In vitro expression of NGN3 identifies RAB3B as the predominant Ras-associated GTP-binding protein 3 family member in human islets

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Abstract

Neurogenin 3 (NGN3) commits pancreatic progenitors to an islet cell fate. We have induced *NGN3* expression and identified upregulation of the gene encoding the Ras-associated small molecular mass GTP-binding protein, RAB3B. RAB3B localised to the cytoplasm of human β-cells, both during the foetal period and post natally. Genes encoding alternative RAB3 proteins and RAB27A were unaltered by *NGN3* expression and in human adult islets their transcripts were many fold less prevalent than those of *RAB3B*. The regulation of insulin exocytosis in rodent β-cells and responsiveness to incretins are reliant on Rab family members, notably Rab3a and Rab27a, but not Rab3b. Our results support an important inter-species difference in regulating insulin exocytosis where RAB3B is the most expressed isoform in human islets.

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Introduction

Understanding normal β-cell development and function underpins various efforts aimed to restore β-cells in patients with type 1 and type 2 diabetes. During the foetal development, the pancreas contains epithelial progenitor cells, which give rise to the adult cell lineages, including β-cells (Murtaugh 2007). Experiments manipulating genes in mice have discovered a multitude of transcription factors that regulate this transition (Wilson *et al.* 2003). Within a subset of progenitors, positive for the transcription factors Sry box 9 (Sox9) and pancreas-duodenal homeobox 1 (Pdx1), the basic helix-loop-helix (bHLH) transcription factor Neurogenin-3 (Ngn3, also known as Neurog3) becomes transiently expressed to commit cells to an endocrine fate (Schwitzgebel *et al.* 2000, Lynn *et al.* 2007, Seymour *et al.* 2007). Without Ngn3, islet differentiation fails (Gradwohl *et al.* 2000). The transcription factor has also been shown necessary for β-cell regeneration from adult precursors (Xu *et al.* 2008). Strategies, such as expression microarray following retroviral ectopic *Ngn3* expression, have identified direct genetic targets of Ngn3 encoding transcription factors, such as NeuroD1, paired homeobox factor 4 (Pax4), Nirenberg and Kim (NK) homeobox family member Nkx2.2 and insulinoma-associated 1, all of which when inactivated in mice impair β-cell differentiation (Sosa-Pineda *et al.* 1997, Sussel *et al.* 1998, Huang *et al.* 2000, Heremans *et al.* 2002, Gasa *et al.* 2004, Smith *et al.* 2004, Mellitzer *et al.* 2006). Pdx1 is also increased following Ngn3 expression (Gasa *et al.* 2004). Several of these transcription factors downstream of Ngn3 are then required for mature β-cell function. For instance, Pdx1 regulates *GLUT2*, *glucokinase* and insulin amyloid polypeptide (*IAPP*) expression (McKinnon & Docherty 2001) and, in association with NeuroD1, it transactivates the *insulin* gene (Babu *et al.* 2008).

Many aspects of the β-cell phenotype are conserved across species. Nevertheless, there are subtle differences in mature β-cell function between mice and humans: the relative roles of glucose transport and phosphorylation as a part of glucose sensing (Schuit 1997); responsiveness to glucokinase activators (Johnson *et al.* 2007); glucose-induced desensitisation (Zawalich *et al.* 1998); responses to galanin (McDonald *et al.* 1994) and melatonin (Ramracheya *et al.* 2008); the roles of Pax4 (Brun *et al.* 2008) and p57Kip2 (Potikha *et al.* 2005) in β-cell proliferation; and, central to β-cell function, regulation of the *insulin* promoter (Hay & Docherty 2006). This makes direct study of human pancreas development and β-cells worthwhile. Studying foetuses from first trimester termination of pregnancy has provided a framework for understanding early human pancreas development with β-cells

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increasing rapidly after 8 weeks post conception (wpc), first as cell clusters and then within islets, where they express other markers of maturity such as prohormone convertase 1/3, IAPP, chromogranin A and some components of the glucose-sensing apparatus (Piper *et al.* 2004, Richardson *et al.* 2007). Similar studies first identified SOX9 as important for pancreatic development and β-cell differentiation; the pancreata of patients with campomelic dysplasia being hypoplastic and composed of poorly formed islets (Piper *et al.* 2002). Human pancreatic progenitor cells expressing PDX1 (Piper *et al.* 2004) and *NGN3* transcripts have been identified at 8 wpc (Castaing *et al.* 2005).

To further address the potential inter-species differences downstream of endocrine commitment, we induced human *NGN3* expression in a cell line with similarities to human foetal pancreatic progenitors, leading to increased expression of the Ras-associated small molecular mass GTP-binding protein, RAB3B. RAB3 proteins regulate intracellular trafficking and exocytosis in a range of cell types (Gonzalez & Scheller 1999) with RAB3B recently implicated in protecting and enhancing the function of dopaminergic nerve terminals (Chung *et al.* 2009). Inactivation of either *Rab3a* or *Rab27*, but not *Rab3b*, in mice causes glucose intolerance (Yaekura *et al.* 2003, Aizawa & Komatsu 2005, Kasai *et al.* 2005). In this study, we have identified RAB3B, rather than RAB27A or other RAB3 isoforms, as the predominant isoform in human islets implying an inter-species difference and providing a new candidate for mutation or abnormal function as a cause of diabetes and as a potential therapeutic target for enhancing insulin secretion in humans.

Materials and Methods

Human tissue collection

The collection of human foetal material under guidelines issued by the Polkinghorne committee has been described previously (Piper *et al.* 2004, Ostrer *et al.* 2006). Ethical approval was granted by the Southampton and South West Hampshire Local Regional Ethics committee. In these experiments, material from at least two foetuses per stage was examined. Human islets were obtained with appropriate ethical approval from the King's College Hospital Islet Transplantation Unit (King's College Hospital, London, UK). Pancreata were removed from non-diabetic cadaver organ donors and islets were isolated under aseptic conditions as described previously (Huang *et al.* 2004).

Immunohistochemistry and immunoblotting

Tissue preparation, immunoblotting, immunohistochemistry and immunofluorescence were performed as described previously (Piper *et al.* 2004, Piper Hanley *et al.* 2008). Antibodies are listed in Supplementary Table 1, see section on supplementary data given at the end of this article with dilutions, catalogue numbers and sources. Exceptions to dilutions for immunoblotting were 1:1000 for NGN3 and RAB3B. For biotinylated secondary antibodies, streptavidin (SA)–HRP (1:200, Vector Laboratories Ltd, Peterborough, UK), SA–FITC (1:150, Sigma–Aldrich Ltd), or SA–Texas Red (1:200; Vector Laboratories Ltd) conjugates were used according to the manufacturers' instructions. For bright-field immunohistochemistry, the colour reaction was developed following SA–HRP with diaminobenzidine (Merck) containing 0·1% hydrogen peroxidase (Sigma–Aldrich Ltd). Negative controls were omission of primary or secondary antibody.

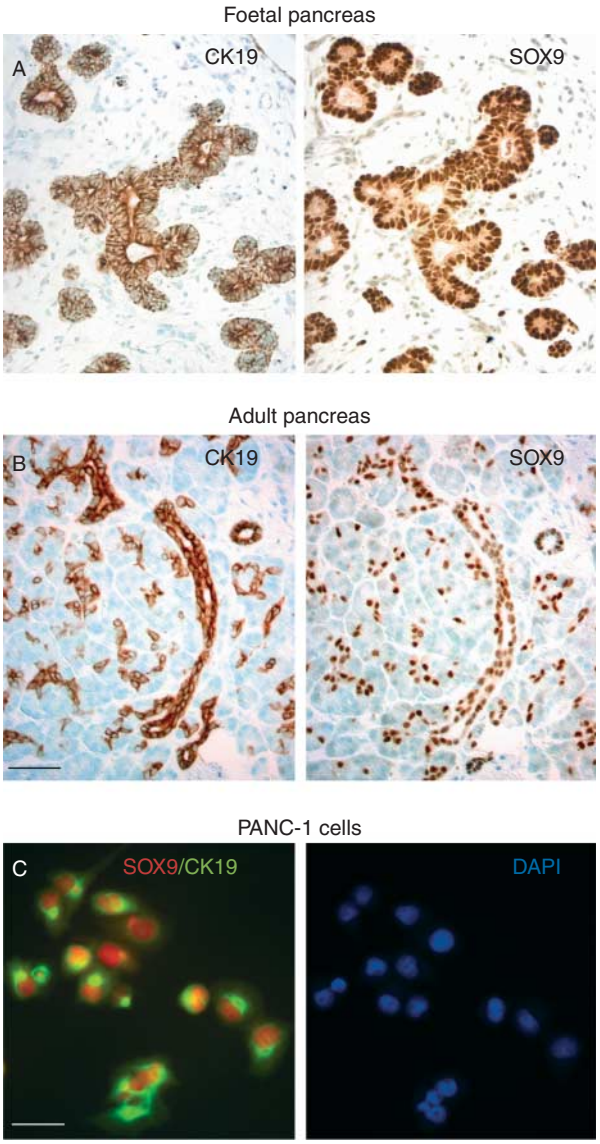


Figure 1 PANC-1 cells express CK19 and SOX9. (A) Consecutive 5 μm sections of the human foetal pancreas at 8 wpc stained for CK19 and SOX9. (B) Consecutive 5 μm sections of the human adult pancreas stained for CK19 and SOX9. (C) Dual immunofluorescence of PANC-1 cells for SOX9 (red) and CK19 (green) counter-stained with DAPI (blue) of the same image is shown to the right. Size bars represent 250 μm (A and B) and 50 μm (C).

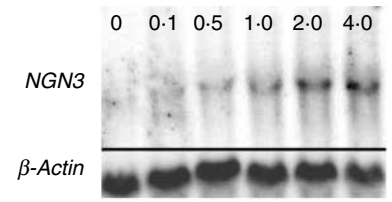
Cloning of the PANC-1 cell line with inducible NGN3 expression

The human PANC-1 cell line (cat. no. 87092802) was purchased from the European Cell and Animal Culture Collection (ECACC, Salisbury, UK) and cultured in DMEM containing 10% foetal bovine serum (FBS) and prokaryotic antibiotics. All vectors were from Clontech Laboratories Inc. The human *NGN3* coding sequence was amplified by PCR using primers containing HindIII and XbaI restriction sites (forward, 5′-CCCAAGCTTGACTCAAACTTACCCTT-CCCTCTG-3′; reverse, 5′-GCTCTAGAGCTCCGGCC-GGGTAGTGCT-3′) and cloned using these restriction sites into the pTRE2 vector to create pTRE2–NGN3. PANC-1 cells were transfected sequentially with pTet-On and pTRE2–NGN3 plus pTK-Hyg using Transfast (Promega Ltd). Stable PANC-1 clones demonstrating inducible NGN3 expression (PANC-1_{iNGN3}) were isolated in DMEM containing tetracycline-free FBS by selection with G418 and hygromycin according to the manufacturer's instructions (Clontech Laboratories). To assess the expression of functional NGN3 protein, the proximal 1613 bp of 5′ flanking region from the mouse *NeuroD1* gene was amplified to create a luciferase construct, p-1613*NeuroD1*-Luc. This construct contains two E-box motifs regulated by Ngn3 (Huang *et al.* 2000). Luciferase assays (Promega Corp.), as described previously (Hanley *et al.* 2001), were conducted 48 h after the addition of doxycycline (Dox) to screen PANC-1_{iNGN3} clones for induction of functional NGN3.

Isolation of RNA, northern blotting, reverse transcription and real-time PCR

Total RNA was isolated from PANC-1_{iNGN3} cells, human foetal pancreas and adult islets using Tri reagent (Sigma–Aldrich Ltd) for subsequent gel electrophoresis and reverse transcription (RT) using Superscript III (Invitrogen Ltd). RNA gel electrophoresis was carried out under denaturing conditions using 5 μg of total RNA per lane followed by washing and transfer overnight to Hybond N+ membrane (Amersham Pharmacia Biotech Ltd). The membrane was

A



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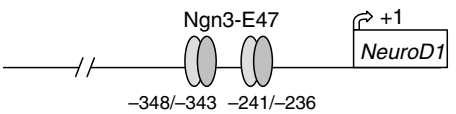


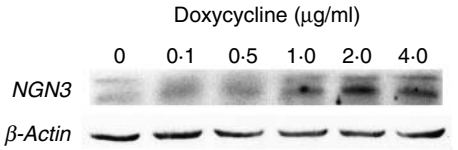
C

PANC-1_{iNGN3} clone	Fold induction	S·E·M.
Clone 9	2·24	±0·48
Clone 15	1·71	±0·39
Clone 40	1·45	±0·48
Clone 51	1·97	±0·27

D

Gene	Fold induction	S.E.M.
NGN3	2·98	±0·28
RAB3B	2·50	±0·08

E



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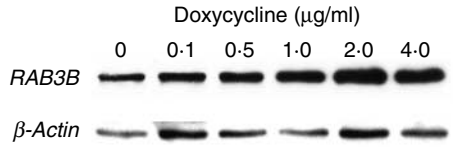
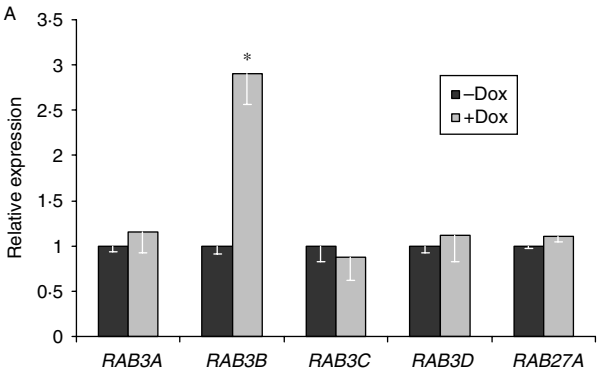


Figure 2 Inducible NGN3 expression in PANC-1 cells. (A) Northern blot analysis for NGN3 expression following the addition of doxycycline for 48 h to a clone of PANC-1_{iNGN3} cells. (B) Schematic of the mouse *NeuroD1* promoter with two E-box motifs at −348/−343 and −241/−236 bp in its 5′ flanking region that are regulated by Ngn3-E47 dimers (Huang *et al.* 2000). (C) Fold induction ±S.E.M. of luciferase activity from transient transfection of p-1613*NeuroD1*-Luc measured 48 h after the addition of 2 μg/ml doxycycline in four PANC-1_{iNGN3} clones. (D) Fold increase ±S.E.M. of *NGN3* and *RAB3B* expression from microarray analysis of PANC-1_{iNGN3} clone 51 by adding 2 μg/ml doxycycline for 48 h. (E and F) Western blot analysis of clone 51 PANC-1_{iNGN3} cells showing NGN3 (E) and RAB3B (F) protein expression following addition of increasing concentrations of doxycycline for 48 h.



B

Gene	Fold induction across all PANC-1_{iNGN3} clones	S.E.M.
RAB3A	1·05	0·03
RAB3B	1·85	0·24*
RAB3C	1·00	0·12
RAB3D	1·15	0·02
RAB27A	1·12	0·03

Figure 3 Expression of RAB3 family members and RAB27A following induction of NGN3 expression in PANC-1_{iNGN3} cells. (A) Expression of *RAB3* isoforms and *RAB27A* in PANC-1_{iNGN3} clone 51 after 2 μg/ml doxycycline for 48 h. Bars show mean ±S.E.M. from at least two experiments. (B) Mean fold induction ±S.E.M. of expression for *RAB3* isoforms and *RAB27A* in the other PANC-1_{iNGN3} clones (Fig. 2B) after 2 μg/ml doxycycline for 48 h. *$P<0·05$ compared to '−dox' treatment.

cross-linked by exposure to ultraviolet light and hybridised at 68 °C overnight with radiolabelled DNA probes to the *NGN3* coding sequence. Following post-hybridisation washes, the membrane was exposed to autoradiography film at −80 °C and developed.

Real-time PCR in the foetal tissue and the PANC-1 cells used pre-designed Taqman Gene Expression assays for each gene (Applied Biosystems, Warrington, UK) and an ABI PRISM 7900HT system with standard cycling conditions. *TBP* and *HPRT1* were used as endogenous controls. Results were analysed with SDS v2.1 software (Applied Biosystems) using the relative quantification method. mRNA was isolated from human pancreatic islets using the RNeasy Mini kit (Qiagen Ltd) according to the manufacturer's instructions and was quantified using a Nanodrop spectrometer (NanoDrop, Rockland, ME, USA). cDNA was synthesised and quantitative RT-PCR standards ranging from 10 to 10^9 copies DNA were prepared as described previously (Persaud *et al.* 2002). Real-time PCR amplification was performed using a LightCycler rapid thermal cycler system. Reactions were performed in 10 μl comprising nucleotides, Taq DNA polymerase and buffer (all included in the LightCycler FastStart Reaction Mix SYBR Green I); template cDNA; 3 mM $MgCl_2$; and 0·5 μM primers. All PCR protocols included an initial 10 min denaturation step and each cycle subsequently included a ramp at 95 °C for denaturation, annealing for 10 s at the temperatures listed in Supplementary Table 2, see section on supplementary data given at the end of this article and a 72 °C extension phase for 14 s (*β-ACTIN*) or 18 s (all other genes). The amplification products of both the primer pairs were subjected to melting point analyses and subsequent gel electrophoresis to ensure specificity of amplification.

Statistical analysis

Data were expressed as means±S.E.M. Statistical analysis used paired *t*-test or one-way ANOVA followed by Dunnett's *post hoc* test, as indicated. Values with $P<0·05$ were considered significant.

Results

Inducible NGN3 *expression by PANC-*1_{iNGN3} *cells*

In the human pancreas, cytoplasmic CK19 and nuclear SOX9 were restricted to the foetal epithelial progenitor cells and adult ductal cells (Fig. 1A and B; Piper *et al.* 2004). A similar profile was identified uniformly in the human pancreatic ductal carcinoma cell line, PANC-1 (Fig. 1C), which was negative for amylase (data not shown), confirming this cell line as a suitable source in which to engineer inducible *NGN3* expression. Sequential stable transfection of vectors for dox-inducible *NGN3* expression resulted in the isolation of more than 60 human PANC-1_{iNGN3} clones for further analysis. These starting clones retained SOX9 and CK19

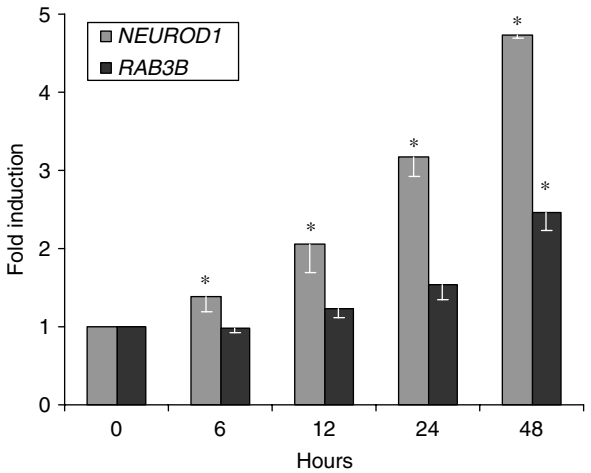


Figure 4 Timing of RAB3B expression following the induction of NGN3 in PANC-1_{iNGN3} cells. The relative expression of *RAB3B* and *NEUROD1* is shown following the addition of 2 μg/ml doxycycline to PANC-1_{iNGN3} clone 51. Bars show mean±S.E.M. from two experiments. *$P<0·05$ following analysis by ANOVA and Dunnett's *post hoc* test.

expression (data not shown). From selected clones, northern blotting revealed dose-responsive *NGN3* expression (Fig. 2A). Four clones, 9, 15, 40 and 51, were analysed in greater detail. The ability of these clones to induce functional NGN3 protein after the addition of dox to the media was assessed using a luciferase construct containing two E-box motifs from the wild-type mouse *NeuroD1* 5′ flanking region (Fig. 2B; Huang *et al.* 2000). All the four clones increased luciferase activity on the addition of dox to the media (Fig. 2C). Clone 51 gave the most reproducible results with least background luciferase activity and, thus, was chosen for the induction of NGN3 and subsequent microarray analysis (see Supplementary Methods, see section on supplementary data given at the end of this article). As expected, *NGN3* upregulation was detected by the array (Fig. 2D). The gene encoding RAB3B was also identified from candidates whose expression was induced at least twofold by 2 μg/ml dox treatment for 48 h. Reassuringly, both NGN3 and RAB3B proteins showed dose-responsive increases following dox treatment of clone 51 (Fig. 2E and F).

Expression of RAB3 family members and RAB27A in NGN3-inducible PANC-1 cells

In the mouse central nervous system, Rab3 proteins functions redundantly due to other co-expressed family members (Schluter *et al.* 2004). In mouse β-cells, inactivation of *Rab3a* and *Rab27a*, but not *Rab3b*, caused glucose intolerance (Yaekura *et al.* 2003, Kasai *et al.* 2005). Therefore, we analysed whether NGN3 expression led to the expression of other *RAB3* genes as well as *RAB27A*. Following the induction of NGN3 for 48 h, neither *RAB3A*, *RAB3C* and *RAB3D* family members nor *RAB27A* was increased in either clone 51 (Fig. 3A) or the other PANC-1_{iNGN3} cell clones (Fig. 3B). *RAB3B* was significantly increased in all the four PANC-1_{iNGN3} clones, albeit to a lesser extent in clones 9, 15 and 40 than in clone 51. Spurious induction of *RAB3B* by the antibiotic was excluded, as dox had no effect on *RAB3B* expression in PANC-1 cells lacking the pTRE2–NGN3 vector (data not shown).

NGN3 is a transient requirement during mouse endocrine cell differentiation (Schwitzgebel *et al.* 2000). As exocytosis of stored hormone granules is a function of mature endocrine cells, NGN3 would not be expected to directly regulate *RAB3B* expression. We examined the timing of *RAB3B* expression in the PANC-1_{iNGN3} cells. By northern blotting, *NGN3* transcripts were induced within 2 h of adding dox (data not shown). mRNA levels of *NEUROD1*, a direct target of NGN3 action (Huang *et al.* 2000), were increased by ~40% at 6 h and doubled at 12 h. In contrast, *RAB3B* transcripts accumulated relatively slowly, levels being increased only by ~50% at 24 h after the addition of

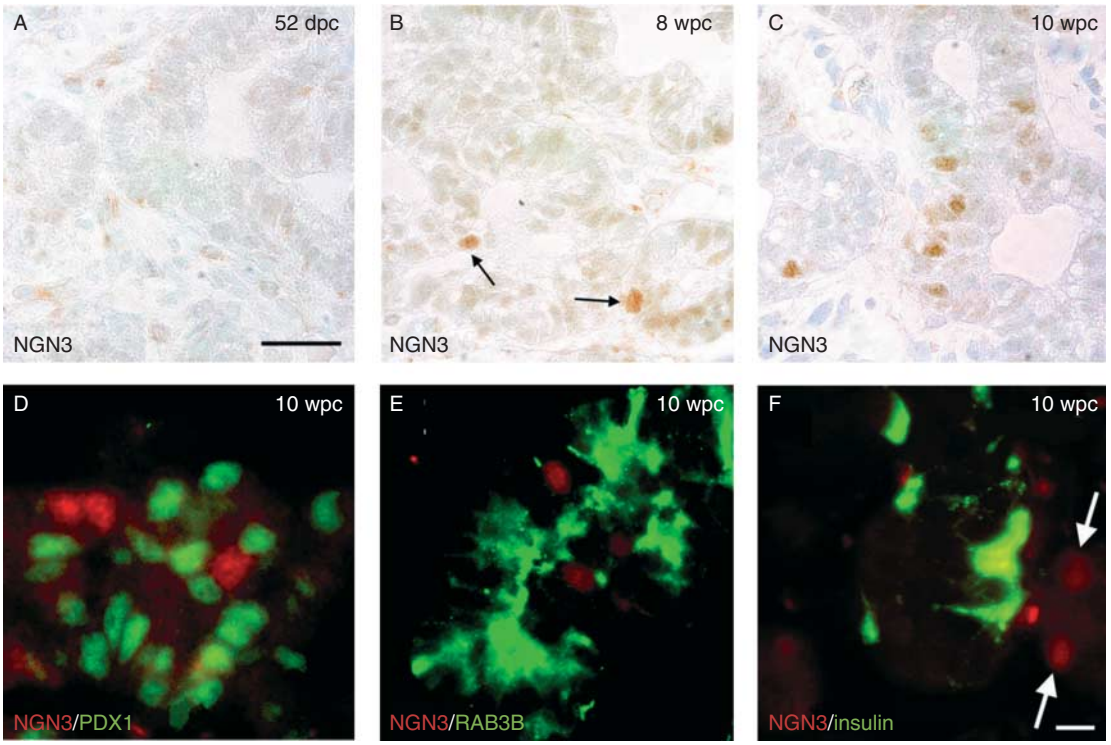


Figure 5 Expression of NGN3 during human pancreas development. (A–F) NGN3 immunohistochemistry in sections of developing human pancreas at 52 dpc (A), 8 wpc (B, arrows point to positive cells) and 10 wpc (C–F). Dual immunofluorescence of NGN3 (red) and PDX1 (green) is shown in (C). (E and F) Dual immunofluorescence of NGN3 with RAB3B (E) and insulin (F). Arrows in (F) point to NGN3-positive nuclei. Size bars represent 150 μm (A–C) and 40 μm (D–F).

Dox (Fig. 4). This implies that the effect of NGN3 on *RAB3B* transcription is indirect and mediated through other transcription factors. We used RNAi to moderate the increase in *NEUROD1* expression (Supplementary Figure 1, see section on supplementary data given at the end of this article). The downstream induction of *RAB3B* was unaltered, indicating that it does not rely on NEUROD1 (data not shown).

Expression of RAB3B *during human pancreas development and in islets*

From analyses of four specimens at 50 and 52 dpc, the human embryonic pancreas was largely devoid of cells positive for NGN3 (Fig. 5A). In two specimens at 8 wpc, isolated epithelial cells with nuclei stained for NGN3 immunoreactivity were apparent centrally within the organ (arrows in Fig. 5B). At this stage, we have previously reported occasional insulin-positive cells in the same location (Piper *et al.* 2004). Similarly positioned NGN3-positive cells were more numerous within two specimens of larger pancreas at 10 wpc (Fig. 5C) and they did not co-localise with PDX1 (Fig. 5D). In keeping with an indirect induction by NGN3, neither RAB3B nor insulin-positive cells stained for NGN3 (Fig. 5E and F). RAB3B was first detected weakly by immunohistochemistry in the cytoplasm of clustered cells adjacent to the duct-like epithelial structures of the foetal pancreas at 10 wpc (Fig. 6A and B). It was detected more robustly in the earliest foetal islets at 12 wpc (Fig. 6C) and in the cells of adult islets (Fig. 6D). RAB3B was not found in ducts or acinar cells (Fig. 6D) of the adult pancreas. At 10 wpc, RAB3B co-localised with insulin and glucagon (Fig 6E–J). In the adult pancreas, RAB3B extensively co-localised with insulin but was somewhat variable in different β-cells (Fig. 7A–C). Some RAB3B was present in some α- and δ-cell but at relatively low level (Fig. 7D–I). In contrast, RAB3B did not co-localise with pancreatic polypeptide in the adult pancreas (Fig. 7G–L). Consistent with Fig. 6D, RAB3B was not detected in CK19-positive duct cells in the adult pancreas (Fig. 7M–O). These data are consistent with the timing of *RAB3B* expression following its induction by NGN3 in PANC-1_{iNGN3} cells (Fig. 4).

By real-time PCR, *RAB3B* was detected robustly in adult islets. Its transcripts were ~500-fold increased compared to

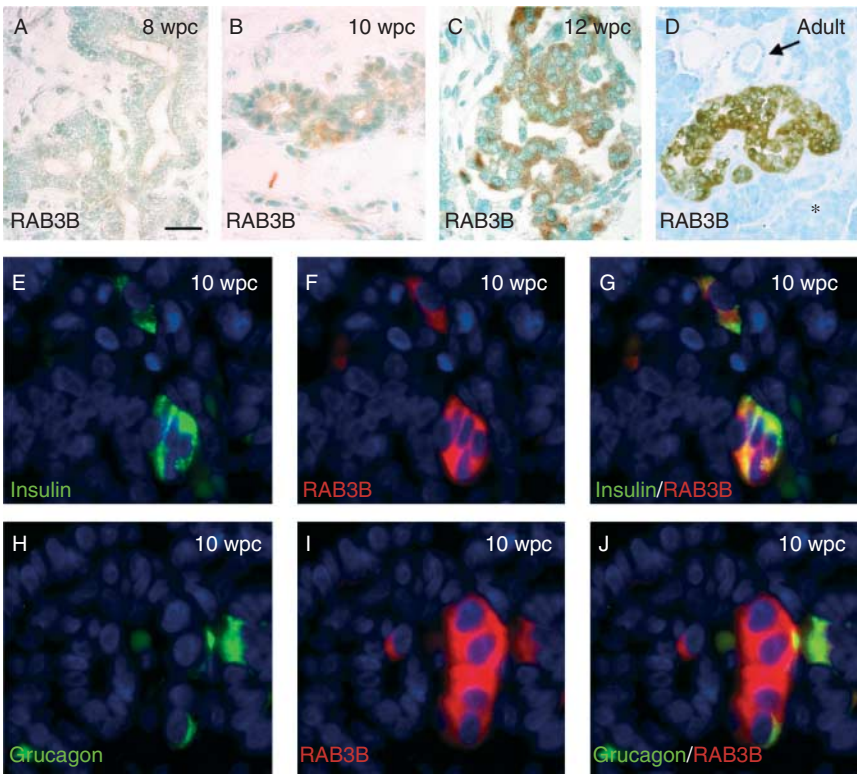


Figure 6 Expression of RAB3B during human pancreas development. (A–D) RAB3B immunohistochemistry in sections of developing human pancreas at 8 wpc (A), 10 wpc (B, arrow points to faintly positive cell cluster) and 12 wpc (C), and in the adult pancreas (D, arrow and star indicate duct and acinar tissue respectively). (E–J) Individual and dual immunofluorescence at 10 wpc counterstained with DAPI (blue). Size bar represents 150 μm (A–C), 250 μm (D) and 40 μm (E–J).

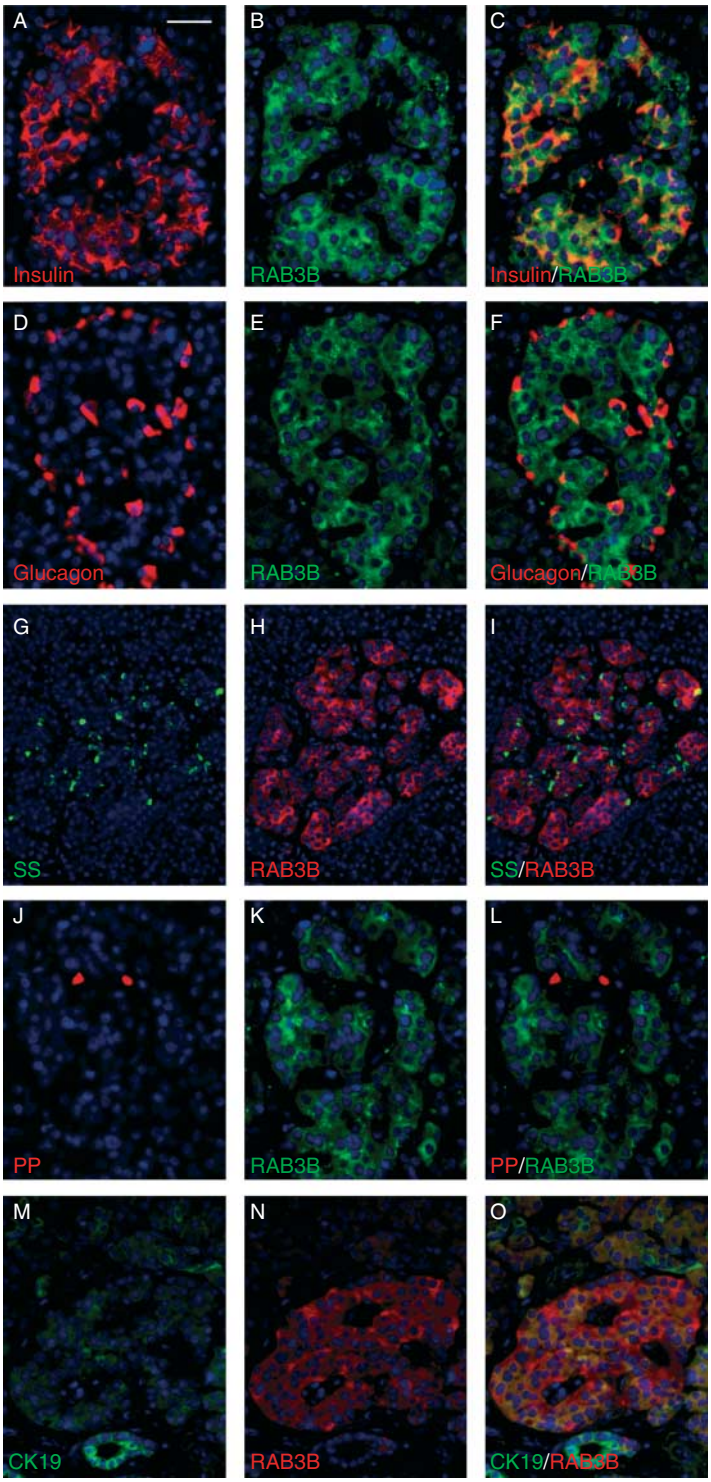


Figure 7 Expression of RAB3B in the adult pancreas. (A–O) Immunofluorescence counterstained with DAPI (blue) in sections of the adult pancreas. Size bar represents 40 μm (A–F and J–O) and 150 μm (G–H).

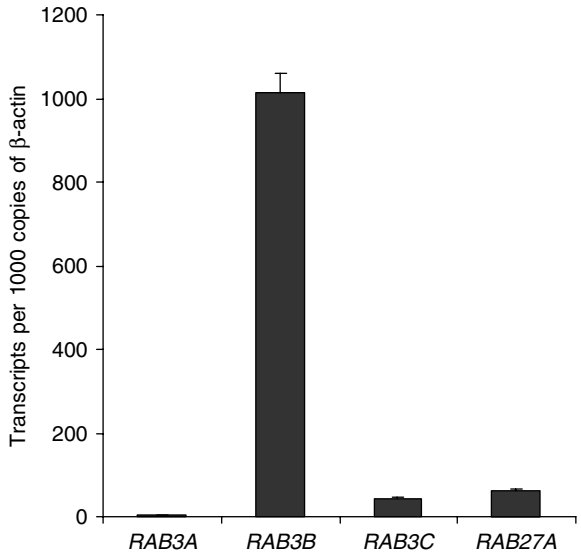


Figure 8 Expression of RAB3 family members and RAB27A in isolated human adult islets. Real-time PCR analysis of *RAB3* isoforms and *RAB27A* in adult islets expressed relative to *β-ACTIN* transcript levels. *RAB3D* did not amplify despite the use of more than 40 cycles of PCR.

RAB3A, 25-fold increased compared to *RAB3C* and 17-fold increased compared to *RAB27A* (Fig. 8). *RAB3D* did not amplify despite 40 cycles of PCR, implying very low or absent expression compared to the other isoforms under investigation.

Discussion

Studies in mice have proven that Ngn3 is required for pancreatic endocrine cell commitment. Without the bHLH transcription factor, the islet differentiation programme fails (Gradwohl *et al.* 2000). Conversely, driving ectopic Ngn3 expression in all pancreatic progenitor cells leads to premature over-commitment to an endocrine cell fate (Apelqvist *et al.* 1999). Lineage tracing of Ngn3-positive cells marks all mature pancreatic endocrine cell types (Gu *et al.* 2002). The transcription factor is extinguished before terminal differentiation and hormone expression (Schwitzgebel *et al.* 2000). Thus, it can be concluded that, in rodents, transient Ngn3 expression in an appropriate number of pancreatic epithelial progenitor cells is the normal mechanism for islet development and also appears important for potential β-cell regeneration in adult mice (Xu *et al.* 2008). In this study, consistent with previous data describing *NGN3* transcripts in the human foetal pancreas at 8 wpc (Castaing *et al.* 2005), we found the transcription factor present at 8 and 10 wpc within central cells of the pancreas where endocrine differentiation is known to predominate away from the pro-proliferative, exocrine-inducing effects of peri-pancreatic mesenchyme (Miralles *et al.* 1998, Polak *et al.* 2000, Elghazi *et al.* 2002, Piper *et al.* 2004). At this stage of development, large numbers of insulin-positive cells are apparent before organisation into islets at the end of the first trimester (Piper *et al.* 2004). Coupled with the identification of hypomorphic *NGN3* mutations in patients with juvenile-onset diabetes (Wang *et al.* 2006, Jensen *et al.* 2007), these findings make *in vitro* models useful in the search for downstream target genes of NGN3 expression in human (Heremans *et al.* 2002, Mellitzer *et al.* 2006) and in mouse cell types (Gasa *et al.* 2004).

Our *in vitro* cell line model to study the downstream consequences of NGN3 expression is an imperfect replica of human foetal pancreatic progenitors. In our experience, PANC-1 cells lack PDX1 protein. On the microarray, hybridisation signal was weak for *PDX1* and unaltered by the dox treatment. However, similar *in vitro* models have been used by others (Gasa *et al.* 2004) and PDX1 was absent in the NGN3-positive foetal pancreatic cells both in this study and in mouse (Schwitzgebel *et al.* 2000). Our cloned cells did uniformly express SOX9 and CK19 mimicking foetal pancreatic progenitor cells and an adult ductal phenotype. Nevertheless, by the ectopic expression of a single gene in a tumour cell line, it is inconceivable to generate bona fide β-cell precursors with complete, faithful gene expression profiles. Hence, our model of inducible NGN3 expression was used as a tool to identify downstream candidate markers of human β-cells for validation in the native cell type. NGN3 expression promptly activated *NEUROD1*, which encodes a transcription factor that has been linked causally with various forms of non-autoimmune diabetes (Frayling *et al.* 2001). Our model also allowed detection of RAB3B, which was subsequently shown to localise robustly to the human foetal and adult β-cells.

There are four RAB3 family members. Regazzi *et al.* (1996) previously showed RAB3B and RAB3C, but not RAB3A, by immunoblotting of whole human islets, comprising multiple hormone-secreting cell types. At that time, RAB3D was not assessed. In rat and mouse, Rab3a and Rab27a are very important regulators of exocytosis, both *in vitro* and *in vivo*, in β-cells (Regazzi *et al.* 1996, Yi *et al.* 2002, Waselle *et al.* 2003, Yaekura *et al.* 2003, Kasai *et al.* 2005, Abderrahmani *et al.* 2006). Through the interaction with a network of interacting proteins, they coordinate the intracellular trafficking of insulin granules that culminate in docking at the cell membrane and insulin release (Yi *et al.* 2002, Waselle *et al.* 2003). *Rab3a* and *Rab27a* knockout mice show defects in glucose-stimulated insulin secretion (GSIS) similar to those observed in patients with type 2 diabetes and in mice lacking the glucagon-like peptide-1 (GLP-1) receptor (Scrocchi *et al.* 1996, Yaekura *et al.* 2003, Aizawa & Komatsu 2005, Kasai *et al.* 2005). Furthermore, hyperglycaemia drastically reduced levels of Rab3a and Rab27a protein in rat β-cells as a consequence of expression of the transcriptional repressor, inducible cAMP early repressor (Abderrahmani *et al.* 2006). It transpires that GLP-1 potentiation of insulin secretion requires a complex between the cAMP sensor protein cAMP–GEFII, bound to the

sulphonylurea receptor 1, a protein called Piccolo and Rab-interacting molecule 2 (RIM2; Ozaki *et al.* 2000, Kashima *et al.* 2001, Fujimoto *et al.* 2002, Shibasaki *et al.* 2004). RIM2 then interacts with the RAB protein at the insulin granule, thus linking GLP-1 signalling and events at the ATP-sensitive potassium channel to insulin secretion. This emerging understanding of an important mechanism underlying GSIS makes the identification of the prevalent RAB isoform(s) in human β-cells important.

In this study, RAB3B was the only family member induced as a consequence of NGN3 expression. Its induction by NGN3 occurred later than that of *NEUROD1*, which, along with the non-overlapping expression profiles in developmental material, implies indirect regulation of *RAB3B* by NGN3. We show that RAB3B was extensively localised to β-cells both during human foetal development and in adult islets with some expression in some adult α- and δ-cells. Analysis of human adult islets demonstrated *RAB3B* expression to be approximately equal to the transcript numbers of *β-ACTIN*, present in all islet cell types, and greatly in excess of those for *RAB3A*, *RAB3C*, *RAB3D* and *RAB27A*. The level of *RAB3B* transcripts detected equates to ~20–50% of those which we have previously found for insulin (data not shown). In the mouse central nervous system, Rab3 family members regulate the exocytosis of neurotransmitters in a redundant manner, all isoforms needing inactivation to generate an epileptic phenotype (Schluter *et al.* 2004). This raises the question of whether redundant function could also affect pancreatic β-cell function. Although feasible, this seems unlikely: *RAB3A* and *RAB3C* transcripts were only weakly detected and *RAB3D* was not detected in islets whereas in the central nervous system, under normal conditions, all *Rab3* isoforms are expressed (Schluter *et al.* 2004); in *in vitro* analysis of rat melanotrophs, Rab3b could not substitute for the function of Rab3a (Rupnik *et al.* 2007); inactivation of either *Rab3a* or *Rab27a* in isolation produced glucose intolerance in mice (implying non-redundant function; Yaekura *et al.* 2003, Kasai *et al.* 2005); over-expression of Rab3a and Rab27a protein in MIN6 cells generated different effects on insulin secretion (Yi *et al.* 2002); and in dopaminergic nerves, over-expressing RAB3B, but not RAB3A, was protective in the models of Parkinson's disease (Chung *et al.* 2009). Interestingly, on searching the Unigene database, *Rab3b* is absent from cDNA libraries generated from mouse or rat insulinoma cell lines. Twelve of the 13 pancreatic clones for human *RAB3B* arise from the islet or insulinoma sources. Conversely, *RAB3A*, inactivation of which causes glucose intolerance in mice (Yaekura *et al.* 2003), has not been identified from Unigene human islet or insulinoma cDNA libraries.

There is a clear involvement of mutations in the pathway between the ATP-sensitive potassium channel and insulin secretion as causes of permanent neonatal diabetes (Gloyn *et al.* 2004). *RAB3B* localises to 1p31–p32, a locus previously linked to glucose intolerance and diabetes (Hsueh *et al.* 2003). We have conducted preliminary screens in three cases of permanent neonatal diabetes with linkage to this locus without identifying causative mutations in the *RAB3B* coding region. Irrespective of this, the potential for agents targeted at RAB function as novel potentiators of GSIS has already been proposed (Aizawa & Komatsu 2005). It will be important to ensure such efforts target the appropriate protein. In this study, we identified *RAB3B* as an indirect target of NGN3 expression. Under normal circumstances, *RAB3B* is clearly the predominant family member in human islets representing a significant difference in gene expression across species. Specifically, neither RAB3A nor RAB27A appears as likely to play the important role in human β-cells that has been described for the equivalent rodent cells (Regazzi *et al.* 1996, Yi *et al.* 2002, Waselle *et al.* 2003, Kasai *et al.* 2005, Abderrahmani *et al.* 2006).

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/JOE-10-0120.

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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