal brain, kidney, and within neuroblasts migrating from the neural crest. However, little expression of this gene is found in adult tissues. Targeted expression of human N-MYC selectively caused pancreatic neuroendocrine tumors in transgenic zebra fish (Yang *et al.* 2004). The expression of both N-MYC and NDRG4 in pancreatic duct cells indicates further similarities in cell differentiation pathways between the pancreas and central nervous system. It is possible that the effects of GLP-1 on NDRG4 in ARIP cells were mediated by an increased expression of N-MYC, but such an indirect pathway cannot be concluded from the present experiments. Exposure of cell clusters derived from primary neonatal rat ductal cultures to GLP-1 caused a small increase in the population of glucagon immunoreactive cells within these clusters, implying that the actions of GLP-1 are not confined to established pancreatic cell lines.

In summary, we have found the expression of NDRG4A2 in rat pancreatic duct cells and in the ARIP pancreatic cell line. The expression of this gene is regulated by GLP-1 and is inversely related to cell proliferation but associated with the expression of PDX-1 and a commitment to cell differentiation. This suggests that NDRG4A2 may play a role in the differentiation of pancreatic cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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