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ARTICLE

Transcription factor expression in the developing human fetal endocrine pancreas

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Abstract
Aims/hypothesis Morphological changes that occur during pancreatic endocrine cell differentiation have been shown in rodent systems to be dependent on sequential alterations in transcription factor expression. However, similar data for humans have been limited. The aim of the present study was to provide a connection between pancreatic morphology, transcription factor gene expression and protein localisation during human fetal development.
Methods Human fetal pancreases were examined at early (8–12 weeks of fetal age), middle (14–16 weeks) and late (19–21 weeks) stages, using immunohistological, microarray and qRT-PCR analyses.
Results We observed a significant decrease in pancreatic duodenal homeobox 1 (PDX-1)$^+$/cytokeratin 19$^+$ cells ($p<0.001$), with a simultaneous increase in PDX-1$^+$/insulin$^+$ cells from 8 to 21 weeks ($p<0.05$). Increased PDX-1/insulin co-localisation within islet clusters was noted, while no co-expression of PDX-1 with glucagon was found, suggesting that loss of PDX-1 is essential for alpha cell formation. Given that neurogenin 3 (NGN3) expression is critical for establishing the endocrine cell programme in the rodent pancreas, we examined its expression pattern and co-localisation in PDX-1$^+$, insulin$^+$ and glucagon$^+$ cells. Co-localisation of NGN3 with PDX-1, insulin and glucagon was noted during early development, with significant decreases in middle and late stages ($p<0.001$). Our microarray and co-localisation analyses of transcription factors linked to NGN3 demonstrated that ISL1 transcription factor (ISL1), neurogenic differentiation 1 (NEUROD1), NK2 related transcription factor related, locus 2 (NKX2-2) and paired box gene 6 (PAX6) were upregulated during development and present in all four endocrine cell types, while NK6 related transcription factor related, locus 1 (NKX6-1) was expressed exclusively in beta cells.
Conclusions/interpretation This study is an important step towards identifying key molecular factors involved in development of the human fetal endocrine pancreas.

Keywords Human fetal pancreas · Islet transcription factors · Microarray analysis · qRT-PCR analysis

B. M. Lyttle and J. Li contributed equally to this work.

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Abbreviations

CK19	cytokeratin 19
ISL1	ISL1 transcription factor
NEUROD1	neurogenic differentiation 1
NKX2-2	NK2 related transcription factor related, locus 2
NKX6-1	NK6 related transcription factor related, locus 1
NGN3	neurogenin 3
PAX6	paired box gene 6
PDX-1	pancreatic duodenal homeobox 1
PP	pancreatic polypeptide
qRT-PCR	quantitative real-time RT-PCR
RMA	robust multichip analysis

Introduction

The endocrine pancreas is a major regulator of glucose homeostasis. Disruption of insulin-secreting beta cells leads to diabetes, a metabolic disorder affecting millions of people today. Therapeutic strategies aimed at repopulating insulin-producing cells show great potential for restoring glucose homeostasis, and extensive research efforts are focused on developing ways of differentiating stem/progenitor cells into beta cells [1–3]. However, before these strategies are developed, a thorough understanding of human pancreatic islet cells, the nature of potential islet progenitors and their genetic profiles is required.

Morphological changes accompanying maturation of the human fetal endocrine pancreas have been previously characterised [4–8]. The human pancreas initially develops as two separate dorsal and ventral domains that bud off the endoderm germ layer by 2 to 3 weeks of fetal age. Dorsal and ventral cells expressing pancreatic duodenal homeobox 1 (PDX-1) appear by 4 weeks, while insulin$^+$ cells emerge by 7 weeks of development [6, 7]. The period of 10 to 12 weeks is characterised by the proliferation of branched tubules into primitive acini, islets and ducts with limited differentiation [7–9]. By 14 to 15 weeks, changes in pancreatic architecture include the appearance of vascularised parenchymous lobules containing small islet aggregations [6, 8, 10]. Adult-like islets containing all four endocrine cell types and a fine capillary network are observed by the second trimester; the appearance of insulin granules in islets coincides with development of the microvasculature, indicating the endocrine nature of the fetal pancreas [6, 8]. Ontogenic changes from this point onwards involve expansion and growth.

Rodent pancreatic organogenesis is governed by extrinsic and intrinsic signalling pathways that promote or restrict morphogenesis and cellular differentiation [11]. Murine loss-of-function phenotypes have been valuable to our understanding of transcription factors in determining pancreatic cell fate: endocrine precursor differentiation into definitive hormone-expressing cells depends on sequential changes in transcription factor gene expression [9, 12]. For example, *Pdx1* expression is essential for all pancreatic cell lineages [13], while *Ngn3* demarcates cells destined to form the endocrine pancreas [14]. *Beta-2/Neurod1* acts downstream of *Ngn3* and, together with *Pdx-1*, regulates several beta cell-specific genes [15]. Lastly, coordinated production of other transcription factors, including *Pax6*, *Nkx2-1*, *Nkx6-1*, *MafB* and *Isl1*, regulates islet cell differentiation [16].

Although rodent models have substantially improved our understanding of genetic and molecular processes regulating pancreatic development [16, 17], transcription factor cascades controlling human pancreatic endocrine cell differentiation and function are largely unknown. Our aim was to characterise developmental changes occurring from 8 to 21 weeks of fetal age in the human fetal endocrine pancreas relating specifically to insulin$^+$ and glucagon$^+$ cell clusters; to this we used immunohistological, microarray and quantitative real-time PCR (qRT-PCR) approaches. Human fetal pancreases were grouped into three developmental stages: (1) early (8–12 weeks), when pancreases contain primarily numerous undifferentiated PDX-1$^+$ ductal cells with a few scattered single endocrine cells and, by 11 weeks, rare small islet clusters; (2) middle (14–16 weeks), when numerous small vascularised islet-clusters are present; and (3) late (19–21 weeks), when adult islet structures with a rich vascular network are observed (Fig. 1b, Electronic supplementary material [ESM] Fig. 1). Here, we report that PDX-1 and neurogenin 3 (NGN3), markers of early pancreatic endocrine cells in the rodent, are also present during early stages in the human endocrine pancreas. In addition, our studies confirm the co-localisation of several transcription factors downstream of NGN3 (e.g. ISL1 transcription factor [ISL1], neurogenic differentiation 1 [NEUROD1], paired box gene 6 [PAX6], transcription factor related, locus 2 [NKX2-2] and NK6 transcription factor related, locus 1 [NKX6-1]) in insulin$^+$ and/or glucagon$^+$ cells. This study provides a connection between alterations in morphology and transcription factor changes during normal development of the human pancreas and should prove useful for future investigations into regenerative capacities of human pancreatic endocrine cells.

Methods

Pancreatic tissue collection Human fetal pancreases (8–21 weeks of fetal age) were collected according to protocols approved by the Health Sciences Research Ethics Board at the University of Western Ontario and the Research Ethics

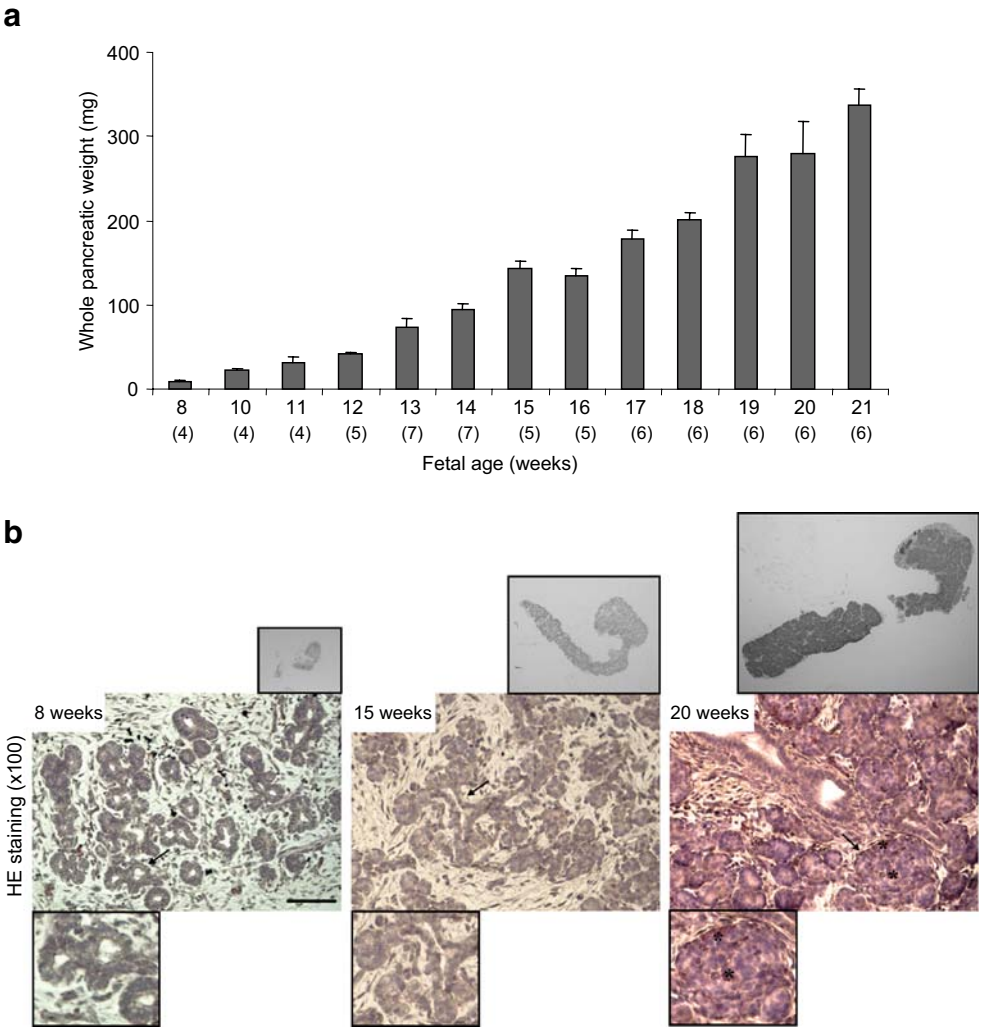

Fig. 1 Developmental changes in the human fetal pancreas. **a** Whole pancreatic weight (mean±SEM) at time specified. Numbers in parentheses, pancreases (*n*) examined per age group. **b** General histology of 8, 15 and 20 week human fetal pancreases. Inserts indicate the whole pancreatic sections (×8). Arrows (large images) indicate the areas of enlargement below (×400). *, capillary networks filled with erythrocytes. Scale bar, 100 μm

Boards of the McGill University Health Centre, in accordance with guidelines of the Canadian Council on Health Sciences Research Involving Human Subjects. Whole pancreatic tissues were weighed and processed for immunohistochemistry, RNA and/or protein extraction, with at least six pancreases per age group.

Immunofluorescence and morphometric analysis Pancreases were fixed in 4% (wt/vol.) paraformaldehyde and embedded in paraffin [18]. Sections, 5 μm thick, were cut throughout the entire length of the pancreas with two sets of six serial sections at 50 μm intervals. Tissue sections were stained with appropriate dilutions of primary antibodies (listed in ESM Table 1) as described previously [19]. FITC and Texas Red secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Images were recorded using a Leica DMIRE2 fluorescence microscope (Leica Microsystems, Richmond Hill, ON, Canada) with Openlab image software (version 4.0.2; Improvision, Lexington, MA, USA). Negative controls were performed by omission of primary antibodies.

Quantitative evaluation of alpha and beta cell mass in whole pancreases was performed using computer-assisted image analysis. Using Openlab software (Improvision), areas positive for insulin and glucagon were manually traced throughout the length of the pancreas. Total alpha and beta cell mass was determined using the following equations: (1) $A_\beta/A_P=M_\beta/M_P$, where A_β and M_β are total insulin$^+$ area and beta cell mass; or (2) $A_\alpha/A_P=M_\alpha/M_P$, where A_α and M_α are total alpha cell area and mass, and A_P and M_P are total pancreatic section area and pancreatic mass, as described previously [18]. A minimum of seven pancreases per age group were analysed.

To identify co-localisation of transcription factors with epithelial and endocrine cell markers, double immunofluorescence staining was performed. Co-expression of PDX-1 with cytokeratin 19 (CK19) and endocrine cell markers, and co-expression of NGN3 with PDX-1, insulin or glucagon was quantified by counting double-labelled cells. To assess proliferation of ductal and endocrine cells, co-expression of Ki67 with CK19, insulin and glucagon was determined by counting double-labelled cells. At least 1500 CK19$^+$ or PDX-1$^+$ cells

and 500 insulin$^+$ or glucagon$^+$ cells (except at 8–10 weeks of age) from at least 12 randomly imaged fields from each pancreatic section were counted with at least five pancreatic sections per age group.

Measurement of plasma insulin and pancreatic insulin content Blood samples from fetal ages 14–21 weeks were collected from fetal umbilical cords. Frozen pancreatic samples from 10–21 weeks were homogenised in an ethanol–acid solution (165 mmol/l HCl in 75% ethanol, vol./vol.). Pancreatic and plasma insulin were measured using a human ultra-sensitive insulin ELISA kit (ALPCO, Salem, NH, USA), according to the manufacturer's instructions. Tissue DNA contents were determined. Plasma insulin levels were expressed as pmol/l and pancreatic insulin content as pmol/μg DNA [20].

Microarray and data analysis Total RNA was extracted immediately after pancreatic tissue collection using TRIZOL reagent (Invitrogen, Burlington, ON, Canada). RNA quality was verified by agarose gel electrophoresis using ethidium bromide staining and by Agilent 2100 Bioanalyzer Scans (Agilent Technologies, Palo Alto, CA, USA) using a kit (RNA 6000 Nano kit RNA Quality; Caliper Life Sciences, Mountain View, CA, USA), as described previously [21]. Biotinylated complimentary RNA was generated and hybridised to Affymetrix HG-U133A GeneChips (Affymetrix, Santa Clara, CA, USA) [21].

Biological replicates at 8 to 10 weeks (n=5), 14 to 16 weeks (n=5) and 19 to 21 weeks (n=6) were used for microarray data analyses (ESM Fig. 2). Affymetrix GCOS 1.2 (Affymetrix) and GeneSpring version 7.1 (Agilent Technologies Canada, Mississauga, ON, Canada) software were used to examine expression levels [21]. Data were then normalised using Robust Multichip Analysis (RMA) to show expression relative to data for 8 to 10 weeks. For replicates within one stage, a median value for the expression of each gene was taken to establish an average expression level (ESM Table 2). RMA data were used for all further analyses. Data are expressed as mean ± SEM fold changes [21].

RT-PCR and real-time RT-PCR To validate gene expression levels obtained from microarray analyses, quantitative real-time RT-PCR assays were performed with a kit (iQ SYBR Green Supermix, VWR, Mississauga, ON, Canada) in Chromo4 Real-Time PCR (Bio-Rad Laboratories, Mississauga, ON, Canada), as described previously [21, 22]. PCR primers are listed in ESM Table 3. Relative gene expression was calculated using the arithmetic formula $2^{-\Delta\Delta C_T}$, where C_T is the difference between the threshold cycle of a given target cDNA and the internal standard gene 18S rRNA subunit cDNA. Relative PCR signals from middle and late stages were normalised to early stage data [21, 23].

Statistical analysis Data are expressed as mean±SEM. Statistical significance was determined using one-way ANOVA followed by the post hoc least significant difference group comparison test. Differences were considered to be statistically significant when $p<0.05$.

Results

Developmental changes in the human fetal pancreas Measurement of whole pancreatic mass demonstrated consistent increases in pancreatic weight from 8 to 21 weeks of fetal age (Fig. 1a). Total pancreatic weight at 8 weeks was 9±1.5 mg, with a 15-fold (16 weeks, 135±8.5 mg) and 35-fold (21 weeks, 337±15 mg) increase by the middle and late stages, respectively. Histological analyses confirmed progressive development of ductal structures in the pancreas during the early and middle stages. By 20 weeks, a significant change in tissue architecture was noted, as adult-like islet clusters with extensive capillary networks were observed (Fig. 1b, ESM Fig. 1) [5].

Using whole human fetal pancreatic sections, we performed morphometric studies to characterise developmental changes in alpha and beta cell mass. A significant sevenfold increase in both was noted from early to middle stages ($p<0.001$; Fig. 2a). However, the increase in alpha cell mass was greater from the middle to late stage (2.6- vs 1.6-fold for the beta cell mass) (Fig. 2a). The ratio of alpha:beta cells during the period of 10 to 12 weeks was 40:60%; by 19 to 21 weeks this ratio had shifted to 55:45%. These differences are likely to be due to alterations in cell proliferation capacity during these developmental stages. A constant low level of beta cell proliferative activity (~3%) was observed from 8 to 21 weeks (ESM Fig. 3). In contrast, alpha cells exhibited higher proliferative activity at all stages: a 1.5-fold increase was observed from the 8 to 12 week period to the 14 to 16 week period (3.9±0.4% vs 5.8±0.5%, $p<0.007$), this level being maintained at 19 to 21 weeks (5.2±0.2%) (ESM Fig. 3).

To verify whether the rise in beta cell mass noted during development affects insulin secretion and content, plasma and pancreatic insulin levels were analysed (Fig. 2b,c). A significant increase in plasma insulin levels from the middle to the late stage was observed ($p<0.05$). However, this only reached 10% of normal adult plasma insulin levels. Insulin content increased in parallel with beta cell mass ($p<0.001$ for early vs middle and late; Fig. 2c), suggesting increased beta cell function through progressive developmental stages. Furthermore, the expression of *PDX1*, *INS* and *GCG* genes was evaluated by microarray and qRT-PCR during early, middle and late stages: *PDX1* mRNA levels remained relatively constant throughout the three stages, while dramatic increases in *INS* and *GCG* expression were observed between

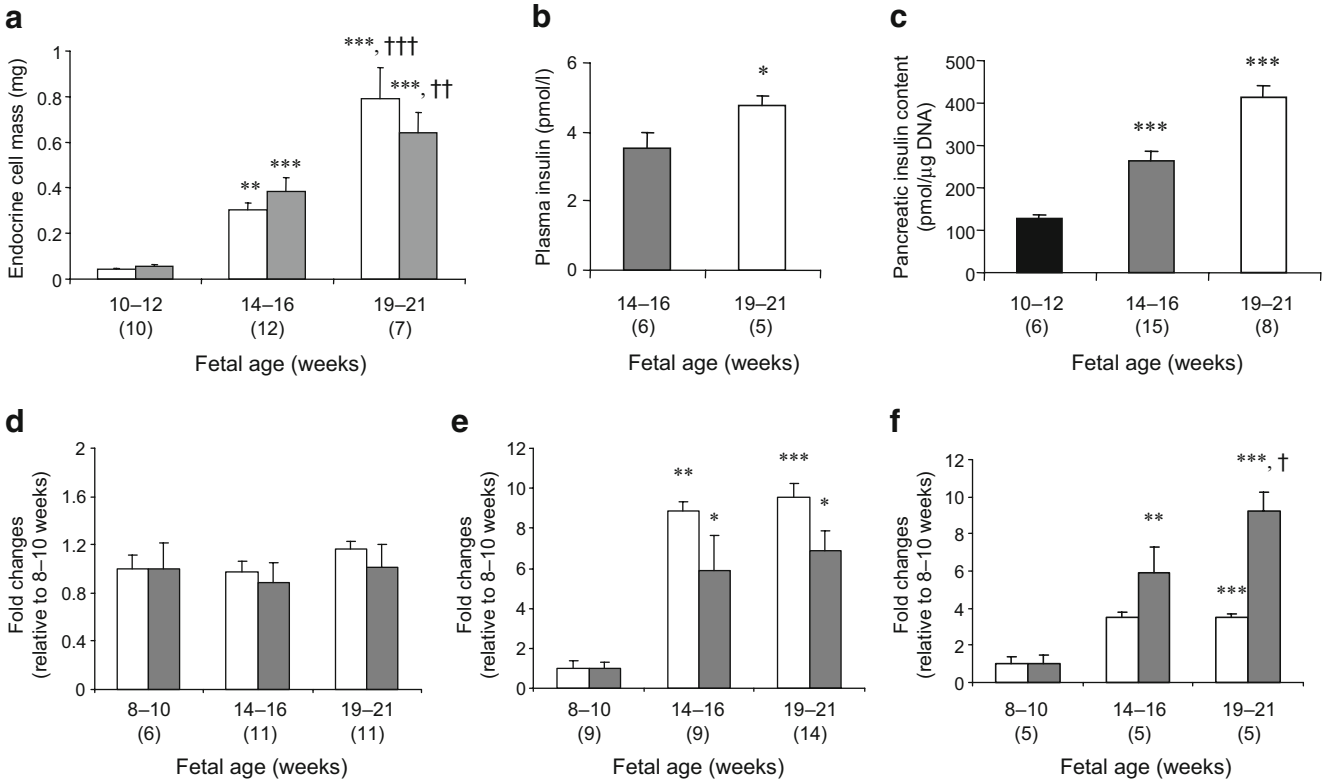

Fig. 2 Quantitative analyses of endocrine cell mass and gene expression. **a** Morphometric analyses of alpha (white bars) and beta (grey bars) cell mass from 10–21 weeks. Values are mean±SEM; $^{**}p<0.01$ and $^{***}p<0.001$ vs 10–12 weeks; $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$ vs 14–16 weeks. **b** Measurement of plasma insulin from 14–21 weeks and insulin content (**c**) from 10–21 weeks. Values mean±SEM; $^{*}p<0.05$ vs 14–16 weeks, $^{***}p<0.001$ vs 10–12 weeks. Microarray (white bars) and qRT-PCR (grey bars) analyses of *PDX1* (**d**), insulin (**e**) and glucagon (**f**) gene expression from 8–21 weeks of human fetal pancreatic development. Data were normalised to 8–10 weeks and are expressed as mean±SEM. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs 8–10 weeks; $^{\dagger}p<0.05$ vs 8–10 and 14–16 weeks. Numbers in parentheses, pancreases (*n*) examined per age group

early and middle stages (Fig. 2d–f). These data suggest that the 12 to 16 week period is especially important for endocrine cell development and hormonal expression.

Expression of PDX-1 in the human fetal pancreas To identify the proportion of PDX-1-expressing cells during human fetal pancreatic development, PDX-1 and CK19 co-expression studies were conducted (Fig. 3a–c). Cells in 8 to 12 week pancreases demonstrated high levels of co-localisation. The numbers had tapered off by 16 weeks ($p<0.05$), and by the late stage cells co-expressing CK19 and PDX-1 had decreased by 50% ($p<0.001$) (Fig. 3d,e). Since PDX-1 expression in ducts is important for specifying progenitor populations that eventually form the endocrine pancreas, co-expression studies were conducted with PDX-1 and insulin, glucagon or somatostatin, respectively (Fig. 3a–c). Our studies revealed that 38% and 58% of the insulin^{+} cells express PDX-1 at 8 to 12 and 19 to 21 weeks, respectively, suggesting its involvement in the maturation and maintenance of beta cells (Fig. 3d,e). Co-expression of somatostatin with PDX-1 was frequently observed, while no co-localisation of glucagon with PDX-1 was detected (Fig. 3c), indicating that loss of PDX-1 expression is likely to be essential for alpha cell formation.

Expression of NGN3 and transcription factors linked to NGN3 in the human fetal pancreas NGN3, a member of the basic helix–loop–helix family, is required for development of all endocrine lineages in the mouse. When it is knocked out, the pancreas fails to develop any endocrine cells [14]. Using dual immunofluorescence staining, we observed that NGN3 was not only co-expressed with PDX-1, but also co-localised with newly differentiated endocrine cells in the developing human pancreas (Fig. 4a–c). A high level of NGN3 in PDX-1^{+} (12±0.5%), insulin^{+} (26±3%) or glucagon^{+} (29±3%) cell populations was observed during early development, but gradually decreased from the middle to late stages with only 3.7±04, 6.6±0.6 and 8.7±0.7%, respectively, at 20 to 21 weeks ($p<0.001$; Fig. 4d–f). Co-expression of NGN3 with insulin or glucagon in adult-like islets was minimal; however, NGN3^{+} cells remained at the edges of the islets during the late stage (Fig. 4a–c). Our qRT-PCR analyses demonstrated a significant decrease in *NGN3* expression as development progressed ($p<0.001$; Fig. 5).

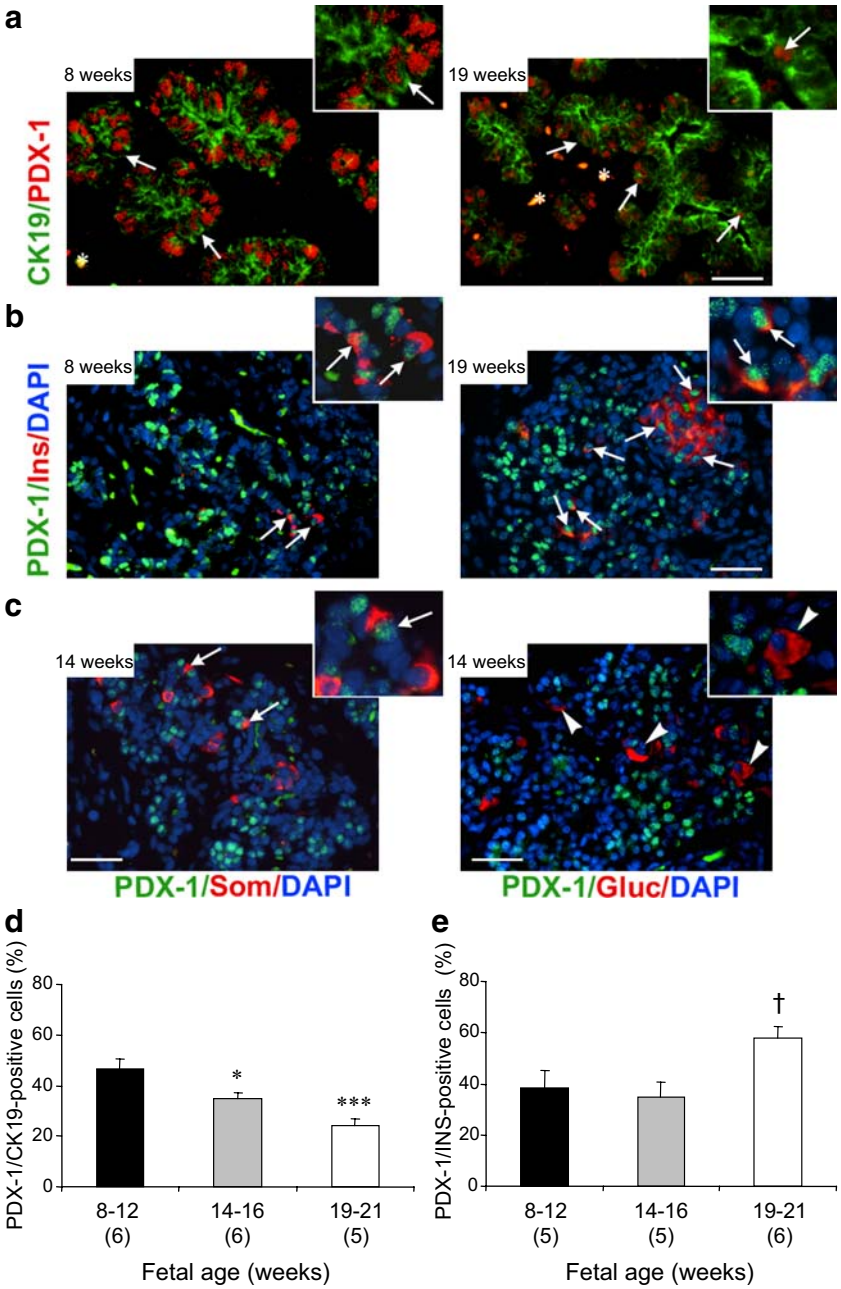

Fig. 3 Expression of PDX-1 during human fetal pancreatic development. **a** Co-expression of PDX-1 (red) with CK19 (green). **b** PDX-1 (green) with insulin (Ins, red) at 8 and 19 weeks. **c** PDX-1 (green) with somatostatin (Som) and glucagon (Gluc) (both red) in 14 week human fetal pancreases. Nuclei were stained by DAPI (blue). Arrows, PDX-1$^+$ co-stained cells; arrowheads, glucagon$^+$ cells without PDX-1; asterisks (*), non-specific staining. Scale bar, 50 μm, inserts 630×. Percentage of (**d**) PDX-1$^+$/CK19$^+$ and (**e**) PDX-1$^+$/insulin$^+$ cells relative to the total number of CK19$^+$ or insulin$^+$ cells counted. Values mean±SEM. Numbers in parentheses, pancreases (*n*) examined per age group. *p<0.05 and ***p<0.001 vs 8–12 weeks; $^\dagger p$<0.05 vs 8–12 and 14–16 weeks

To better understand the role of NGN3 and its associated transcription factors in morphological and functional changes occurring during the 8 to 21 week period of human fetal pancreatic development, microarray analyses were conducted. Using the Ingenuity Pathway Analysis system (http://www.ingenuity.com), we identified genes of pancreatic hormones and transcription factors that act upstream and downstream of NGN3. Of the 25 genes revealed, ten were upregulated, two downregulated and thirteen were unaltered during human fetal pancreatic development (Table 1). As expected, we noted a highly significant increase in the expression of pancreatic islet genes (e.g. *INS*, *GCG*, somatostatin, islet amyloid polypeptide) from early stages through to late stages. The majority of *NGN3*-related upstream transcription factor genes, e.g. *PDX1*, *ONECUT1*, *TCF1* (also known as *HNF1A*) and *FOXA2*, were relatively

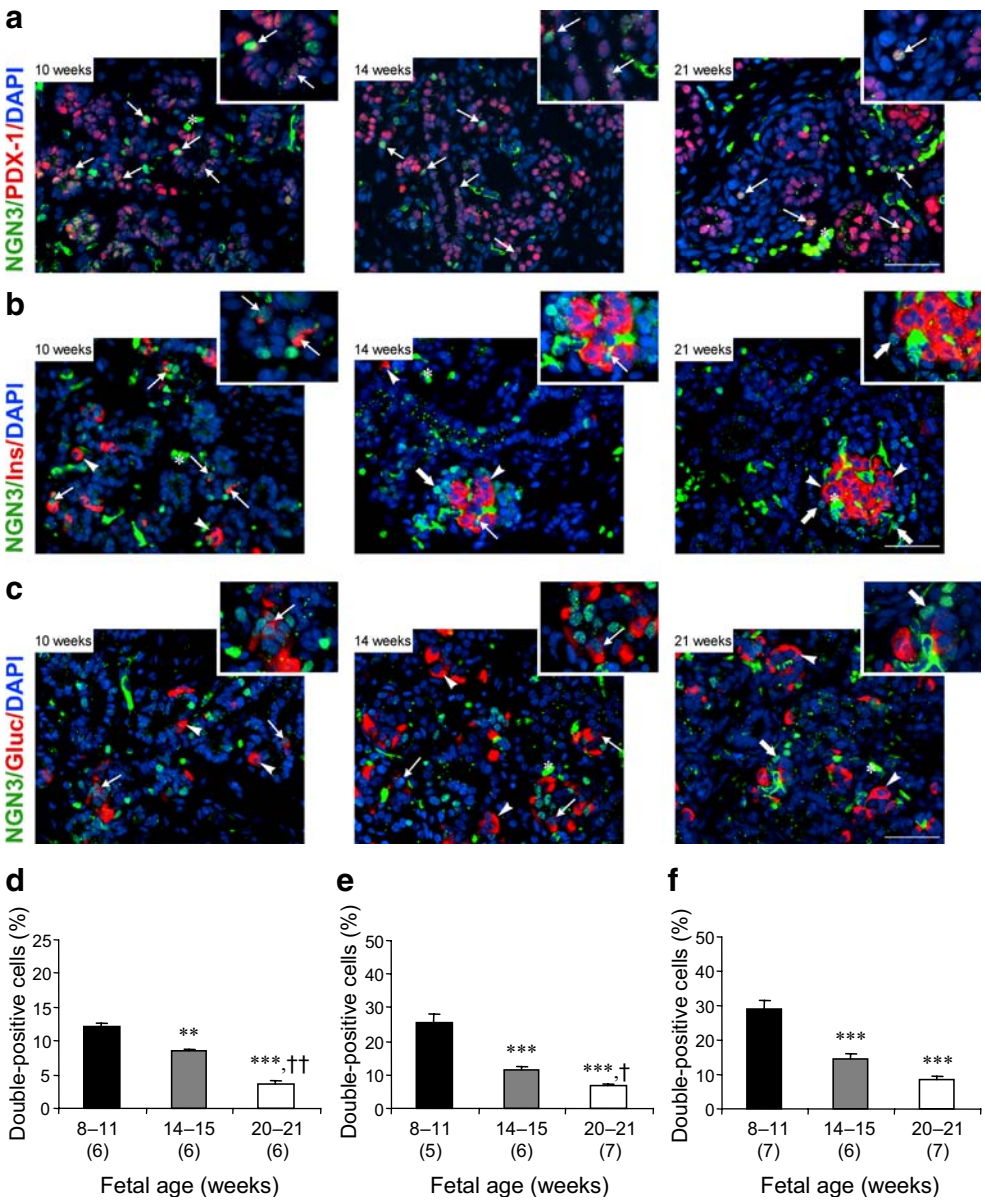

Fig. 4 Expression of NGN3 during human fetal pancreas development. Co-expression of NGN3 (green) with (**a**) PDX-1, (**b**) insulin (Ins) or (**c**) glucagon (both red) at times shown (weeks of human fetal pancreas development). Nuclei were stained by DAPI (blue). Arrows, $NGN3^+$ co-stained cells; arrowheads, $insulin^+$ or $glucagon^+$ cells without NGN3; bold arrows, $NGN3^+$ cells at the edges of the islet clusters; white asterisks (*), non-specific staining. Scale bar: 50 μm, inserts ×630. Percentage of $NGN3^+$/$PDX\text{-}1^+$ (**d**), $NGN3^+$/$insulin^+$ (**e**) and $NGN3^+$/$glucagon^+$ (**f**) cells relative to the total number of $PDX\text{-}1^+$, $insulin^+$ and $glucagon^+$ cells counted. Values mean± SEM. Numbers in parentheses, pancreases (*n*) examined per age group. $^{**}p<0.01$ and $^{***}p<0.001$ vs 8–11 weeks; $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ vs 14–15 weeks

constant throughout the 8 to 21 weeks, while *HLXB9* (also known as *MNX1*) and *SNAI2* were downregulated. A significant increase in the expression of several *NGN3* downstream transcription factors was noted (e.g. *ISL1, Neurod1, MAFB* and *PAX6*); in contrast, no changes were detected in *PAX4, NKX2-2* and *NKX6-1* expression.

To validate our microarray findings, qRT-PCR analyses were performed on 13 of the transcription factors most commonly linked to NGN3 (Figs 2d–f and 5). We noted similar significant increases in *ISL1*, *NEUROD1*, *MYT1L*, *MAFB* and *PAX6* gene expression and constant expression of *PDX1*, *FOXA2*, *ONECUT1*, *TCF1*, *PAX4*, *NKX2-2* and *NKX6-1* in both qRT-PCR and microarray analyses. While our microarray studies demonstrated consistently low levels of *NGN3* expression, the qRT-PCR assays showed a significant decrease at the middle and late stages. These differences probably reflect different hybridisation kinetics of the microarray probe set for *NGN3* mRNA and/or the greater sensitivity of the qRT-PCR assays [24].

Co-localisation of transcription factors with endocrine cell markers in the developing human fetal pancreas To confirm our qRT-PCR and microarray findings and identify transcription factors exclusively involved in specific islet cell development, dual immunofluorescence for ISL1, NEUROD1, NKX2-2, PAX6 and NKX6-1 with insulin (Figs 6 and 7a,b) or glucagon (Fig. 7a, ESM Fig. 4) were performed. ISL1, NEUROD1 and PAX6 appeared to show increased abundance as development progressed, co-localising with insulin, glucagon, somatostatin and pancreatic polypeptide (PP), but not with amylase (Fig. 6, ESM Figs 4 and 5). Although relatively constant expression of *NKX2-2*

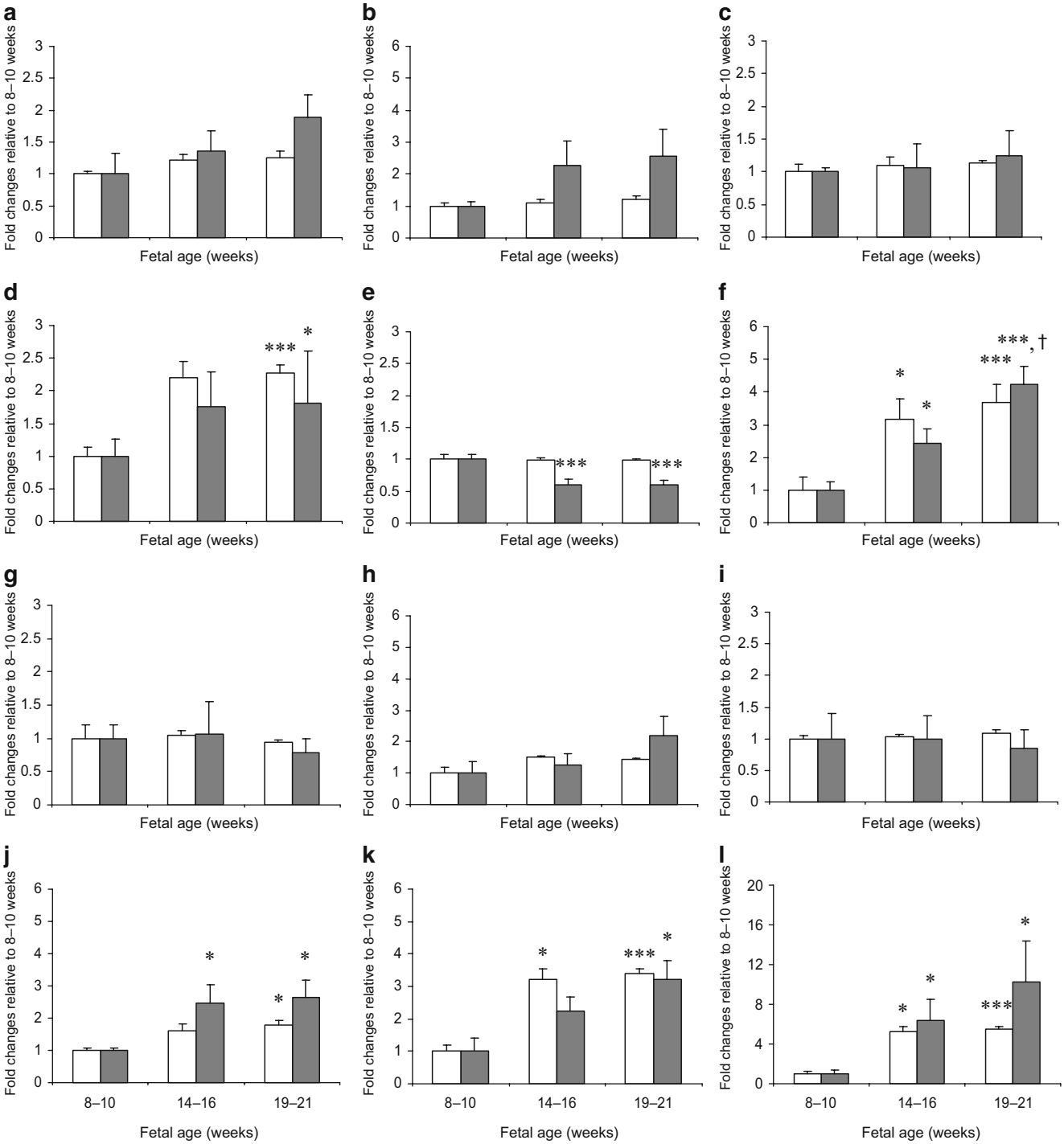

Fig. 5 Validation of microarray expression profiles with real-time RT-PCR. Expression patterns of the following transcription factor genes implicated in murine endocrine pancreas cell development and identified by microarray analyses (white bars) of human fetal pancreases: **a** *FOXA2*, **b** *ONECUT1*, **c** *TCF1*, **d** *ISL1*, **e** *NGN3*, **f** *NEUROD1*, **g** *PAX4*, **h** *NKX2-2*, **i** *NKX6-1*, **j** *MYT1L*, **k** *MAFB* and **l** *PAX6*. qRT-PCR values (grey bars) represent gene expression as determined by analysis, normalised to 18S and relative to 8–10 weeks (qRT-PCR analysis: $n=5$ per age group). Data are expressed as mean± SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs 8–10 weeks; $^{\dagger}p<0.05$ vs 14–16 weeks

and *NKX6-1* was noted throughout development by microarray and qRT-PCR assays, our co-localisation studies suggest an increase in expression from early through to late stages (Figs 6 and 7, ESM Fig. 4). Interestingly, NKX2-2 was present in all endocrine cell types (Fig. 6, ESM Fig. 4), while NKX6-1 co-localised only with insulin^{+} cells (Fig. 7a,b). Furthermore, NKX6-1 was not present in somatostatin^{+}, pancreatic polypeptide^{+} or amylase^{+} cells (Fig. 7c),

Table 1 List of 25 genes of pancreatic hormones and NGN3-related transcription factors expressed during human fetal pancreatic development

Genes	Gene symbol	Description	14–16 weeks	19–21 weeks
Upregulation (probe ID)				
206598_at	*INS*	Proinsulin	9.68±0.42	10.39±0.71
213921_at	*SST*	Somatostatin	6.85±0.86	6.42±0.40
206915_at	*IAPP*	Islet amyloid polypeptide	6.35±3.28	12.90±3.14
205646_s_at	*PAX6*	Paired box gene 6 (aniridia, keratitis)	5.23±0.55	5.55±0.19
206282_at	*NEUROD1*	Neurogenic differentiation 1	3.84±0.63	4.46±0.56
218559_s_at	*MAFB*	V-maf musculoaponeurotic fibrosarcoma oncogene homologue B (avian)	3.23±0.32	3.41±0.13
206422_at	*GCG*[a]	Glucagon	3.19±0.21	3.17±0.16
206104_at	*ISL1*	ISL1 transcription factor, LIM/homeodomain, (islet-1)	2.21±0.24	2.27±0.12
210016_at	*MYT1L*	Myelin transcription factor 1-like	1.62±0.19	1.79±0.15
206915_at	*NKX2-2*	NK2 transcription factor related, locus 2	1.51±0.03	1.42±0.07
No changes (probe ID)				
210103_s_at	*FOXA2*[b]	Forkhead box A2	1.22±0.09	1.26±0.10
208497_x_at	*NGN1*	Neurogenin1	1.12±0.06	1.11±0.05
210745_at	*ONECUT1*[c]	One cut domain, family member 1	1.11±0.10	1.22±0.08
210515_at	*TCF1* [d]	Transcription factor 1	1.10±0.12	1.13±0.04
210673_x_at	*TTF1*	Thyroid transcription factor 1	1.08±0.14	1.27±0.13
208559_at	*PDX1*	Pancreatic duodenal homeobox factor 1	1.07±0.09	1.28±0.06
219884_at	*LHX6*	LIM homeobox 6	1.06±0.05	0.94±0.05
220559_at	*EN1*	Engrailed homologue 1	1.06±0.02	1.04±0.01
207867_at	*PAX4*	Paired box gene 4	1.04±0.07	−1.06±0.03
221366_at	*NKX6-1*	NK6 transcription factor related, locus 1	1.03±0.04	1.09±0.06
219537_x_at	*DLL3*	Delta-like 3	1.02±0.02	1.01±0.03
207965_at	*NGN3*	Neurogenin 3	−1.02±0.04	−1.00±0.01
205253_at	*PBX1*	Pre-B cell leukaemia transcription factor 1	−1.02±0.15	−1.04±0.06
Downregulation (probe ID)				
214614_at	*HLXB9*	Homeobox HB9	−1.31±0.05	−1.56±0.03
213139_at	*SNAI2*	Snail homologue 2	−1.81±0.08	−2.69±0.02

Biological replicates: 8–10 weeks (n=5), 14–16 weeks (n=5) and 19–21 weeks (n=6)

Data are expressed as mean fold changes±SEM and normalised using the RMA to show expression relative to 8–10 weeks data, with downregulation indicated by preceding minus sign

The up- and downregulated genes in this list show at least a 1.5-fold change in expression in at least one stage of development relative to early development

[a] Also known as *GRPP*, *GLP1*, *GLP2*; [b] Also known as *HNF3β*; [c] Also known as *HNF6*; [d] Also known as *HNF1*

indicating that it is a beta cell-specific transcription factor in the human fetal pancreas.

Discussion

The present study describes associations between morphological changes occurring during human fetal pancreatic development and the expression of transcription factor genes and proteins known to be crucial for normal pancreatic endocrine cell differentiation in rodent models.

The observed increase in pancreatic weight from 8 to 21 weeks of fetal life suggested numerous internal changes. Histological studies confirmed progressive development during this period of ductal and islet structures in the human fetal pancreas that were paralleled by increases in alpha and beta cell populations. The changes in alpha cell mass coincided with significant increases in $Ki67^+$/$glucagon^+$ cells, suggesting that rapid expansion of alpha cells is mainly due to replication. Changes in beta cell mass correlated with increased pancreatic insulin content and secretion, indicating that these developmental stages serve as a critical window when beta cells initiate their function. By 14 weeks, the well-defined endocrine pancreas contains small clusters of cells producing insulin, glucagon, somatostatin and PP.

Our morphological data corroborate and expand on many of the previous investigations of human fetal pancreatic development [4–8]. Polak et al. also demonstrated that the increases in endocrine cell mass from 7 to 11 weeks are most likely to be due to the differentiation of precursor cells residing within pancreatic epithelia [7], while Piper et al. reported a similar parallel increase in $insulin^+$ cells and decrease in $CK19^+$ cells [6]. However, Bouwens et al. reported a peak in proliferation (~3%) in alpha and beta cells at 12 to 14 weeks, with a sudden decrease to 0.2% after 16 weeks [5]. The latter data are in sharp contrast with

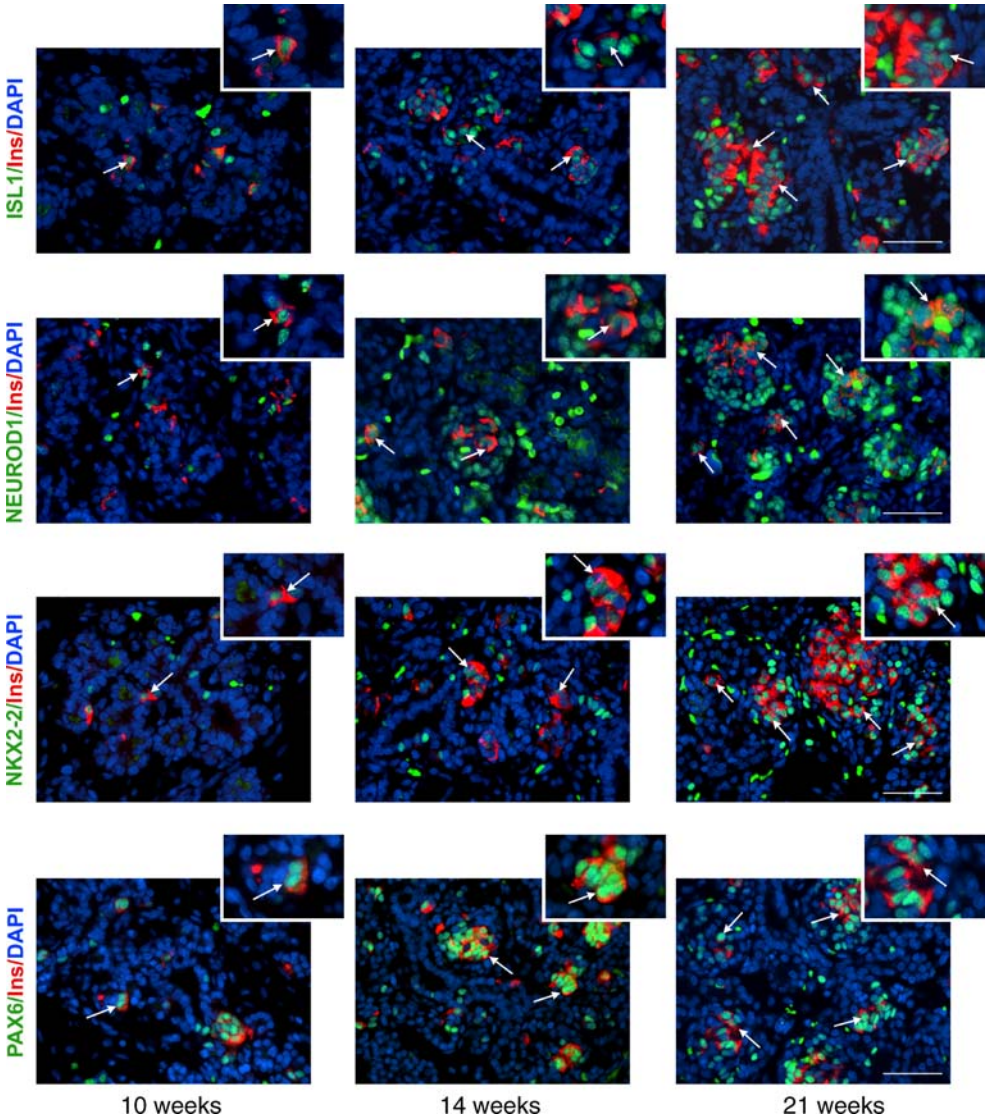

Fig. 6 Double immunofluorescence staining for ISL1, NEUROD1, NKX2-2 and PAX6 (all green) with insulin (red) at 10, 14 and 21 weeks of human fetal pancreas development. Nuclei were stained by DAPI (blue). Arrows, co-stained cells. Scale bar: 50 μm; inserts ×630

our findings, which indicate no significant changes in beta cell proliferation (i.e. consistently ~3%) and a significant increase in alpha cell proliferative activity (5–6%) at 14 to 21 weeks of development. Our increase in alpha cell proliferation was accompanied by significant increases in alpha cell mass from the first to second trimester of development, which has also been reported by Stefan et al. [4]. Their analyses of 8 to 40 week fetal pancreases demonstrated increases in beta cell volume from 8 weeks to term, with a significant peak in alpha cell mass occurring between 17 and 20 weeks [4]. Although their data parallel ours, their absolute values are much lower, probably due to the fact that they examined cell volumes in fragments of pancreatic tissue while our measurements are from total pancreases.

CK19, a marker of fetal pancreatic epithelial cells, becomes restricted to ductal cells in the adult pancreas [6]. PDX-1, initially a specific marker of pancreatic lineage, is expressed in cells forming the ducts, acini and islets; however, its expression is eventually limited to beta cells [6]. Co-localisation of CK19 with PDX-1 was highest at early stages and significantly decreased by 14 weeks. By the late stage, co-expression levels were only half that noted during the early stage, indicating a steady decrease in ductal islet precursors from 8 to 21 weeks.

Co-expression of PDX-1 with insulin or glucagon was examined to identify the proportion of ductal cells committed to endocrine lineage. PDX-1 and insulin co-expression significantly increased by the late stage, while no co-localisation of PDX-1 and glucagon was ever observed, indicating that inactivation of PDX-1 in islet progenitors may be involved in alpha cell differentiation. These results are in agreement with previous studies on the mouse pancreas [17, 25] and support the concept that PDX-1 expression is critical for specifying beta cell fate and maintaining its phenotype.

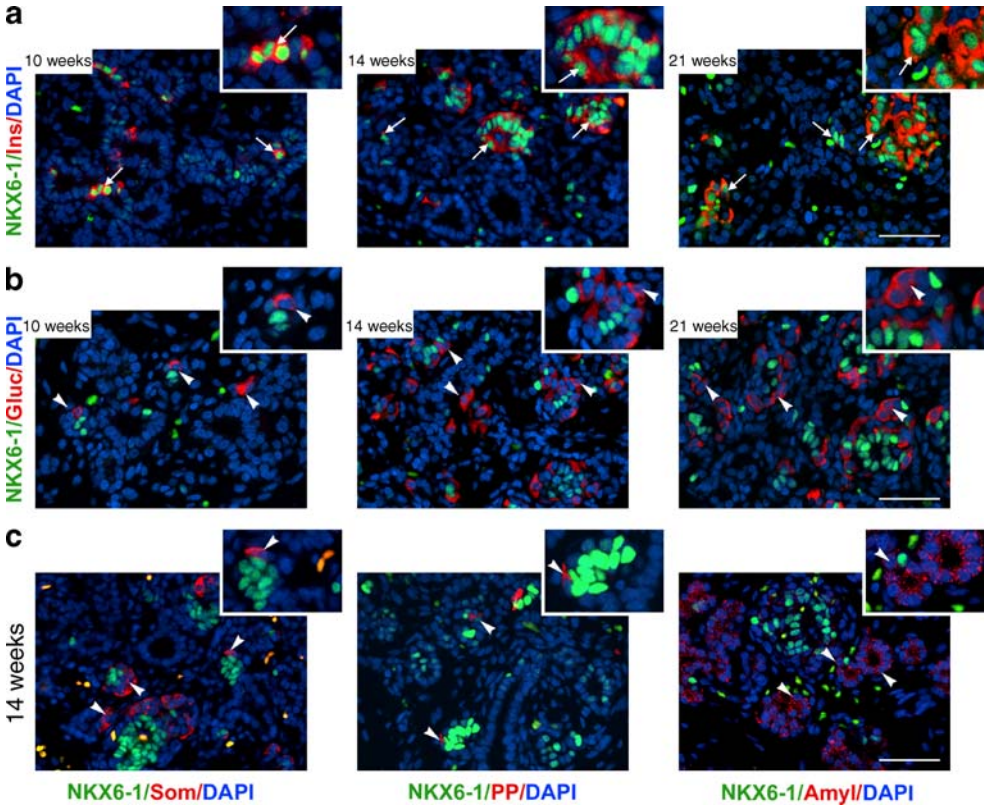

Fig. 7 Double immunofluorescence staining for NKX6-1 (green) with insulin (Ins) (**a**) and glucagon (Gluc) (**b**) (both red) at 10, 14 and 21 weeks of human fetal pancreas development. c Double immunostaining for NKX6-1 (green) with somatostatin (Som), PP and amylase (Amyl) (all red) at 14 weeks of human fetal pancreas development. Nuclei were stained by DAPI (blue). Arrows, co-stained cells; arrowheads, somatostatin$^+$, PP$^+$ and amylase$^+$ cells without staining of NKX6-1. Scale bar: 50 μm; inserts ×630

Using microarray and qRT-PCR assays, we analysed the gene expression patterns of transcription factors known to be involved in rodent pancreatic development [16, 17]. We focused on 25 genes of pancreatic hormones and transcription factors identified as linked to NGN3 in regulating proliferation, differentiation and survival of pancreatic endocrine precursors. Although little change was noted in the expression of genes functioning upstream of NGN3, several downstream targets demonstrated dynamic alterations in expression. These data suggest that transcription factors upstream of NGN3 may be important in establishing endocrine cell progenitors prior to 8 weeks, while genes acting downstream of NGN3 are more active during the 8 to 21 week period, and are likely to be involved in the final differentiation and maintenance of endocrine cell phenotypes and functions.

Because loss and gain of function studies in the rodent have demonstrated the critical role of NGN3 in specifying all four endocrine cell fates [12, 14, 26], co-expression of NGN3 with PDX-1, insulin and glucagon was assessed in the human fetal pancreas. Highest expression of *NGN3* was observed at 8 to 10 weeks, with a significant decrease at the middle and late stages, as determined by qRT-PCR and immunostaining. NGN3$^+$ cells were found to co-express PDX-1, insulin and glucagon during the early stage; however, the appearance of well-defined insulin$^+$ and glucagon$^+$ cell clusters coincided with a decrease in NGN3 co-localisation. The majority of NGN3$^+$ cells remained at the outer edges of these endocrine cell clusters, suggesting that they may be a precursor population with the potential to differentiate into endocrine cells. Interestingly, co-localisation of NGN3 with insulin and glucagon are not observed in rodent model systems [14]. Using 'add-back' *Ngn3* mice, Johannasen et al. observed that *Ngn3* is capable of inducing insulin$^+$, glucagon$^+$, somatostatin$^+$ and PP$^+$ cells from Pdx-1$^+$ pancreatic epithelium [26]. Rodent studies have also found that *Ngn3* can induce endocrine gene expression, including insulin [14, 15]. A recent report of an individual with a homozygous *NGN3* mutation [27] noted diarrhoea as the main initial symptom, due to a deficiency in gastrointestinal enteroendocrine cells, with diabetes developing at 8 years. Taken together with our findings, these studies suggest that NGN3 is critical for the establishment of islet cell types, not only in rodents but also in humans.

Transcription factors reported to act downstream of NGN3 in the rodent [16], including *ISL1, NEUROD1, MAFB, NKX2-2* and *PAX6*, were upregulated in second trimester pancreases. Co-localisation of ISL1, NEUROD1, NKX2-2 and PAX6 with insulin and glucagon during the 8 to 21 week period (Figs. 6 and 7, ESM Figs. 4 and 5) and in adult human islets (data not shown) suggest that these transcription factors may be involved in differentiation of endocrine cell types and maintenance of their function. Interestingly, NKX2-2 production was high in both alpha and beta cells by the middle stage, similar to findings seen in the rodent pancreas [28–30], while NKX6-1 co-localised only with insulin cells, indicating that NKX6-1 is exclu-

sively involved in human beta cell development. In support of our observations, Schisler et al. found that NKX6-1 suppresses glucagon expression and enhances insulin secretion from rodent beta cells [31]. In contrast, studies using murine knockout models have reported NKX6-1 involvement in alpha and beta cell differentiation [31, 32].

In summary, our study is the first detailed examination of morphological changes in the human fetal pancreas from 8 to 21 weeks with parallel quantification and localisation of transcription factor gene expression. Our findings indicate that major development of the human fetal endocrine pancreas occurs during the first and second trimesters of pregnancy. Moreover, our analyses provide evidence that transcription factor gene expression patterns in human fetal pancreatic development are, in general, similar to those reported in the rodent. Further mechanistic and functional studies are now required to understand the specific role of each transcription factor in the differentiation and regulation of endocrine cells in the human fetal pancreas.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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