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

SHORT RUNNING TITLE: NGN3 & RAB3B IN HUMAN β-CELL DIFFERENTIATION


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Abstract

Neurogenin 3 (NGN3) commits pancreatic progenitors to an islet cell fate. We induced NGN3 expression and identified up-regulation of the gene encoding the small molecular mass GTP-binding protein, RAB3B. RAB3B localized to the cytoplasm of human beta-cells, both during the fetal 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 beta-cells and responsiveness to incretins is reliant upon 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 beta-cells.
Introduction

Understanding normal beta-cell development and function underpins various efforts aimed at restoring beta-cells in patients with type 1 and type 2 diabetes. During fetal development, the pancreas contains epithelial progenitor cells, which give rise to the adult cell lineages, including beta-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 (Lynn, et al. 2007; Schwitzgebel, et al. 2000; Seymour, et al. 2007). Without Ngn3, islet differentiation fails (Gradwohl, et al. 2000). The transcription factor has also been shown necessary for beta-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), NK family member Nkx2.2 and Insulinoma associated 1 (Ia1), all of which when inactivated in mice impair beta-cell differentiation (Gasa, et al. 2004; Heremans, et al. 2002; Huang, et al. 2000; Mellitzer, et al. 2006; Smith, et al. 2004; Sosa-Pineda, et al. 1997; Sussel, et al. 1998). Pdx1 is also increased following Ngn3 expression (Gasa et al. 2004). Several of these transcription factors downstream of Ngn3 are then required for mature beta-cell function. For instance, Pdx1 regulates GLUT2, glucokinase and IAPP expression (McKinnon and Docherty 2001) and, in association with NeuroD1, it transactivates the insulin gene (Babu, et al. 2008).

Many aspects of the beta-cell phenotype are conserved across species. Nevertheless, there are subtle differences in mature beta-cell function between mice and humans: the relative roles of glucose transport and phosphorylation as part of glucose sensing (Schuit 1997); responsiveness to glucokinase activators (Johnson, et al. 2007); glucose-induced desensitization (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 beta-cell proliferation; and, central to beta-cell function, regulation of the insulin promoter (Hay and Docherty 2006). This makes direct study of human pancreas development and beta-cells worthwhile. Studying fetuses from first trimester termination of pregnancy has provided a framework for understanding early human pancreas development with beta-cells 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 (PC) 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 beta-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 express PDX1 (Piper et al. 2004) and NGN3 transcripts have been identified at 8 wpc (Castaing, et al. 2005).

To further address potential inter-species differences downstream of endocrine commitment, we induced human NGN3 expression in a cell line with similarities to human fetal 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 and Scheller 1999) with RAB3B recently implicated in protecting and enhancing function of dopaminergic nerve terminals (Chung, et al. 2009). Inactivation of either Rab3a or Rab27 but not Rab3b in mice causes glucose intolerance (Aizawa and Komatsu 2005; Kasai, et al. 2005; Yaekura, et al. 2003). Here, we have identified RAB3B, rather than RAB27A or other RAB3 isoforms, as the predominant isoform in human beta-cells 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 fetal material under guidelines issued by the Polkinghorne committee has been described previously (Ostrer, et al. 2006; Piper et al. 2004). Ethical approval was granted by the Southampton and South West Hampshire Local Regional Ethics committee. In these experiments, material from at least two fetuses 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 isolated under aseptic conditions as described previously (Huang, et al. 2004).

Immunohistochemistry and immunoblotting

Tissue preparation, immunoblotting, immunohistochemistry, and immunofluorescence and were performed as described previously (Piper Hanley, et al. 2008; Piper et al. 2004). Antibodies are listed in Supplementary Table 1 with dilutions, catalogue numbers and sources. Exceptions to dilutions for immunoblotting were 1:1000 for NGN3 and RAB3B. For biotinylated secondary antibodies, streptavidin (SA) horseradish peroxidase (SA-HRP; 1:200, Vector Laboratories Ltd, Peterborough, UK), SA-FITC (1:150, Sigma-Aldrich Ltd, Poole, UK), 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 (DAB, Merck) containing 0.1% hydrogen peroxidase (Sigma- Aldrich Ltd). Negative controls were omission of primary or secondary antibody.

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 Dulbecco’s Modified Eagle’s Media (DMEM) containing 10% Fetal Bovine Serum (FBS) and prokaryotic antibiotics. All vectors were from Clontech Laboratories Inc (Mountain View, CA). The human NGN3 coding sequence was amplified by PCR using primers containing HindIII and XbaI restriction sites (forward, 5’ CCCAAGCTTGACTCAAACTTACCCTCTG 3’; reverse, 5’ GCTCTAGAGCTCCGGCGGATGTGCT 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, Chilworth, UK). Stable PANC-1 clones demonstrating inducible NGN3 expression (PANC-1\textsubscript{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-1613NeuroD1-Luc. This construct contains two E-box motifs regulated by Ngn3 (Huang et al. 2000). Luciferase assays (Promega Corp, Chilworth, UK), as described previously (Hanley, et al. 2001), were conducted 48 h after the addition of doxycycline (Dox) to screen PANC-1\textsubscript{INGN3} clones for induction of functional NGN3.

**Isolation of RNA, Northern blotting, reverse transcription and realtime PCR**

Total RNA was isolated from PANC-1\textsubscript{INGN3} cells, human fetal pancreas and adult islets using Tri reagent (Sigma-Aldrich Ltd, UK) for subsequent gel electrophoresis and reverse transcription using Superscript III (Invitrogen Ltd, Paisley, UK). 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., Amersham, UK). The membrane was crosslinked 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 fetal 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 analyzed 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, Crawley, UK) according to the manufacturer’s instructions and was quantified using a Nanodrop spectrometer (NanoDrop, Rockland, ME). cDNA was synthesized and quantitative RT-PCR standards ranging from 10 copies 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 \(10\mu l\) comprising: nucleotides, Taq DNA polymerase and buffer (all included in the LightCycler FastStart Reaction Mix SYBR Green I); template cDNA; 3mM MgCl\(_2\); and 0.5µM primers. All PCR protocols included an initial 10 min denaturation step and each cycle subsequently included a ramp to 95°C for denaturation, annealing for 10s at the temperatures listed in Supplementary Table 2, and a 72°C extension phase for 14 sec (\(\beta\)-ACTIN) or 18 sec (all other genes). The amplification products of both primer pairs were subjected to melting point analyses and subsequent gel electrophoresis to ensure specificity of amplification.
Statistical analysis

Data were expressed as means + / - SE. Statistical analysis used paired t-test or 1-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\textsubscript{iNGN3} cells

In the human pancreas cytoplasmic CK19 and nuclear SOX9 were restricted to the fetal epithelial progenitor cells and adult ductal cells (Fig. 1A-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 over sixty human PANC-1\textsubscript{iNGN3} clones for further analysis. These starting clones retained SOX9 and CK19 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 wildtype mouse NeuroD1 5' flanking region (Fig. 2B) (Huang et al. 2000). All four clones increased luciferase activity upon 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). As expected, NGN3 up-regulation was detected by the array (Fig. 2D). The gene encoding Ras-associated GTP-binding protein (RAB) family member 3B (RAB3B) was also identified from candidates whose expression was induced at least 2-fold by 2 µg/ml dox treatment for 48h. Reassuringly, both NGN3 and RAB3B proteins showed dose-responsive increases following dox treatment of clone 51 (Fig 2E-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 beta-cells, inactivation of Rab3a and Rab27a, but not Rab3b, caused glucose intolerance (Kasai et al. 2005; Yaekura et al. 2003). Therefore, we analyzed whether NGN3 expression led to the expression of other RAB3 genes as well as RAB27A. Following the induction of NGN3 for 48h, neither RAB3A, RAB3C and RAB3D family members, nor RAB27A were increased in either clone 51 (Fig. 3A) or the other PANC-1\textsubscript{iNGN3} cell clones (Fig. 3B). RAB3B was significantly increased in all four PANC-1\textsubscript{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<sub>IGN3</sub> 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 approximately 40% at 6 h and doubled at 12 h. In contrast, RAB3B transcripts accumulated relatively slowly, levels being increased only by approximately 50% 24 h after the addition of Dox (Fig. 4). This implies that the effect of NGN3 upon RAB3B transcription is indirect and mediated via other transcription factors. We used RNAi to moderate the increase in NEUROD1 expression (Supplementary Fig. 1). The downstream induction of RAB3B was unaltered, indicating that it does not rely upon 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 colocalise with PDX1 (Fig. 5D). In keeping with an indirect induction by NGN3, neither RAB3B nor insulin-positive cells stained for NGN3 (Fig. 5E, F). RAB3B was first detected weakly by immunohistochemistry in the cytoplasm of clustered cells adjacent to the duct-like epithelial structures of the fetal pancreas at 10 wpc (Fig. 6A, B). It was detected more robustly in the earliest fetal islets at 12 wpc (Fig. 6C) and in 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 beta-cells (Fig. 7A-C). Some RAB3B was present in some alpha and delta-cells but at relatively low level (Fig. 7D-I). In contrast, RAB3B did not co-localise with pancreatic polypeptide in adult pancreas (Fig. 7G-L). Consistent with Fig. 6D RAB3B was not detected in CK19 positive duct cells in adult pancreas (Fig. 7M-O). These data are consistent with the timing of RAB3B expression following its induction by NGN3 in PANC-1<sub>IGN3</sub> cells (Fig. 4).

By realtime PCR, RAB3B was detected robustly in adult islets. Its transcripts were approximately 500-fold increased compared to RAB3A, 25-fold increased compared to RAB3C and 17-fold increased
compared to \textit{RAB27A} (Fig. 8). \textit{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 prior to 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 beta-cell regeneration in adult mice (Xu et al. 2008). Here, consistent with previous data describing NGN3 transcripts in the human fetal 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 (Elghazi, et al. 2002; Miralles, et al. 1998; Piper et al. 2004; Polak, et al. 2000). At this stage of development, large numbers of insulin-positive cells are apparent prior to organization 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 (Jensen, et al. 2007; Wang, et al. 2006), 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 fetal pancreatic progenitors. In our experience PANC1 cells lack PDX1 protein. On the microarray, hybridisation signal was weak for PDX1 and unaltered by dox treatment. However, similar in vitro models have been employed by others (Gasa et al. 2004) and PDX1 was absent in the NGN3-positive fetal pancreatic cells both in this study and in mouse (Schwitzgebel et al. 2000). Our cloned cells did uniformly express SOX9 and CK19 mimicking fetal 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 beta-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 beta-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 localize robustly to human fetal and adult beta-cells.

There are four RAB3 family members. Regazzi and colleagues previously showed RAB3B and RAB3C, but not RAB3A, by immunoblotting of whole human islets, comprised of multiple hormone-secreting cell-types (Regazzi, et al. 1996). 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 beta-cells (Abderrahmani, et al. 2006; Kasai et al. 2005; Regazzi et al. 1996; Waselle, et al. 2003; Yaekura et al. 2003; Yi, et al. 2002). Via interaction with a network of interacting proteins, they coordinate the intracellular trafficking of insulin granules that culminates in docking at the cell membrane and insulin release (Waselle et al. 2003; Yi et al. 2002). 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 (Aizawa and Komatsu 2005; Kasai et al. 2005; Scrocchi, et al. 1996; Yaekura et al. 2003). Furthermore, hyperglycaemia drastically reduced levels of Rab3a and Rab27a protein in rat beta-cells as a consequence of expression of the transcriptional repressor, Inducible cAMP Early Repressor (ICER) (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 (SUR1), a protein called Piccolo and Rab-interacting molecule 2 (RIM2) (Fujimoto, et al. 2002; Kashima, et al. 2001; Ozaki, et al. 2000; 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 beta-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 beta-cells both during human fetal development and in adult islets with some expression in some adult alpha and delta-cells. Analysis of human adult islets demonstrated RAB3B expression to be approximately equal to 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 approximately 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 fashion, 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 beta-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) (Kasai et al. 2005; Yaekura et al. 2003); 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 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 thirteen pancreatic clones for human *RAB3B* arise from 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 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* localizes 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 and Komatsu 2005). It will be important to ensure such efforts target the appropriate protein. Here, we identify *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 appear as likely to play the important role in human beta-cells that has been described for the equivalent rodent cells (Abderrahmani et al. 2006; Kasai et al. 2005; Regazzi et al. 1996; Waselle et al. 2003; Yi et al. 2002).
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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Figure legends

**Figure 1.** PANC-1 cells express CK19 and SOX9.

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

**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 base pairs (bp) in its 5' flanking region that are regulated by Ngn3-E47 dimers (Huang et al. 2000). C. Fold induction +/- standard error (S.E.) of luciferase activity from transient transfection of p-1613NeuroD1-Luc measured 48 h after the addition of 2 µg/ml doxycycline in four PANC-1_iNGN3 clones. D. Fold increase +/- S.E. of NGN3 and RAB3B expression from microarray analysis of PANC-1_iNGN3 clone 51 by adding 2 µg/ml doxycycline for 48 h. E-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.

**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. from at least two experiments. B. Mean fold induction +/- S.E. 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.

**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. from two experiments. *P = <0.05 following analysis by ANOVA and Dunnett’s post hoc test.
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-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).

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 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 adult pancreas.
A-O. Immunofluorescence counterstained with DAPI (blue) in sections of adult pancreas. Size bar represent 40 µm (A-F, J-O) and 150 µm (G-H).

Figure 8. Expression of RAB3 family members and RAB27A in isolated human adult islets.
Realtime 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 over 40 cycles of PCR.
Figure 1, Piper Hanley et al

A

Fetal pancreas

CK19

SOX9

B

Adult pancreas

CK19

SOX9

C

PANC-1 cells

SOX9 / CK19

DAPI
Figure 2, Piper Hanley et al

A

Doxycycline (µg/ml)

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<td></td>
</tr>
<tr>
<td>2.0</td>
<td></td>
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</tr>
<tr>
<td>4.0</td>
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</tr>
</tbody>
</table>

\[ NGN3 \]

\[ RAB3B \]

\[ Ngn3-E47 \]

\[ NeuroD1 \]

B

\[ Ngn3-E47 \]

\[ NeuroD1 \]

\[ +1 \]

C

<table>
<thead>
<tr>
<th>PANC-1_{iNGN3} clone</th>
<th>Fold induction</th>
<th>S.E.</th>
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</thead>
<tbody>
<tr>
<td>Clone 9</td>
<td>2.24</td>
<td>+/- 0.48</td>
</tr>
<tr>
<td>Clone 15</td>
<td>1.71</td>
<td>+/- 0.39</td>
</tr>
<tr>
<td>Clone 40</td>
<td>1.45</td>
<td>+/- 0.48</td>
</tr>
<tr>
<td>Clone 51</td>
<td>1.97</td>
<td>+/- 0.27</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold induction</th>
<th>S.E.</th>
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</thead>
<tbody>
<tr>
<td>NGN3</td>
<td>2.98</td>
<td>+/- 0.28</td>
</tr>
<tr>
<td>RAB3B</td>
<td>2.50</td>
<td>+/- 0.08</td>
</tr>
</tbody>
</table>

E

Doxycycline (µg/ml)

\[ NGN3 \]

\[ β-ACTIN \]

F

Doxycycline (µg/ml)

\[ RAB3B \]

\[ β-ACTIN \]
Figure 3, Piper Hanley et al

**A**

![Bar chart showing relative expression of RAB3A, RAB3B, RAB3C, RAB3D, and RAB27A with and without Dox.]

**B**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold induction across all PANC-1_INGN3 clones</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAB3A</td>
<td>1.05</td>
<td>0.03</td>
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<tr>
<td>RAB3B</td>
<td>1.85</td>
<td>0.24*</td>
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<tr>
<td>RAB3C</td>
<td>1.00</td>
<td>0.12</td>
</tr>
<tr>
<td>RAB3D</td>
<td>1.15</td>
<td>0.02</td>
</tr>
<tr>
<td>RAB27A</td>
<td>1.12</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Figure 4, Piper Hanley et al
Figure 5, Piper Hanley et al
Figure 6, Piper Hanley et al
Figure 7, Piper Hanley et al
Figure 8, Piper Hanley et al