The effect of neurogenin3 deficiency on pancreatic gene expression in embryonic mice

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Abstract

To understand the molecular mechanisms regulating pancreatic endocrine development and function, pancreatic gene expression was compared between *Ngn3*-deficient mice and littermate controls on embryonic days 13 and 15. Microarray analysis identified 504 genes with significant differences in expression. Fifty-two of these showed at least twofold reduction in *Ngn3* knockouts compared to controls. Many of them were previously described to be involved in endocrine development and function. Among the genes not previously characterized were *Rhomboid veinlet-like 4*, genes involved in tetrahydrobiopterin biosynthesis and the Iroquois-type homeobox gene *lrx1*, the latter was selected for further investigation. *In situ* hybridisation demonstrated that two Iroquois genes, *lrx1* and *lrx2*, were expressed in pancreatic endoderm of wild-type, but not *Ngn3* mutant embryos. Furthermore, ectopic *Ngn3* induced prominent *lrx2* expression in chicken endoderm. Co-labelling established that *lrx1* and *lrx2* mRNA is located to glucagon-, but not insulin- or somatostatin-producing cells in mice and chicken. These data suggest that *lrx1* and *lrx2* serve an evolutionary conserved role in the regulation of α -cell-specific gene expression.

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Introduction

The pancreas is an organ located next to the duodenal part of the intestine and serves two main functions. It produces and secretes enzymes required for the digestion of proteins, carbohydrates, nucleic acids, as well as fats in the small intestine, and hormones including insulin and glucagon required for the regulation of blood glucose levels (Slack 1995). Three distinct populations of cells make up the adult pancreas. The acinar cells produce and secrete enzymes, the duct cells form a highly branched tube system facilitating delivery of enzymes to the intestine and the endocrine cells produce hormones. In adult pancreas, the endocrine cells constitute approximately 2% of the pancreas and are grouped into cell clusters known as islets of Langerhans. Five distinct cell types are discernible in the endocrine compartment. The majority of cells are insulin-producing β -cells, whereas glucagon-producing α -cells, somatostatin-producing δ -cells, pancreatic polypeptide-producing PP-cells and ghrelin-producing ϵ -cells constitute a minor fraction

(Slack 1995, Wierup *et al.* 2002, Prado *et al.* 2004, Heller *et al.* 2005).

At embryonic day 9 (e9), distinct patches of primitive gut epithelium start to evaginate and give rise to the dorsal and the ventral pancreatic buds (Slack 1995). These buds are characterized by homogeneous expression of pancreatic-duodenal homeobox 1 (Pdx1; Ohlsson *et al.* 1993) and these Pdx1-positive cells give rise to all cell types of the adult pancreas (Gu *et al.* 2003). As the buds grow, they invade the surrounding mesenchyme and the resulting epithelial–mesenchymal interactions facilitate further proliferation and differentiation (Kim & Hebrok 2001). Several cell fate choices are required for the development of a complex organ like the pancreas from initially identical cells, and research done during the last decade has revealed several important steps of pancreas development (Murtaugh & Melton 2003, Wilson 2003).

The basic helix–loop–helix transcription factor neurogenin3 (*Ngn3*) is a central player in the choice between exocrine and endocrine cell fate. The absolute

requirement of this transcription factor for endocrine development was demonstrated by Gradwohl *et al.* (2000) who showed that mice lacking functional Ngn3 fail to develop endocrine cells, but develop an apparently normal exocrine pancreas although accumulation of secretory granules was observed at postnatal day 1 (Gradwohl *et al.* 2000). Transgenic mice expressing Ngn3 under the Pdx1 promoter have small underdeveloped pancreata consisting primarily of endocrine cells, showing that ectopic expression of Ngn3 is sufficient to drive Pdx1+ pancreatic precursor cells to an endocrine fate, thereby depleting the pool of pancreatic precursors capable of differentiating into exocrine tissue (Apelqvist *et al.* 1999, Jensen *et al.* 2000b, Schwitzgebel *et al.* 2000). Further evidence for the pro-endocrine nature of Ngn3 was provided by Grapin-Botton *et al.* (2001) by showing that *in ovo* electroporation of chicken endoderm with Ngn3-expressing plasmids promoted endocrine differentiation, not only in the pancreas region but also in the entire primitive gut tube. However, both studies found that endocrine cells with an α -cell fate was the predominant cell type produced by forced expression of Ngn3, indicating that additional factors are required for the development of β -cells. Ngn3 expression is turned off prior to terminal differentiation (Jensen *et al.* 2000a) and is thus not co-expressed with hormone-producing islet cells. Functional studies have identified several transcription factors acting downstream of Ngn3, which are necessary for proper development of the endocrine cells.

Here we present the microarray analysis of *Ngn3*-deficient mice. A cDNA chip containing 20 188 probes was used to compare a major part of the transcriptome in dorsal pancreas buds from wild-type and *Ngn3*-deficient embryos isolated at e13 and e15. In wild-type mice, these days mark the beginning and the end of the secondary transition, a stage of pancreas development where the majority of endocrine cells differentiate (Murtaugh & Melton 2003). The aim of this study is to identify genes expressed in endocrine cells and their precursors and to map important pathways that operate during the formation of the endocrine cells.

Materials and methods

Tissue isolation

Time-mated *Ngn3*-mutant mice (Gradwohl *et al.* 2000) were killed by cervical dislocation at e13 and e15. The dorsal pancreatic bud was isolated from embryos and stored in RNAlater (Ambion, Austin, TX, USA) at 4 °C overnight. The next day, RNAlater was removed and the tissue was stored at −80 °C.

Genotyping

Genotyping of the embryos was done by PCR analysis on genomic DNA isolated from forelimbs, as described by Gradwohl *et al.* (2000), using BioTaq polymerase (Bioline GmbH, Luckenwalde, Germany) according to the manufacturer's recommendations. Genomic DNA was extracted by incubating 50 mM Tris (pH 8), 100 mM EDTA and 0.5% SDS, in 10 mg/ml proteinase K, at 55 °C overnight. Proteins were precipitated by the addition of NaCl to a final concentration of 1.3 M and the lysate was cleared by centrifugation at 10 000 *g* for 5 min. Supernatants were transferred to a clean tube and nucleic acids precipitated by the addition of 0.5 vol isopropanol, followed by gentle mixing and centrifugation at 10 000 *g* for 10 min. The pellet was washed in 70% EtOH followed by 96% EtOH and finally resuspended in 100 μ l Milli-Q water. Thermal cycling was done in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) for 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR products were visualized by agarose gel electrophoresis in the presence of EtBr.

Total RNA isolation

Mini RNA isolation kit (Zymo Research, Orange, CA, USA) was used to isolate total RNA from pancreas tissue using manufacturer's instructions with a few changes. After addition of lysis buffer, the tissue was homogenized using disposable pistils for 1.5 ml Eppendorf tubes (Buch&Holm, Herlev, Denmark). The wash buffer was substituted with 80% EtOH and the total RNA was eluted with Milli-Q water. Subsequently, the integrity and the quantity of the purified RNA were determined on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the Eukaryotic Total RNA Nano chip (Agilent) as described by the manufacturer.

Sample labelling

The total RNA from dorsal pancreata was amplified using a combination of the RiboAmp kit (Arcturus, Mountain View, CA, USA) and T7 MEGAscript kit (Ambion). Briefly, generation and purification of double-stranded cDNA was done with components from the Arcturus kit following the manufacturer's recommendations. The resulting cDNA was *in vitro* transcribed using components from the MEGAscript kit and aminoallyl-modified UTP (aa-UTP; Sigma). Briefly, the generated double-stranded cDNA was *in vitro* transcribed in 40 μ l reactions (1 $\times$ T7 reaction buffer, 7.5 mM ATP, 7.5 mM CTP, 7.5 mM GTP, 3.75 mM UTP, 3.75 mM aa-UTP and 4 μ l T7 Enzyme Mix) at 37 °C for 5 h. Subsequently, the cDNA template was digested with 4 U DNaseI for 15 min at 37 °C and amino allyl cRNA

was purified on RNeasy columns (Qiagen). Manufacturer's recommendations were modified by replacing the RPE wash buffer with 80% EtOH, adding an additional wash step and eluting the aa-cRNA from the columns in two times 30 µl Milli-Q water. The purified aa-cRNA was split into 2 µg aliquots, completely dried in a SpeedVac (Holm and Halby, Brøndby, Denmark) and stored at -20°C . Coupling of fluorescent dyes to aa-cRNA was done by mixing 2 µg aa-cRNA resuspended in 4.5 µl freshly made 0.1 M sodium carbonate buffer (pH 9.0) with Alexa Fluor 555 or Alexa Fluor 647 succinimidyl ester (Molecular Probes, Eugene, OR, USA) resuspended in 4.5 µl dimethyl sulfoxide (DMSO) (Sigma) and incubating at 25°C for 1 h. Subsequently, uncoupled succinimidyl ester dyes were quenched by the addition of 4.5 µl, 4 M hydroxylamine and incubating for 15 min at 25°C . Labelled cRNA was purified on RNeasy columns with the following modifications to manufacturer's protocol. An additional wash step with 500 µl RPE buffer was added and the dye-coupled cRNA was eluted two times in 40 µl Milli-Q water. Labelling efficiency and concentration of nucleic acid were determined by measuring absorbance of the cRNA-dye conjugate at λ -max of the dye and 260 nm respectively. All incubation steps were done in a MJ Research PTC-200 Peltier Thermal Cycler.

Microarray

The ArrayTAG 20k murine gene collection (LION Bioscience AG, Heidelberg, Germany) was spotted in duplicate on CodeLink slides (GE Healthcare, Hillerød, Denmark) using a BioRobotics Microgrid II spotter (Genomic Solutions Ltd, Cambridgeshire, UK). Probes contained an amino modification at the 5' end of the sense strand allowing specific covalent coupling of the sense strand to *N*-hydroxysuccinimide ester groups on the slide. Prior to hybridisation, residual reactive groups on the slide were blocked by incubating, 0.1 M Tris (pH 9), in 50 mM ethanolamine, at 50°C for 30 min. Slides were rinsed twice with deionised water and incubated in $4\times\text{SSC}$, 0.1% SDS for 30 min. Subsequently, slides were rinsed in deionised water and placed in boiling water for 2 min to render the covalently attached probes single stranded. Following two additional rinses with water, the slides were pre-hybridised in digoxigenin (DIG) Easy Hyb buffer (Roche) for 30 min at 42°C . Finally, slides were rinsed twice in water and dried using compressed air. Dye-conjugated cRNA corresponding to 30 pmol of each fluorescent dye was mixed with 7 µg mouse Cot-1 DNA (Invitrogen) and 1 µg poly dA (DNA technology, Århus, Denmark). The hybridisation mix was dried in a SpeedVac and resuspended in 80 µl digoxigenin (DIG) Easy Hyb buffer. Nucleic acids of the hybridisation mix were denatured at 95°C for 2 min

immediately prior to setting up the hybridisation. Labelled cRNA was hybridised to the arrays under LifterSlips (Erie Scientific Company, Portsmouth, NH, USA) in GeneMachines HybChambers (Genomic Solutions Ltd, Cambridgeshire, UK) immersed in a 42°C water bath for 16 h.

The LifterSlips were removed by immersing slides in $2\times\text{SSC}$, 0.1% SDS, and the slides were placed subsequently in a slide rack immersed in $2\times\text{SSC}$, 0.1% SDS. All post-hybridisation washes were performed on a shaker to ensure gentle agitation of the wash solution. Slides were washed twice in $2\times\text{SSC}$, 0.1% SDS at room temperature for 5 min, twice in $0.5\times\text{SSC}$, 0.1% SDS at 55°C for 15 min, and finally a quick rinse in water immediately prior to drying with compressed air. Slides were scanned in an Axon 4000B scanner at a resolution of 5 µm/pixel and image analysis was done in GenePix Pro 5 (Molecular Devices, Sunnyvale, CA, USA).

Experimental setup

The experiment was designed to analyse the expression of genes in dorsal pancreatic cells at two temporally separated stages of pancreas development. This was accomplished by comparing expression profiles of embryonic dorsal pancreas tissue from *Ngn3* null mice with wild-type littermates at days 13 and 15 of embryonic development. The comparison of gene expression in mutant and wild-type pancreas was primarily expected to show genes that are lower expressed/missing in the mutant, as *Ngn3* null mice have no endocrine pancreas tissue. From each developmental stage, five wild-type and five mutant samples were chosen, representing embryos from at least three different litters. Wild-type and mutant samples from the common stage of development were paired randomly and analysed in flipped colour. Probes were spotted in duplicate on each slide in a randomised (fixed) layout, effectively distributing the duplicate spots randomly over the slide.

Data analysis

Analysis of the microarray data was done using R-2.2.0 (R Development Core Team 2005) and Bioconductor 1.7 packages (Gentleman *et al.* 2004). The limma package (Smyth *et al.* 2005) was used for the normalisation and identification of differentially expressed transcripts essentially as described in the *Limma User's Guide*. The arrays were normalised by print-tip loess normalisation followed by between slide-scale normalisation. After averaging within slide duplicates, a linear model was fitted to the data and empirical Bayesian smoothing was applied to the standard errors and

finally, the Benjamini and Hochberg procedure of controlling false-discovery rate (Benjamini & Hochberg 1995) was used to adjust *P*-values and a false-discovery rate of 0.01 was chosen. The dye-swap replicates were treated as described in the user's guide. The AnnBuilder package (Zhang *et al.* 2003) was used to build annotation database for the LION array; GO, GOstats and Rgraphviz packages were used for GO analysis and construction of the GO graph.

DNA constructs

The mouse cDNA for Ngn3 was a gift from F Guillemot. Mouse cDNA for Irx1 was a gift from Dr Vincent M Christoffel, University of Amsterdam, Holland. The full-length mouse Irx2 was purchased as an IMAGE EST clone. The chicken Irx genes were purchased from MRC Gene Service, Cambridge, UK. For electroporation, the Ngn3 cDNA was subcloned into the pCAGGS-IRES-EGFP vector (a gift from Dr Anne Grapin-Botton). Restriction digests and sequencing confirmed the correct orientation and reading frames of the constructs. Plasmids were purified using Endofree Maxiprep system (Qiagen) according to manufacturer's instructions.

In ovo endoderm electroporation

This method was performed as described earlier (Grapin-Botton *et al.* 2001, Pedersen & Heller 2005). After 72 h, surviving embryos were recovered and fixed in fresh 4% paraformaldehyde (PFA) in PBS overnight. Only highly electroporated embryos, as determined by the abundance of green fluorescent protein (GFP⁺) cells in the embryos, were further processed. Embryos were embedded in OCT compound (Sakura Finetech Europe, Zoeterwoude, The Netherlands) and frozen on dry ice and stored at -80 °C until sectioned.

Immunocytochemistry and in situ hybridisation

In situ hybridisations were performed on 10 µm frozen sections with DIG-labelled antisense RNA probes for Irx1-5 created using the DIG RNA synthesis kit from Roche, following the manufacturer's instructions. Mouse cDNAs for Irx1-5 were a gift from Dr Vincent M Christoffel, University of Amsterdam, Holland. The chicken Irx genes were purchased from MRC Gene Service, Cambridge, UK. Probes were hybridised to the tissues at 68 °C followed by an AP-conjugated anti-DIG antibody (Roche). Deposition of the Nitro-Blue Tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) chromophore (Sigma) was catalysed in 100 mM NaCl 1 ml 1 M 100 mM Tris, pH 9, 51 ml 1 M 50 mM MgCl 1ml 0.5 M 0.1% Tween 20 10 µl 100% (NTMT) buffer at pH 9.5 and fast red (Sigma) signals

for the fluorescent *in situ* were developed in 0.1 M Tris (pH 8.2). Immunostaining reactions were performed after antigen retrieval by microwave treatment in 10 mM sodium citrate (pH 6.0). The primary antiserum was incubated overnight at 4 °C. Immunoreactivity was visualised with a Tyramide Signal Amplification (TSA) kit (Perkin-Elmer Life Science, Wellesley, MA, USA) or with the Alexa-series of secondary antibodies (Molecular Probes)

	Species	Dilution	Source
Antigen			
Pdx1 253	Rabbit	1:5000	Joel Habener, MGH, Boston, MA
Pax6	Rabbit	1:2000	Babco, Richmond, CA.
Nkx6-1	Rabbit	1:5000	Jensen <i>et al.</i> (1996)
Ngn3	Rabbit	1:1000	Michael German, UCSF
ISL-1	Rabbit	1:1000	Helena Edlund, Umeå, Sweden
Insulin	Mouse	1:75	Novo Nordisk A/S, Denmark
Insulin	Guinea Pig	1:200	DAKO, Glostrup, Denmark
Glucagon	Mouse	1:50	Novo Nordisk A/S, Denmark
PP	Rabbit	1:500	DAKO, Glostrup, Denmark
Somatostatin	Mouse	1:10	Novo Nordisk A/S, Denmark
Somatostatin	Rabbit	1:100	DAKO, Glostrup, Denmark
Ghrelin	Goat	1:500	Santa Cruz Biotechnology, CA
cMYC (A14)	Rabbit	1:80	Santa Cruz Biotechnology, Santa Cruz, CA
cMYC (9E10)	Mouse	1:50	Santa Cruz Biotechnology, Santa Cruz, CA

RT-PCR

Quantitative reverse transcriptase (RT)-PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Assays-on-Demand Gene Expression Products (Applied Biosystems). Assays for verification of microarray results were delivered in low-density arrays. Total RNA was reverse-transcribed using Reverse Transcriptase Reagents (Applied Biosystems) according to manufacturer's recommendations. Relative quantification of gene expression measured on low-density arrays was performed using the ΔΔC_t method. Critical threshold (C_t) cycle is defined as the cycle in which the reporter signal crosses a given threshold. The C_t-values were normalized to 18S levels by subtracting the 18S C_t values resulting in ΔC_t values. The ΔΔC_t values were

obtained by subtracting ΔC_t values from mutant and wild type, resulting in a $\log_2$ ratio of target gene expression in mutant over wild type (equivalent to the M value determined in the microarray data analysis). The following assays were ordered as low-density arrays: Mm00514341_m1 (*Chga*), Mm00517617_m1 (*Cryba2*), Mm00432448_m1 (*Cdkn1a*), Mm00484138_m1 (*Cyp4b1*), Mm00456190_m1 (*Myt1*), Mm00440465_g1 (*Neurod2*), Mm00440480_m1 (*Nnat*), Mm00499982_m1 (*Pcdh21*), Mm00485697_m1 (*Resp18*), Mm00803266_m1 (*Rbp4*), Mm00460511_m1 (*Rhbd14*), Mm00460679_m1 (*Sesn2*), Mm00600526_m1 (*Syt13*), Mm00443220_m1 (*Tcp11*), Mm00451001_m1 (*Tm4sf2*), Mm00491265_m1 (*2510004L01Rik*), Mm00549967_m1 (*C81234*), Mm00731595_gH (*Ins2*), Mm00437606_s1 (*Neurog3*).

Results

Microarray analysis of *Ngn3*-deficient mice

To examine the difference in transcriptomes of dorsal pancreas from wild-type and *Ngn3*-deficient mice, a spotted cDNA microarray comprising 20 188 probes covering 12 140 genes plus ESTs was used. The comparison was made at two different stages of pancreas development, allowing monitoring of large-scale parallel gene expression at the beginning and end of the secondary transition. Total RNA was isolated from homozygous wild-type and mutant pancreata, and a single round of RNA amplification yielded sufficient material for microarray analysis of individual pancreas.

Spatial analysis of the foreground and background intensities obtained after image analysis of the hybridised arrays did not reveal any major flaws (data not shown; Petri *et al.* 2004). Data analysis was performed as described in 'Materials and methods' and resulted in identification of 504 transcripts showing differential expression between *Ngn3*-deficient and wild-type mice at e13 and/or e15 (see Fig. 1). The MA plots show that many of the identified genes show only relatively small differences in expression levels ($|M| < 1$ corresponding to less than twofold). Furthermore, the majority of genes show decreased expression levels in mutant mice compared to wild type. To identify genes that exhibited a robust difference between wild type and mutant, we defined an M value cut-off corresponding to at least twofold difference. This filtering strategy reduced the list of genes to 52 showing significant difference at e13 and/or e15 (Table 1), all with decreased expression in *Ngn3*-deficient mice. Expression of several neuropeptide genes (*Ghrl*, *Gip*, *Gal*, *Cck* and *Npy*) as well as genes whose products are involved in hormone processing (*Pcsk2*, *Pcsk1n*, and *7B2*) are lacking in the mutant mice. Multiple members of the granin family (*Chga*, *Chgb*, *Scg3*), which are known to be expressed in endocrine cells of the pancreas, displayed large differences between mutant and wild-type mice ($M < -2$). Two members (*Syt7* and *Syt13*) of the synaptotagmin family of vesicle proteins, which are believed to mediate calcium-dependent regulation of membrane trafficking (Yoshihara & Montana 2004) and transthyretin, which has an important role in the plasma transport of thyroid hormone T4 and retinol

Figure 1 MA plots of microarray data from e13 (A) and e15 (B). Transcripts showing significant difference between wild-type and *Ngn3*-deficient mice at a false discovery rate of 1% are emphasized with large coloured dots. Transcripts showing higher levels in wild type compared to mutant ($M > 0$) are red and transcripts showing lower levels ($M < 0$) are green.

Table 1 List of genes showing robust difference in expression levels between *Ngn3*-deficient and wild-type mice at e13 or e15

	Gene name	Symbol	e13.M	e13.Q.Value	e15.M	e15.Q.Value
ID						
Hormone, hormone processing						
MG-6-2n7	Cholecystokinin	Cck	-2.18	3.19×10^{-9}	-1.99	3.21×10^{-7}
MG-8-92o10	Galanin	Gal	-1.40	5.34×10^{-7}	-0.57	9.32×10^{-6}
MG-12-168g11	Gastric inhibitory polypeptide	Gip	-1.26	1.28×10^{-4}	-0.55	3.10×10^{-4}
MG-3-30i2	Ghrelin	MGI:1930008	-2.56	1.23×10^{-7}	-2.23	1.96×10^{-8}
MG-6-55p2	Neuropeptide Y	Npy	-0.94	3.45×10^{-4}	-2.26	1.16×10^{-8}
MG-6-22i12	Proprotein convertase subtilisin/kexin type 1 inhibitor	Pcsk1n	-1.84	7.25×10^{-7}	-1.37	4.66×10^{-9}
MG-3-76n17	Proprotein convertase subtilisin/kexin type 2	Pcsk2	-2.22	4.34×10^{-7}	-2.22	9.20×10^{-8}
MG-14-40o13	Secretory granule neuroendocrine protein 1, 7B2 protein	Sgne1	-1.26	2.52×10^{-7}	-1.69	2.21×10^{-8}
Binding						
MG-6-3j21	Chromogranin A	Chga	-3.02	1.07×10^{-9}	-2.50	2.21×10^{-8}
MG-14-45h10	Chromogranin B	Chgb	-3.17	7.47×10^{-8}	-2.38	1.18×10^{-8}
MG-6-55a14	Secretogranin III	Scg3	-1.98	3.72×10^{-7}	-2.03	6.61×10^{-8}
MG-8-101o4	Retinol binding protein 4, plasma	Rbp4	-1.62	6.37×10^{-7}	-1.51	4.00×10^{-9}
MG-6-25p16	Secretagoin, EF-hand calcium binding protein	Scgn	-1.99	4.07×10^{-6}	-2.15	2.45×10^{-8}
MG-16-118p10	Protocadherin 21	Pcdh21	-0.97	2.77×10^{-4}	-1.23	2.41×10^{-7}
Transcription, translation						
MG-6-4c22	Iroquois related homeobox 1 (<i>Drosophila</i>)	Irx1	-1.35	2.39×10^{-7}	-0.78	2.28×10^{-5}
MG-6-27f14	Myelin transcription factor 1	Myt1	-1.03	1.32×10^{-5}	-1.21	2.21×10^{-8}
MG-6-2g18	Ribosomal protein S12	Rps12	-1.04	3.31×10^{-8}	-1.38	4.37×10^{-8}
Transport						
MG-6-64j8	Synaptotagmin 7	Syt7	-0.67	5.84×10^{-5}	-1.25	5.59×10^{-10}
MG-6-64b7	Synaptotagmin 7	Syt7	-0.90	5.34×10^{-7}	-1.40	5.59×10^{-10}
MG-8-12f2	Synaptotagmin 13	Syt13	-1.65	3.68×10^{-6}	-1.71	8.88×10^{-9}
MG-14-119n19	Transthyretin	Ttr	-1.73	6.57×10^{-7}	-0.40	7.86×10^{-4}
MG-11-1e9	Transthyretin	Ttr	-1.74	4.07×10^{-6}	-0.41	4.44×10^{-4}
Signalling						
MG-6-36e16	Rhomboid, veinlet-like 4 (<i>Drosophila</i>)	Rhbdl4	-0.98	2.35×10^{-4}	-1.00	2.56×10^{-8}
MG-8-89p12	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	Gnas	-1.90	1.15×10^{-7}	-2.18	2.21×10^{-8}
MG-6-30c20	Protein phosphatase 1, regulatory (inhibitor) subunit 1A	Ppp1r1a	-0.83	3.72×10^{-7}	-1.60	4.23×10^{-8}
Kinase/phosphatase						
MG-6-15i10	Ribosomal protein S6 kinase, polypeptide 4	Rps6ka4	-1.17	8.45×10^{-6}	-0.25	9.39×10^{-3}
MG-12-165c14	Serum/glucocorticoid regulated kinase 2	Sgk2	-1.07	3.68×10^{-6}	-0.38	2.03×10^{-2}
MG-14-3k11	Cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	-1.14	2.14×10^{-4}	-0.33	2.02×10^{-3}
MG-6-47c24	Protein tyrosine phosphatase, receptor type, N	Ptpn	-1.46	6.80×10^{-6}	-0.55	4.16×10^{-5}
MG-6-67o19	Proviral integration site 2	Pim2	-1.07	9.83×10^{-6}	-1.13	5.08×10^{-9}
Not classified						
MG-19-1k5	Ureidopropionase, beta	Upb1	-1.16	1.53×10^{-6}	-0.52	4.92×10^{-5}
MG-6-16i16	Nucleosome assembly protein 1-like 5	Nap1l5	-1.75	1.79×10^{-6}	-0.69	5.24×10^{-6}
MG-12-140m7	Serine protease inhibitor, Kazal type 3	Spink3	-1.53	1.76×10^{-4}	-0.53	1.76×10^{-1}
MG-8-72n2	Fibrinogen, B beta polypeptide	Fgb	-1.33	3.20×10^{-3}	-0.28	1.03×10^{-1}
MG-60-1p11	Crystallin, beta A2	Cryba2	-0.74	1.76×10^{-4}	-1.61	1.89×10^{-8}
MG-6-38n22	Regulated endocrine-specific protein 18	Resp18	-1.59	1.37×10^{-7}	-1.23	2.16×10^{-7}
MG-3-89b2	Solute carrier family 38, member 5	Slc38a5	-3.84	1.15×10^{-7}	-2.21	2.79×10^{-9}
EST						
MG-6-60f22	RIKEN cDNA 1700012H17 gene	1700012H17Rik	-1.86	7.72×10^{-7}	-0.43	1.08×10^{-3}

MG-6-44a1	RIKEN cDNA 2610028H07 gene	2610028H07Rik	-1.45	4.34 × 10 ⁻⁷	-0.31	7.55 × 10 ⁻³
MG-3-32/24	RIKEN cDNA 3100002J23 gene	3100002J23Rik	-1.50	2.59 × 10 ⁻⁷	-1.84	2.53 × 10 ⁻⁷
MG-6-51m20	RIKEN cDNA A930033C01 gene	A930033C01Rik	-2.00	9.24 × 10 ⁻⁸	-1.86	4.00 × 10 ⁻⁹
MG-6-43b23	RIKEN cDNA 1810043H04 gene	1810043H04Rik	-1.88	6.50 × 10 ⁻⁶	-0.44	7.06 × 10 ⁻⁴
MG-15-252p15	RIKEN cDNA 2310015B20 gene	2310015B20Rik	-1.15	3.08 × 10 ⁻⁵	-1.13	1.82 × 10 ⁻⁶
MG-3-911f6	RIKEN cDNA 4932418K24 gene	4932418K24Rik	-1.46	5.88 × 10 ⁻³	-0.97	4.24 × 10 ⁻²
MG-3-45i10	RIKEN cDNA 4930488E11 gene	4930488E11Rik	-0.90	1.53 × 10 ⁻⁶	-1.81	6.98 × 10 ⁻⁸
MG-3-45m4	RIKEN cDNA 1700086L19 gene	1700086L19Rik	-2.56	3.00 × 10 ⁻¹⁰	-2.82	4.84 × 10 ⁻⁹
MG-6-76j9	RIKEN cDNA 0610007P22 gene	0610007P22Rik	-2.01	4.27 × 10 ⁻⁹	-1.92	3.60 × 10 ⁻⁹
MG-6-1o1	EST (seq not found in Genbank)	EST	-1.94	1.37 × 10 ⁻⁷	-1.63	7.80 × 10 ⁻⁷
MG-6-86f9	EST (seq not found in Genbank)	EST	0.04	8.51 × 10 ⁻¹	-1.43	4.02 × 10 ⁻⁸
MG-3-220d22	EST (seq not found in Genbank)	EST	-1.24	1.60 × 10 ⁻⁶	-0.30	2.07 × 10 ⁻³
MG-12-149k8	EST (seq not found in Genbank)	EST	-1.10	4.38 × 10 ⁻⁶	-0.37	3.54 × 10 ⁻⁴
MG-6-43i24	Gene model 1052, (NCBI)	Gm1052	-1.11	1.43 × 10 ⁻⁵	-0.33	2.36 × 10 ⁻¹

(Soares *et al.* 2003) are expressed at decreased levels in the mutant. Several genes encoding proteins involved in phosphorylation reactions are showing robust differences between mutant and wild type. Expression of genes involved in signalling pathways is also affected by Ngn3 deficiency. *Rhomboid veinlet-like 4*, a vertebrate homologue of *Drosophila Rhomboid* (Jaszai & Brand 2002) encoding a protein involved in regulated intramembrane proteolysis, as well as *Stimulatory G protein alpha subunit*, are expressed at lower levels in mutant mice. Several ESTs and transcripts of genes with unknown function (RIKEN clones) are found at lower levels in dorsal pancreas from mutant mice, some of which show large differences. Interestingly, in this list of robustly differentially expressed transcripts, we detect two transcription factors, namely *Myt1*, which was recently reported to be expressed in endocrine precursor cells (Gu *et al.* 2004) and *Iroquois related homeobox 1* (*Irx1*). *Irx1* belongs to a family of homeodomain proteins, which is conserved from nematodes to humans (Cavodeassi *et al.* 2001). Six different *Irx* genes have been identified in mouse, of which *Irx1*, *Irx3* and *Irx5* are present on the array, but differential expression between mutant and wild type is only detected for *Irx1*.

Differential expression of several transcripts showing lower expression in mutant mice was verified by RT-PCR on total RNA isolated from another batch of mice ($n=3$). We verified the expression of a few genes showing more than twofold difference as well as some genes showing less than twofold difference in expression levels at either e13 or e15 (see Fig. 2). The data from these experiments have been summarised in Fig. 2 and the actual values are shown in Supplementary Table 1 (See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>). The RT-PCR results corroborated the microarray data. Only *Cyp4b1* shows disagreement between the two platforms at both e13 and e15, whereas *Cdkn1a* does so at e15.

Further microarray data analysis

The filtration of microarray data described in the previous section resulted in identification of genes showing significant and robust difference in expression between mutant and wild type. However, the genes presented in Table 1 represent only a small subset (52 out of 504 genes) of the transcripts shown by microarray analysis to differ significantly between mutant and wild type at e13 and/or e15. The complete list of genes can be found in Supplementary Tables 2 and 3 (e13 and e15 data respectively: See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>). In particular, the additional filtration results in omission of a substantial fraction of genes showing increased expression, albeit small, at e13 (Fig. 1, red dots). The full sets of genes identified at e13 and e15 were divided

Figure 2 RT-PCR verification of selected gene expression differences. The scatter plots show the correlation between $\log_2$ fold differences determined by microarray analysis (M values) and RT-PCR ($\Delta\Delta C_t$) at e13 and e15 for 16 genes. There is a positive correlation between the two platforms with R^2 values of 0.62 and 0.58 at e13 and e15 respectively. Inconsistencies were observed for *Cyp4b1* at both e13 and e15, and for *Cdkn1a* at e15.

into groups of genes showing increased or decreased expression in the mutant. Subsequently, these gene lists were analysed for over-representation of gene ontology (GO) terms in the biological process (BP) branch of the ontology (see Table 2). The number of genes showing increased expression at e15 is quite small, not many terms are identified and those identified contain few genes. However, the other groups of genes show several notable terms. Not surprisingly, we find 'peptide hormone processing' and 'neuropeptide-signalling pathway' represented by genes showing decreased expression in the mutant at both e13 and e15. At e15, we further identify genes involved in 'transport' and 'G-protein-coupled receptor protein-signalling pathway' in the group of genes showing reduced expression. The most significant finding at e13 among decreased transcripts is terms related to tetrahydrobiopterin biosynthesis. Tetrahydrobiopterin is an obligatory co-factor of nitric oxide synthases (Kwon *et al.* 1989, Tayeh & Marletta 1989) and *in vitro* experiments in INS-1 cells have shown that tetrahydrobiopterin synthesis precedes nitric oxide-dependent inhibition of insulin secretion (Laffranchi *et al.* 1997). The terms that are associated with the largest number of genes showing reduced expression at e13 are related to apoptosis. Interestingly, genes involved in 'cell communication' and 'cell adhesion' show increased expression in the mutant at e13 and there is also an over-representation of genes involved in 'neurogenesis'. To further compare the difference between increased and decreased genes at e13, we computed the induced GO graph of genes identified at e13 and used a green–black–red colour gradient to indicate whether genes annotated

at a particular node (either directly or by inheritance) showed increased expression, decreased expression, or a mix of both (Fig. 3 and Supplementary Figure S1 See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>). GO terms that were identified as significantly over-represented (Table 2) have been emphasized in Fig. 3. While only few terms are identified as significantly over-represented, this graph identifies processes, which are only affected by decreasing gene expression. Some of these terms have been labelled in Fig. 3.

α -Cell-specific *Irx1/2* expression depend on and is induced by NGN3

Irx1 and *Irx2* have previously been shown to be expressed in the pancreas (Houweling *et al.* 2001) but their function and cell-specific expression pattern are unknown. Our microarray data suggest that *Irx1* is expressed in the endocrine lineage. To determine whether *Irx1* and *Irx2* were expressed in mature endocrine cells or their precursors, we performed fluorescent *in situ* hybridisations for the *Irx1/2* genes followed by immunofluorescent stainings with a marker specific for these two populations. *Irx1*- and *Irx2*-expressing cells were found to be ISL1⁺ (Fig. 4A and C), a marker of mature endocrine cells (Thor *et al.* 1991, Ahlgren *et al.* 1997), but negative for the precursor marker NGN3 (Gradwohl *et al.* 2000, Jensen *et al.* 2000a, Schwitzgebel *et al.* 2000, Gu *et al.* 2002) although *Irx1/2*-expressing cells were often found in close association with NGN3⁺ cells (Fig. 4B and D).

Table 2 Over-represented GO BP terms

GO id	Term	Genes	P value
Over-represented Gene Ontology terms, e13 decreased expression			
GO:0046146	Tetrahydrobiopterin metabolism	3	7.02×10^{-5}
GO:0006729	Tetrahydrobiopterin biosynthesis	3	7.02×10^{-5}
GO:0042559	Pteridine and derivative biosynthesis	3	5.56×10^{-4}
GO:0042558	Pteridine and derivative metabolism	3	7.84×10^{-4}
GO:0019438	Aromatic compound biosynthesis	3	1.06×10^{-3}
GO:0016486	Peptide hormone processing	2	3.62×10^{-3}
GO:0008219	Cell death	12	4.15×10^{-3}
GO:0016265	Death	12	4.69×10^{-3}
GO:0043064	Flagellum organization and biogenesis	2	5.36×10^{-3}
GO:0007218	Neuropeptide-signalling pathway	4	5.52×10^{-3}
GO:0000082	G1/S transition of mitotic cell cycle	3	5.57×10^{-3}
GO:0006915	Apoptosis	11	6.68×10^{-3}
GO:0012501	Programmed cell death	11	7.10×10^{-3}
GO:0007585	Respiratory gaseous exchange	2	9.76×10^{-3}
GO:0006558	L-phenylalanine metabolism	2	9.76×10^{-3}
Over-represented Gene Ontology terms, e13 increased expression			
GO:0009880	Embryonic pattern specification	3	3.01×10^{-4}
GO:0007224	Smoothed receptor-signalling pathway	2	1.61×10^{-3}
GO:0009953	Dorsal/ventral pattern formation	2	1.61×10^{-3}
GO:0007389	Pattern specification	4	1.83×10^{-3}
GO:0007399	Neurogenesis	7	3.23×10^{-3}
GO:0007420	Brain development	3	4.52×10^{-3}
GO:0009799	Determination of symmetry	2	4.70×10^{-3}
GO:0009855	Determination of bilateral symmetry	2	4.70×10^{-3}
GO:0007368	Determination of left/right symmetry	2	4.70×10^{-3}
GO:0000059	Protein-nucleus import, docking	2	5.71×10^{-3}
GO:0007155	Cell adhesion	8	5.97×10^{-3}
GO:0007417	Central nervous system development	3	6.31×10^{-3}
GO:0007154	Cell communication	22	7.51×10^{-3}
Over-represented Gene Ontology terms, e15 decreased expression			
GO:0006816	Calcium ion transport	8	3.02×10^{-6}
GO:0016486	Peptide hormone processing	3	5.63×10^{-5}
GO:0007017	Microtubule-based process	9	7.93×10^{-5}
GO:0015674	Di-, tri-valent inorganic cation transport	8	8.10×10^{-5}
GO:0006810	Transport	38	4.44×10^{-4}
GO:0042445	Hormone metabolism	4	1.49×10^{-3}
GO:0006812	Cation transport	11	1.62×10^{-3}
GO:0016485	Protein processing	3	2.24×10^{-3}
GO:0030001	Metal ion transport	9	2.55×10^{-3}
GO:0007186	G-protein-coupled receptor protein-signalling pathway	10	3.34×10^{-3}
GO:0006811	Ion transport	13	5.09×10^{-3}
GO:0007026	Microtubule stabilisation	2	8.47×10^{-3}
Over-represented Gene Ontology terms, e15 increased expression			
GO:0016049	Cell growth	2	2.94×10^{-3}
GO:0008361	Regulation of cell size	2	3.21×10^{-3}
GO:0040007	Growth	2	5.88×10^{-3}

To determine if *Irx1/2* gene expression was restricted to a particular endocrine subtype, we performed *in situ* hybridisation for *Irx1/2* (Fig. 5A and F) followed by fluorescent antibody staining for the major pancreatic hormones. We found *Irx1* and *Irx2* to be co-expressed with glucagon (Fig. 5D and I), but not insulin (Fig. 5C and H) and somatostatin at e18 (Fig. 5E and J). *Irx1/2* did co-localize with a subpopulation of PP cells, most of which also expressed glucagon (data not shown). The α -cell-specific expression of *Irx1/2* was also seen in E4

chicken pancreas (Fig. 5A–D). Additionally, RT-PCR analysis demonstrated prominent *Irx1/2* expression in the mouse glucagonoma cell line, α TC1-9 (Hamaguchi & Leiter 1990) while the insulinoma cell line β TC3 (Efrat *et al.* 1988) had barely detectable levels (data not shown).

Consistent with our microarray data, expression of *Irx1/2* was lost in mice deficient in *Ngn3* (Fig. 6A–D). Furthermore, forced expression of NGN3 in chick endoderm induced the expression of both *Irx1* (data

Figure 3 Induced GO tree of all genes showing significant difference between *Ngn3*-deficient and wild-type mice. Each node in the tree corresponds to a particular GO term, which might be child to one or more GO terms and in turn be parent to the same. The root of the GO tree corresponds to very generic terms and progressing upwards from parent term to child term results in increasingly more specific GO terms. Some nodes in this induced GO graph are not represented by probes on the chip and these have been coloured grey.

Figure 4 *Irx1*- and *Irx2*-expressing cells belong to the endocrine lineage (A–D) Localisation of *Irx1* and *Irx2* with *ISL1* and *NGN3* at e16 visualised by fluorescent *in situ* hybridisations for *Irx1* and *Irx2* (in red) followed by immunofluorescent stainings for *NGN3* and *ISL1* (in green). *ISL1* is detectable in *Irx1*- (A) and *Irx2*- (C) positive cell clusters showing their endocrine nature. (B, D) Some *NGN3*-positive endocrine precursors lie close to *Irx1*- and *Irx2*-expressing cells. A and C are 100 × magnification. B and D are 250 × magnification.

Figure 5 *Irx1* and *Irx2* are expressed in the α -cells but not in β -cells or δ -cells. (A–N). *In situ* hybridisation at e18 for *Irx1* and *Irx2* as indicated combined with double immunofluorescent stainings for insulin and glucagon (A–D and F–I) or immunofluorescent stainings for somatostatin (E and J). B and G show the emerging islets with insulin immunoreactive cells in the core and glucagon immunoreactive cells at the periphery. C and H show merged images of glucagon and *Irx1* and *Irx2* respectively. D and I show merged images of insulin and *Irx1* and *Irx2* respectively. Note that *Irx1* and *Irx2* are co-expressed with glucagon but not with insulin. E and J show that *Irx1*- and *Irx2*-positive cells respectively, do not express somatostatin. (*Irx* is shown in false colour in C–E and H–J). *In situ* hybridisation for the chicken *Irx1* and *Irx2* genes followed by immunocytochemistry for Nkx6.1 and glucagon on the same sections of E4 chick embryos (K–N) *Irx1* and *Irx2* are expressed in the pancreatic area (black arrowheads) where the glucagon cells (white arrowheads) are expressed. Nkx6.1 is used as a marker for the pancreatic region. Small black dots in panels A and B are small bubbles and an artefact of the *in situ* hybridisation procedure.

not shown) and *Irx2* and a subset of the *Ngn3/Irx* positive cell clusters were also glucagon immunoreactive (Fig. 6E–H).

Discussion

The microarray experiment described here was set up to analyse changes in gene expression in the pancreas of *Ngn3*-deficient mice. Owing to the absence of *Ngn3*, cells of the developing pancreas cannot adopt an endocrine cell fate resulting in mice lacking endocrine pancreatic cells (Gradwohl *et al.* 2000). The transcription profiling was done on spotted cDNA microarrays containing 20 188 probes from LION Bioscience's

murine arrayTAG set. A large fraction of the clones in the murine LION clone set were derived from tissues such as brain, testis, embryos and embryonic stem cells, but not pancreas. Therefore, several pancreas-related genes including *insulin*, *glucagon*, *Ngn3*, *Pdx1*, *NeuroD*, *Isl1* and *Pax4* are not present on the array. Nonetheless, due to the many similarities between pancreatic endocrine cells and neuronal cells, both in terms of developmental mechanisms and expressed genes (Slack 1995, Wilson 2003), the murine arrayTAG clone collection is well suited for the analysis of gene expression in pancreas lacking endocrine cells. Initially, we analysed the data to find genes showing significant and more than twofold difference between mutant and wild type at e13 and/or e15. This strict filtering

Figure 6 *Irx1*- and *Irx2*-expressing cells belong to the endocrine lineage and can be induced by exogenous Ngn3. (A–D) *Irx1* and *Irx2* expression in e14 wild-type (WT) and *Ngn3*-deficient (*Ngn3*^{-/-}) pancreata visualised by *in situ* hybridisation. (A–B) *Irx1* and *Irx2* are expressed in a characteristic pattern resembling endocrine cell clusters, which mainly consist of α -cells at this time point and specific expression is denoted by arrows (C and D). The expression of *Irx1* and *Irx2* is lost in the *Ngn3* KO demonstrating that these genes are expressed in the endocrine lineage. Black arrows denote areas of *Irx* expression, while white arrows depict areas with glucagon-expressing cells. Asterisks denote autofluorescent red blood cells. (E–H) Expression of Ngn3 in chicken endoderm induces the expression of *Irx2*. (E) Expression of the pEMPTY-IRES-EGFP plasmid does not induce *Irx2* expression (F), while expression of pNgn-3-IRES-EGFP (G) induces glucagon and *Irx2* expression (H). White and black arrows denote the areas on the section where glucagon and *Irx2* are expressed in the same area. A–D, F, and H are 100 $\times$ magnification. E and G are 250 $\times$ magnification.

approach resulted in identification of a small set of 52 genes, all showing lower transcription levels in mutant compared to wild-type mice (see Fig. 1 and Table 1). The simplest explanation for the reduced level of

transcription of these genes in the mutant is that they are normally expressed in the endocrine lineage of the pancreas and due to the lack of this lineage in *Ngn3*-deficient mice, the transcript levels compared to wild

type appear lower. Indeed, many of the genes in Table 1 are known from the literature to be expressed in the endocrine lineage (*Npy* (Ding *et al.* 1997), *Ghrl* (Wierup *et al.* 2002, Prado *et al.* 2004, Heller *et al.* 2005), *Pcsk1n* (Guest *et al.* 2002), *Pcsk2* (Furuta *et al.* 1997), *Myt1* (Gu *et al.* 2004), *Chga*, *Chgb* (Karlsson 2001), *Scg3* (Sakai *et al.* 2004), *Cdkn1a* (Kaneto *et al.* 1999)). However, several genes in Table 1 have not yet been described in pancreas biology. Rhomboid, veinlet-like 4 is a vertebrate homolog of *Drosophila* Rhomboid. In *Drosophila*, Rhomboid-1 is a key regulator of EGF-receptor signalling (Bier *et al.* 1990), controlling cleavage of the membrane-bound EGF-receptor ligand Spitz. The components of EGFR signalling are ubiquitously expressed during *Drosophila* development, whereas the rhomboid proteases are not and the regulated intramembrane cleavage of Spitz by Rhomboid is thus the primary mechanism for controlling EGFR signalling in developing *Drosophila* embryos (Rutledge *et al.* 1992, Urban *et al.* 2002). Interestingly, EGF pathway components are expressed in the developing pancreas in mice (Miettinen *et al.* 2000) and *ErbB1* as well as *ErbB2* is required for normal endocrine pancreas development (Miettinen *et al.* 2000, Huotari *et al.* 2002). Further studies are needed to determine the location and function of Rhomboid, veinlet-like 4 in endocrine pancreas development. Additionally, many ESTs and genes encoding proteins with unknown functions were identified in our analysis. Some of the ESTs identified in this experiment do not have sequence homology to any sequences in GenBank and since the sequences on this array are derived from the 3' UTR, homology search might not reveal homology to known families of proteins with conserved motifs. RT-PCR verifications of microarray data showed good agreement between the two platforms (Fig. 2 and Supplementary Table 1; See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>). Slight discrepancies were only observed for *Cyp4b1* and *Cdkn1a*. BLAST analysis of the probe sequences for *Cyp4b1* revealed that, in addition to *Cyp4b1*, the probe showed homology to a predicted gene similar to *Cyp4b1* (CYPIVB1, XM_983069; data not shown). This suggests that the *Cyp4b1* discrepancy between microarray and RT-PCR could be caused by cross-hybridisation. *Cdkn1a* data only show discrepancy at e15, where microarray data shows 0.8-fold reduction, while RT-PCR shows 1.2-fold increase in mutants. BLAST analysis of the *Cdkn1a* probe sequence did not show any unexpected hybridisation partners and discrepancy at only e15 suggests minor inaccuracies in one of the platforms. In a previous study of gene expression in pancreas, it was shown that *Cdkn1a* is enriched in Ngn3+ cells versus pancreatic tissue of other stages (Gu *et al.* 2004) which is in line with the microarray data presented here.

Several transcription factors (*Myt1*, *Hes1*, *Hes6*, *MafB*, *Tcf2*, *Pax6* and *Irx1*) show reduced expression in Ngn3 mutants compared to wild type and identification of some of these serve as positive control for the analysis. Previous studies have shown that *Pax6* is expressed in all cells of the endocrine lineage and loss of this transcription factor leads to arrest of endocrine development prior to final differentiation (Sander *et al.* 1997, St Onge *et al.* 1997). *Myt1* was recently identified by Gu *et al.* (2004) in a microarray experiment involving distinct populations of cells from different stages of pancreatic development. *Myt1* was shown to be expressed in endocrine progenitor cells and the ability of Ngn3 to promote α - and β -cell development likely depends on *Myt1* (Gu *et al.* 2004). The transcription factor, *Irx1*, belongs to a family of homeodomain proteins known as *Iroquois* and includes six members. The *Irx* family of transcription factors have been shown to be required early during development to define large territories, but also play a role later on for subdividing these domains (Cavodeassi *et al.* 2001). We confirmed and extended previous observations (Houweling *et al.* 2001, Lebel *et al.* 2003) showing that *Irx1* and 2 but not 3, 4 and 5 (data not shown) are expressed in the pancreas. We demonstrate here that *Irx1* and *Irx2* are specifically expressed in α -cells. Furthermore, our data suggest that *Irx1* and *Irx2* could be operating at early stages of endocrine subtype specification soon after commitment to the endocrine lineage as *Irx1/2* was induced by NGN3 in many cells that did not yet express glucagon. The identical expression of the two *Irx* genes and their highly related homeodomains suggest that they might serve redundant functions during α -cell development. It was recently demonstrated that MAP kinase-mediated phosphorylation can change *Irx2* from a repressor to an activator in developing midbrain tissue (Matsumoto *et al.* 2004), but the transcriptional activity and function of *Irx1/2* proteins in α -cells is unknown. Further, gain- and loss-of-function experiments will be required to dissect the precise role of this family of transcription factors in the pancreas.

The MA plots in Fig. 1 show that genes exhibiting a significant difference in expression level of more than twofold between wild type and mutant constitute only a minor fraction of the significant results. In an attempt to provide an unbiased analysis of the microarray data, we carried out additional analysis based on all genes found to show statistically significant difference between the wild type and the mutant. The genes identified at e13 and e15 were divided into groups of genes showing increased expression and those showing decreased expression. For each of these groups of genes, we used the GOstats package to carry out a hypergeometric test to determine whether there was over-representation of genes at particular GO terms in the Biological Process branch of the ontology (Table 2). The genes showing decreased expression levels in the

mutant might primarily be expected to be expressed in endocrine cells. While 'peptide-hormone processing' and 'neuropeptide signalling' are common to both e13 and e15, there are differences in the terms that are over-represented at the two stages of development. It is tempting to speculate that the difference in BP terms reflects that Ngn3 lineage cells at e13 and e15 are at different developmental stages. At e13, a large group of apoptosis-related genes are identified (*Rnf130*, *Gadd45g*, *Cdkn1a*, *Btg2*, *Txn1l*, *Sphk1*, *1700020C11Rik*, *Smndc1*, *Pim2*, *Unc5a* and *E2f2*). While cell death has been demonstrated to be a common event in the development of several organs (Barres *et al.* 1992, Coles *et al.* 1993, Coucouvanis & Martin 1995), nothing is known about the role of apoptosis in formation of the pancreas. Several negative regulators of apoptosis are identified (*Pim2*, *Sphk1*, *Btg2*, and *Cdkn1a*), which might indicate that suppression of apoptotic signals is necessary for proper development of endocrine cells. At e15, many of the genes showing decreased expression encode proteins involved in transport or 'G-protein-coupled receptor protein-signalling pathway'. It is well known that insulin secretion is triggered by Ca^{2+} influx through voltage-dependent Ca^{2+} channels (reviewed in (Mears 2004)) and recently G-protein-coupled receptor signalling has been shown to influence insulin secretion (Briscoe *et al.* 2003, Itoh *et al.* 2003). Thus both, 'transport' and offspring terms as well as 'G-protein-coupled receptor protein-signalling pathway' are reconcilable with a predominantly mature phenotype of the Ngn3-lineage cell at e15.

Interestingly, 116 probes on the array detect slightly increased expression levels in *Ngn3*-deficient mice at e13. It has been shown that Ngn3 is both required and sufficient for inducing endocrine differentiation in pancreatic cells (Gradwohl *et al.* 2000, Grapin-Botton *et al.* 2001); thus many genes showing increased expression in the mutant could suggest that the absence of endocrine and progenitor cells has an effect on parts of the remaining pancreatic cells. The fact that only 21 genes with increased expression are found at e15 could be caused by a dilution effect where cells that are potentially affected by the lack of endocrine progenitor or mature endocrine cells is outnumbered by non-endocrine cells which are more abundant at e15 than at e13. Among the genes showing increased expression in the mutant at e13, we identified GO terms 'neurogenesis', 'cell adhesion', and 'cell communication' as significantly over-represented. Whether these GO terms reflect some sort of compensatory mechanism or a response to lacking endocrine and precursor cells, requires further study.

The entire list of genes identified at e13 was used to compute the induced GO graph in the BP branch. Colour coding of this graph shows that there are

biological processes that appear to be represented only in the list of increasing or decreasing genes. The group of genes showing decreased expression in the mutant is larger than those showing increased expression and consequently, there is a clear overweight of different shades of green. 'RNA metabolism' and offspring terms like 'nuclear mRNA splicing, via spliceosome' are only represented by genes showing decreased expression. In a recent paper, Gunton *et al.* (2005) presented microarray data showing that a group of RNA processing genes are significantly altered in islets from type-2 diabetic patients. Whether RNA processing plays a crucial role in endocrine cell development or function requires further analysis. The interactive version of the GO graph presented in the Supplementary Data (Figure S1; See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>) can be used to browse the data and give more detailed GO annotation of the genes identified at e13.

A few issues should be kept in mind when using GO annotations to summarise microarray results, as done in this paper. First, only 50% of the genes in the LION murine arrayTAG set have GO annotations and some of these are solely inferred from electronic annotation, meaning that no human judgement was used in the process. Secondly, the GO database is subject to constant change as our understanding of biology and biological processes changes meaning that redoing the analysis in the future will probably give slightly different results. Thirdly, the fact that development of the pancreas involves differentiation of several different cell types differentiating asynchronously, makes analysis of large gene lists difficult, and identification of multiple genes with similar GO annotations does not imply that the encoded proteins interact physically. Nevertheless, the use of GO terms provides an unbiased way of analysing large data sets like microarray data and importantly, brings together the combined knowledge from several different model organisms.

In conclusion, using advanced microarray technology and data mining, we have revealed *Irx-1/2*, as new pancreatic glucagon-producing cell-specific genes, whose function in the pancreas remains unknown. Additionally, our microarray data strongly suggest that the absence of Ngn3 positive and mature endocrine cells results in changes in gene expression in embryonic exocrine/ductal cells. GO-based analysis of genes showing reduced expression in the mutant and genes showing increased expression revealed multiple biological processes that are affected solely by increased or decreased gene expression, suggesting that these processes are involved in the development or the function of pancreatic cells or are important in the cross-talk between the formation of endocrine tissue and the other pancreatic cell types during development.

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