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ARTICLE

Global gene expression profiling and histochemical analysis of the developing human fetal pancreas

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Abstract
Aims/hypothesis An immunohistochemical and genomic analysis of human pancreatic development from 9–23 weeks of fetal age was undertaken to provide a comparative analysis of human and murine islet development.
Methods Human fetal pancreases obtained at gestational ages 9–23 weeks were processed in parallel for immunohistochemistry and gene expression profiling by Affymetrix microarrays.
Results By 9–11 weeks, the pancreas was made up principally of mesenchymal tissue infiltrated by branched epithelial structures containing scattered hormone-negative neurogenin3-positive endocrine cells. Protoacinar structures emerged by 15–19 weeks, along with clusters of endocrine cells producing either glucagon or insulin. By 20–23 weeks, vascularised islet-like structures appeared. More than 70% of endocrine cells produced a single hormone at any age. Analysis of Ki67 immunoreactivity showed that the replicative rate of endocrine cells was low and suggested that the endocrine expansion was derived from hormone-negative precursors. Insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide transcripts were present at 9–10 weeks and increased progressively, commensurate with the expansion of endocrine cell volume. The human equivalent of a mouse endocrine secondary transition was not evident, neither in terms of morphology nor in dramatic changes in endocrine-specific transcriptional regulators. By contrast, exocrine genes showed a marked transition at around 11 weeks, associated with a greater than sixfold increase in exocrine gene transcripts.
Conclusions/interpretation The observed extension of terminal differentiation of human endocrine tissue into late gestation is in contrast with findings in the mouse. It indicates that the human fetal pancreas could provide an abundant islet precursor cell population that could be expanded ex vivo for therapeutic transplantation.

Keywords Fetal pancreas · Gene expression profiling · Human · Immunohistochemistry · Islet development · Microarray

Abbreviations

AMCA	7-amino-4-methyl-3-coumarinylacetic acid
CEL	carboxyester lipase
E	embryonic day
CK19	cytokeratin 19
NGN3	neurogenin3
PCA	principal component analysis
SOM	self-organising map
TSA	tyramine signal amplification

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Introduction

There is a paucity of information on the development of the human pancreas at the morphological and genomic level

because of ethical constraints on procurement of fetal tissue and problems with preservation of mRNA. Like the mouse pancreas, the human pancreas develops from two endodermal diverticula, the dorsal and ventral [1], which fuse around 56 days post coitum of development [2]. The morphogenesis of the endocrine tissue, however, is unlikely to be equivalent given the differences in gestation (260 vs 20 days) and the larger relative volume of the human pancreas [3]. Extrapolation from our knowledge of transcriptional regulation of rodent pancreatic development to the human is thus difficult and needs to be evaluated in the context of morphological events. There are also a number of discrepancies in published accounts of endocrine cell development in the human pancreas regarding whether early endocrine cells produce multiple hormones and at which stage the fetal cells are developmentally or functionally 'mature' [2, 4–9].

Available gene expression data on the human pancreas have tended to focus on subpopulations of endocrine cells [10] or individual transcription factors like neurogenin3 (NGN3) [11] and PDX1 [2, 10, 11] but not the global or dynamic picture. Pancreatic acinar cell development has been documented at the ultrastructural and histochemical level [12] and an early marker of exocrine cell differentiation has been identified as carboxyester lipase (CEL) [13, 14]. However, again, there are discrepant reports on the expression of the genes for major terminal acinar cell markers during human development [15, 16]. We report here microarray profiling of human fetal pancreatic tissue from age 9–23 weeks in conjunction with an immunohistochemical analysis of the same samples. The morphological data presented as high-resolution montages are accessible online along with the microarray data at http://genespeed.ccf.org and http://www.uchsc.edu/misc/diabetes/Sarkar/Sarkar.htm, last accessed in October 2007. Many of the same molecules appear to be involved in human and mouse pancreatic patterning and cellular differentiation. However, an abrupt secondary transition in human endocrine development is not observed and is reflected in differences in the onset and the kinetics of the expression of the genes for key transcriptional regulators. This has implications for the impact of fetal nutrition and intrauterine growth restriction with type 2 diabetes [17, 18] and the use of the tissue as a source of endocrine progenitor cells.

Methods

Fetal pancreas procurement

Human fetal pancreases at gestational ages 9–23 weeks were obtained from Advanced Bioscience Resources (Oakland, CA, USA) and the Birth Defect Research Laboratory, University of Washington (Seattle, WA, USA). The procurement centres obtained informed consent for tissue donation and approval by the institutional review boards. Gestational age was determined from biparietal diameter, femur length and fetal foot measurements. Each pancreas was freed of extraneous tissue and divided, one portion for fixation in 4% paraformaldehyde (wt/vol.) in PBS for 30 min at 4°C, the other frozen in liquid nitrogen for isolation of RNA.

Immunohistochemistry of fetal tissue

Paraffin-embedded pancreases were sectioned (6 μm) and de-paraffinated by standard procedures. Antigen retrieval was performed for 30 min at 97°C in 10 mmol/l Tris, 1 mmol/l EDTA (pH 9.0) or 0.01 mol/l citrate buffer (pH 6.0). The antibodies used are listed in the electronic supplementary material (ESM). For immunofluorescence microscopy, sections were incubated for 1 h at room temperature with blocking buffer (tyramine signal amplification [TSA] system; Zymed, Invitrogen Corporation, Carlsbad, CA, USA) and then with primary antibodies overnight in a humid chamber. Secondary antibodies (1:200) conjugated to cyanine 3, cyanine 2 or 7-amino-4-methyl-3-coumarinylacetic acid (AMCA) fluorophores (Jackson Immunoresearch Laboratories, West Grove, PA, USA) were applied at room temperature for 60 min. The sections were rinsed in PBS and mounted in a glycerol-based medium. Adjacent sections were stained either with haematoxylin and eosin for morphology or with Ki67 antibodies followed by immunoperoxidase development to detect dividing cells. The latter were viewed both in bright field and by immunofluorescence. NGN3 was visualised on 9–13 week fetal pancreases with mouse monoclonal (F25A1B3; DSHB, University of Iowa, IA, USA) using the TSA kit before incubation with insulin and β-catenin antibodies. Image panels were acquired with ×40 oil lens using an Olympus 1X70 inverted microscope equipped with a motorised stage and Quantix monochromatic CCD camera (KAF 1400 chip) (Photometrix, Tucson, AZ, USA). Image arrays are accessible at http://genespeed.ccf.org and http://www.uchsc.edu/misc/diabetes/Sarkar/Sarkar.htm, last accessed in October 2007 (including ESM Figs 1, 2, 3 and 4).

RNA isolation and microarray analysis

Total RNA was extracted from fetal pancreases using Trizol reagent (Invitrogen, Carlsbad, CA, USA), purified by RNeasy columns (Qiagen, Valencia, CA, USA) and quantified and assessed for quality by capillary electrophoresis (Agilent-2100 Bioanalyzer; Agilent, Palo Alto, CA, USA). Biotin-labelled cRNA was synthesised from total RNA 6 μg (Batch 1) and 2.6 μg (Batch 2 and 3) according to the standard Affymetrix protocol and 15 μg of the final product

hybridised to Human Genome HG U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) containing 54,675 probe-sets representing around 22,000 unique genes.

Genome-wide expression and statistical analysis

Quality control and normalisation (MAS5.0) of microarray data were conducted using Bioconductor Project software (Available from http://www.bioconductor.org, last accessed in October 2007). Principal component analysis (PCA) (Partek, St Louis, MO, USA) was undertaken to elucidate data distribution, which was subsequently grouped by five time-points: (1) 9–9.5 weeks, n=3 samples, and 10 weeks, n=2; (2) 11 weeks, n=3; (3) 15 weeks, n=2; (4) 18–19 weeks, n=3; and (5) 20 weeks, n=2, and 23 weeks, n=3. Self-organising maps (SOMs) were used to determine the temporal expression profiles within each group. A three-step gene-filtering process was applied to the derived data sets to exclude: (1) control probe sets on the Affymetrix chips; (2) genes with least moderate variation; and (3) genes with call of 'Absent' in all experiments. Differential expression analysis was assessed by ANOVA using a cut-off threshold p value of 0.05 and a false detection rate of 0.1 (Table 1). The analyses (cluster, genome-wide statistical, volcano plots and corresponding gene lists) can be found at http://genespeed.ccf.org and http://www.uchsc.edu/misc/diabetes/Sarkar/Sarkar.htm, last accessed in October 2007.

Quantitative RT-PCR

cDNA was prepared from total RNA (1 μg) using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Templates (0.1 μg of cDNA) were amplified over the linear range in a 5′ nuclease assay-based system using 6-carboxy-fluorescein dye-labelled Taqman minor groove binding probes (Applied Biosystems, Foster City, CA, USA) on a 96 well ABI 7000 PCR instrument. *GAPDH* was selected for sample normalisation based on preliminary experiments with the ABI control plate (part number 430 9199). The cycle threshold values were determined in triplicate and the data normalised to 9 week samples (control group/calibrator).

Table 1 False detection rate report

Group	Cut-off p value	Number of significant genes
9–10 weeks vs 11 weeks	0.0023555	476
9–10 weeks vs 15 weeks	0.0145289	2,936
9–10 weeks vs 18–19 weeks	0.0234462	4,738
9–10 weeks vs 20–23 weeks	0.0341944	6,910

Differential expression analysis of microarray data was assessed by ANOVA. Genes were considered significant if adjusted p values (corrected by Benjamini and Hochberg's procedure for multiple hypothesis testing) were below 0.1. The human fetal tissue was grouped as 9–10 weeks (n=5), 11 weeks (n=3), 15 weeks (n=2), 18–19 weeks (n=2) and 20–23 weeks (n=5) after initial PCA

Results

Characterisation of fetal human pancreatic development (morphological and histological)

Evaluation of human fetal pancreas from 9–23 weeks of gestational age was initially performed on haematoxylin/eosin stained paraffin sections (Fig. 1 and Fig. 1 on our web site: http://www.uchsc.edu/misc/diabetes/Sarkar/Sarkar.htm, last accessed in October 2007). At 9–10 weeks, the tissue was typified by islands of epithelial tissue within a much larger volume of mesenchymal tissue. The relative epithelial to mesenchymal areas increased from 26% at 9 weeks to >70% by 23 weeks. The growth of epithelium over this period was typified by multilobulate expansion from a number of foci suggestive of a pattern of branching morphogenesis. The majority of epithelial cells, especially at early fetal ages, were characterised by a high nuclear/cytoplasmic ratio and a lack of basal to apical polarity (Fig. 2a) suggesting that they were not terminally differentiated. They were typically arranged around a lumen that in fortuitous sections were seen to extend to branches that terminated in a bulb of closely packed cells (Fig. 2b,c). The cells lining the lumen ranged in morphology from pseudo-stratified epithelial layers (Fig. 2b) surrounding a closed lumen to unilamellar closely packed polarised cells typical of pancreatic ductal epithelium. The mesenchymal tissue was typified with a very low cellular density (Fig. 2a) except in areas adjacent to the epithelia where cellular density was higher. A striking feature was that, at any given fetal age, all these structures could be observed within the one section, suggestive of independent multifocal morphogenic events (Fig. 2a–d). This feature contrasts with the appearance of the rodent pancreas, in which the mesenchymal compartment is less extensive and more densely populated and where the epithelial morphology is more uniform.

Endocrine tissue Immunohistochemical staining for insulin, glucagon (Fig. 3a–f) and somatostatin (data not shown) at 9–10 weeks demonstrated the presence of sparse endocrine cells within clusters of epithelial cells. The latter were marked by strong β-catenin immunoreactivity that was confined to the cell periphery, presumably the plasma membrane (Fig. 4a). By 15 weeks, the endocrine cells remained interspersed throughout the pancreatic epithelium, although they often localised towards the periphery of the cluster and stained less strongly for β-catenin (Fig. 4b).

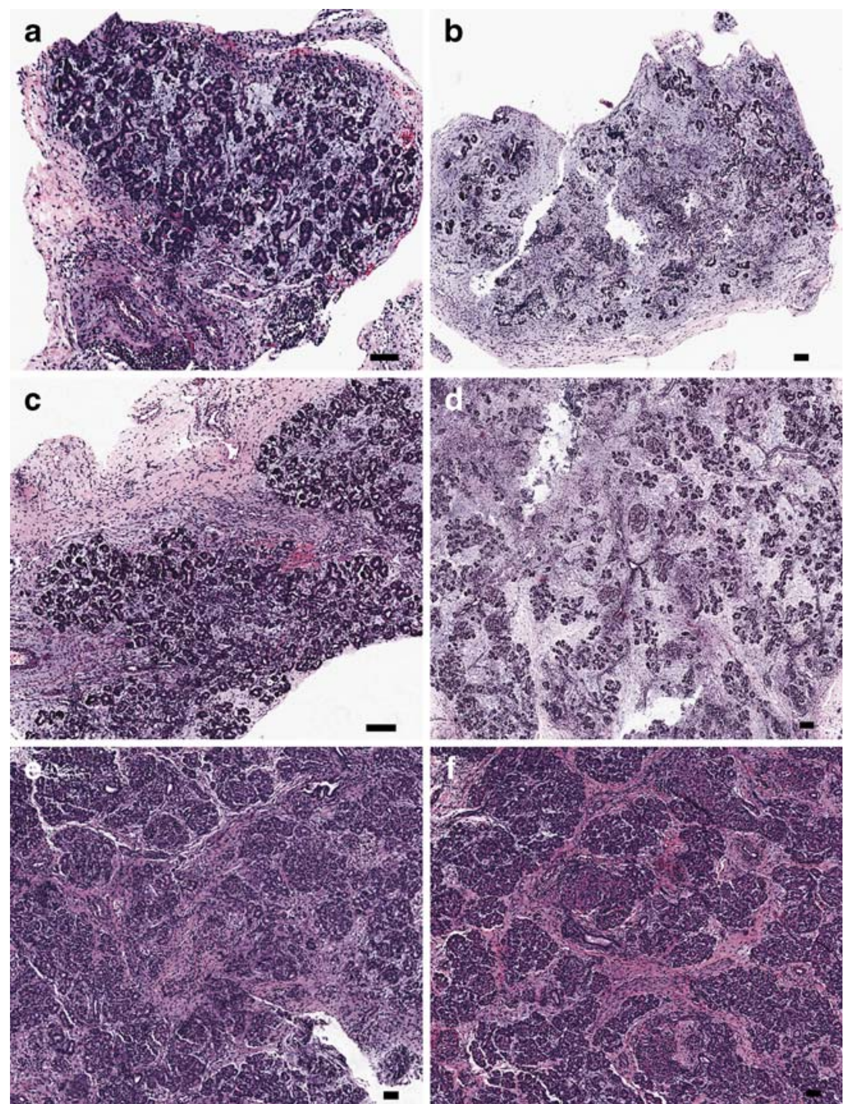

Fig. 1 Transverse sections of individual developing human pancreases counterstained with haematoxylin and eosin at 9 weeks (**a**), 10 weeks (**b**), 11 weeks (**c**), 15 weeks (**d**), 20 weeks (**e**) and 23 weeks (**f**). Epithelial structures distinguished by their dark heterochromatic nuclei are interspersed within mesenchyme that has lower nuclear density and pale cytoplasm. High-resolution images can be seen at http://genespeed.ccf.org and http://www.uchsc.edu/misc/diabetes/Sarkar/Sarkar.htm, last accessed in October 2007. Scale bars, 50 μm

The majority of endocrine cells expressed only glucagon, insulin or somatostatin, although occasionally co-expression of insulin and glucagon was evident (<5% cells). As reported previously [19], the double-hormone-positive cells, however, do not co-label with Ki67, thus indicative of their non-proliferative status. Chromogranin A was observed in all hormone-positive cells (data not shown) and more prominently in glucagon-expressing cells, as is the case in adult islets [20]. From 11 weeks and onwards, small numbers of cells immunoreactive for ghrelin and pancreatic polypeptide were also noted (data not shown).

A progressive increase in the endocrine cell population was evident from 11–23 weeks, reflecting both the progressive expansion of the pancreatic epithelium (Fig. 1) and an increase in the density of endocrine cells within the epithelium (Fig. 3). With increasing fetal age there was evidence of formation of endocrine cell clusters especially from 15 weeks onwards (Fig. 4c,d), although solitary endocrine cells were still observed at all time-points. The endocrine cell clusters at early time-points displayed a significant homotypic association of either insulin- or glucagon-positive cells (Fig. 3e,f). At later time-points, heterotypic endocrine cell clustering and the appearance of typical islet-like structures were a feature. NGN3-positive cells were scattered among these epithelial clusters. Such cells exhibited heterogeneity with respect to the level of staining (9–13 weeks; see Fig. 9b) and were β-catenin and hormone-negative, a striking observation given the suggestion that β-catenin may be required for the development of the exocrine and not endocrine pancreas [21]. The endocrine transcription factor PDX1 was produced within the early pancreatic progenitor epithelium from 9 weeks onwards, although initially with a cytoplasmic localisation. From 19 weeks both nuclear and cyto-

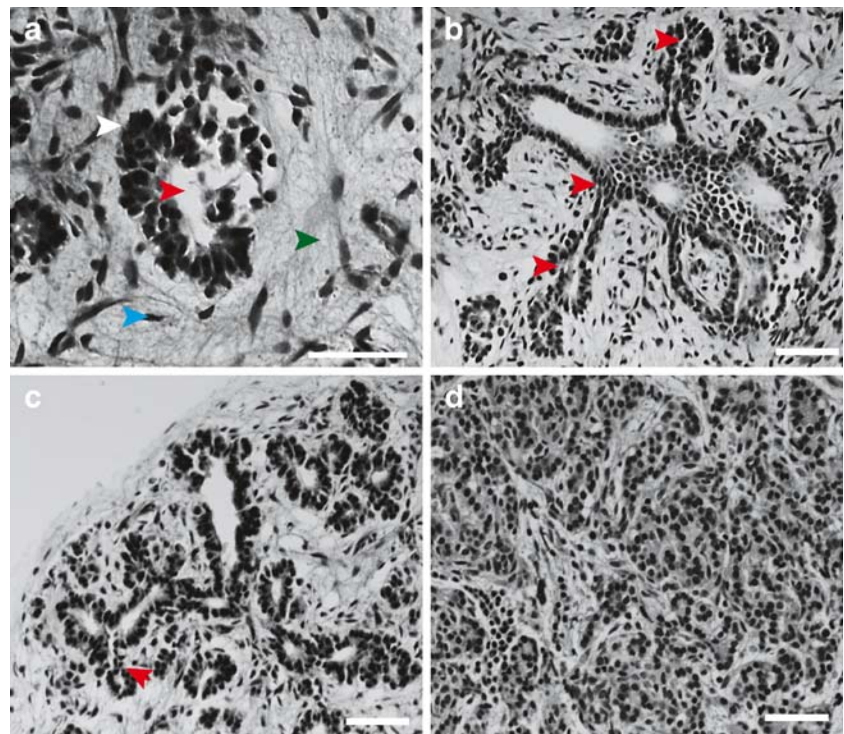

Fig. 2 Relationship of the mesenchymal to epithelial structures and tubulogenesis in fetal human pancreas (haematoxylin and eosin stain). **a** Ten weeks: showing pseudo-stratified epithelial structure with a small lumen (red arrow). A small epithelial structure with no lumen is also seen (white arrow). The nuclei lack polarity and stain intensely. The surrounding mesenchyme (green arrow) is mesh-like and has a low nuclear density compared with the epithelial structures. The flattened cells may be vascular elements (blue arrow). **b** Eleven weeks: showing branching tubules. Red arrows indicate lumen formation and branching. **c** Fifteen weeks: showing well-developed branching structures. The cells lining the lumen have acquired polarity and columnar morphology (red arrow). **d** Twenty-three weeks: epithelial structures are predominant. Scale bars, 50 μm

plasmic PDX1 staining of the pancreatic epithelium and islet-like clusters was seen (see Fig. 9c) more typical of the adult human pancreas (results not shown).

The relationship between cell division and endocrine cell differentiation was evaluated by simultaneous immunoperoxidase staining for Ki67 combined with immunofluorescence for insulin, glucagon and somatostatin (ESM Fig. 1). Ki67-positive nuclei were seen at all time-points in both epithelial and mesenchymal structures with apparent random distribution, except in the region of the mesenchymal–epithelial boundary where it was common to see a string of adjacent cells each with a Ki67-positive nucleus (ESM Fig. 1). None of the latter stained positively for endocrine markers and it was difficult to ascertain whether they were epithelial or mesenchymal in nature. In older fetal ages it was evident that the regions of clustered endocrine cells had far fewer Ki67-positive nuclei than adjacent non-immunoreactive epithelia. Quantitative analyses at 9–10 weeks showed that 2% of the endocrine cells were Ki67-positive (ESM Fig. 1) and increased to 5.2% at 23 weeks. The relative ratio of endocrine to non-endocrine epithelial replication was low at all ages but increased significantly with time from an early (10–11 weeks) value of 0.10–0.29 at later gestation (19–23 weeks; *t* test $p=0.041$). These data suggested that a large majority of endocrine cells are G0-arrested, and thus post-mitotic indicating that the endocrine compartment is predominantly generated through de novo differentiation from a precursor population over the second trimester rather than by replication of an established early set of endocrine cell-type populations.

Exocrine tissue Amylase, a marker of the mature exocrine pancreas, was detected only at 23 weeks in typical acinar structures (ESM Fig. 2). The protoacinar marker CEL was not detectable at 9 weeks (Fig. 4e) but emerged in the β-catenin-positive epithelium at 11 weeks. It peaked at 15 weeks (Fig. 4f) and thereafter appeared in both luminal and peripheral locations of the epithelium in β-catenin-positive cells (Fig. 4g,h).

Ductal tissue Cytokeratin 19 (CK19) showed diffuse immunolabelling of all epithelial clusters at 9 weeks but by 11 weeks was associated with cells on the luminal aspects of epithelial clusters. Later, the luminal cells became more columnar in appearance and retained a CK19 staining pattern typical of the adult human pancreas. Triple-positive immunostaining of CK19 with the endocrine markers insulin and chromogranin was observed in the pancreas from early gestational ages; however, the intensity of CK19 staining was lower relative to hormone-negative epithelial structures that had luminal openings and columnar-like epithelium (ESM Fig. 3).

Pancreatic mesenchyme A proportion of mesenchymal structures showed immunoreactivity for vimentin and smooth muscle actin (data not shown) but no colocalisation

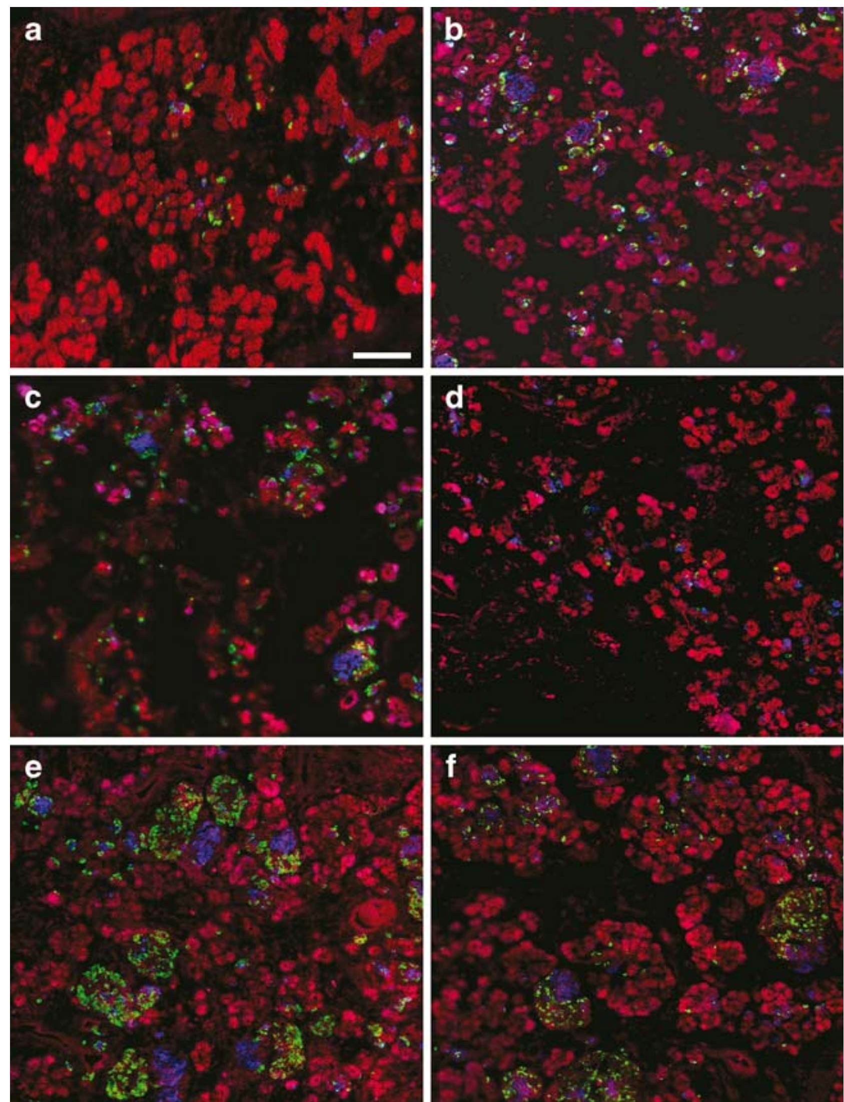

Fig. 3 Immunofluorescence detection of endocrine differentiation in the developing human fetal pancreas at 9.5 weeks (**a**), 11 weeks (**b**), 15 weeks (**c**), 19 weeks (**d**) 20 weeks (**e**) and 23 weeks (**f**). Transverse sections of fetal pancreases were stained for insulin (AMCA, blue) and glucagon (cyanine 2, green) and β-catenin (cyanine 3, red). High-resolution images can be seen at http://genespeed.ccf.org and http://www.uchsc.edu/misc/diabetes/Sarkar/Sarkar.htm, last accessed in October 2007. Scale bar, 50 μm

of endocrine, exocrine and the mesenchymal markers was observed at any developmental stage. Vascular endothelium detected by CD34 antibodies was observed in the mesenchyme from 9 weeks and by 10–23 weeks was closely associated with large or small islet-like clusters (ESM Fig. 4).

Global gene expression analysis

PCA of the microarray data obtained from 17 pancreas samples of 9–23 weeks of gestational age defined three cluster sets corresponding to 9–10, 11 and 15–23 weeks (Fig. 5a). Overlap between the 15, 18–19 and 20-23 week clusters suggested that organ development proceeded through gradual growth in the 15–23 week window without any major changes of the constituent cell types. Assuming progressive development, we used SOMs to take into account relationships between the adjacent temporal points of developmental series and a 4×3 cluster was subsequently used to display gene expression clustering (Fig. 5b) for five age groups [9, 9.5–10 (n=5), 11 (n=3), 15 (n=2), 18–19 (n=2) and 20–23 (n=5) weeks].

Expression of genes for endocrine cells The endocrine genes insulin, glucagon and somatostatin showed a steady increase from 9–15 weeks tapering off by 18–23 weeks (Figs 5 and 6a). The signal intensity for these markers probably reached saturation at the later time-points, a feature that masked further possible increases that was typical of other dense core secretory granule markers such as *PCSK1*, *PCSK1N*, *PTPRN*, *SLC30A8* (Fig. 7), *IAPP*, *CHGA* and *CHGB* (Fig. 6a). Components of the stimulus-secretion coupling machinery such as *GCK* and *GLUT2* (*SLC2A4*) were nevertheless more variable (Figs 7a and 9a). The high signal intensity observed for insulin, glucagon and somatostatin corresponded to the abundance

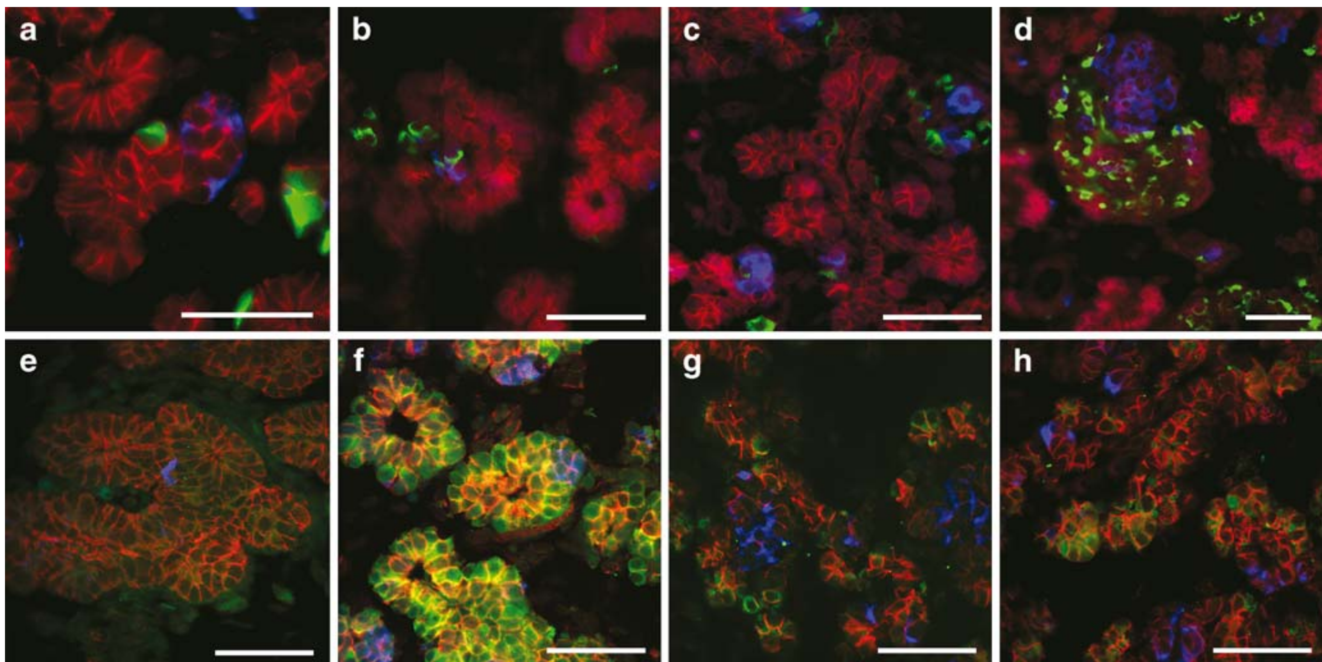

Fig. 4 Immunofluorescence detection of endocrine differentiation in the developing human fetal pancreas. Transverse sections of fetal pancreases were stained for insulin (AMCA, blue), glucagon (cyanine 2, green) and β-catenin (cyanine 3, red). **a** Ten weeks: the epithelium initially has a narrow lumen and cells appear pseudo-stratified. Endocrine cells peripherally localised and few endocrine cells stain for both insulin and glucagon. **b** Fifteen weeks: branching epithelium. **c** Nineteen weeks: further endocrine cell clustering and islet formation. **d** Twenty-three weeks: islet-like structures with a mixed endocrine population. **e**–**h** Progression of immunoreactivity for the exocrine marker CEL (cyanine 2, green), endocrine cells marked by chromogranin A (AMCA, blue) and the epithelium is marked by β-catenin (cyanine 3, red). CEL staining is initially absent at 9 weeks (**e**), peaks at 15 weeks (**f**) and then declines from 19–23 weeks (**g**, **h**). β-Catenin reactivity is diminished in endocrine structures. Scale bars, 50 μm

of immunopositive cells detected by immunohistochemistry. Low signals for ghrelin (*GHRL*) and pancreatic polypeptide (*PPY*; Fig. 6a) corresponded to the low frequency of these cells in the histological analysis.

Expression of the genes for exocrine cells The exocrine-specific genes show a distinct transition around 11–15 weeks (Fig. 6b). At 9–10 weeks low signals were detected for the fetal acinar cell marker *CEL* and other exocrine markers *CPA1*, *CPA2*, *CTRB1*, *CTRC*, *elastase* (*ELA*) (*2A*, *3A*, *3B*), *PRSS1*, *REG1A* and *REG3G* (Figs 6b and 7b). In every case, dramatic increases were observed at 11 weeks reaching a plateau (or saturation) at 15–19 weeks (Figs 6b and 7b). Other markers of differentiated acinar cells such as amylase (*AMY2A*) are not detected by arrays but could be shown by immunohistochemistry at later gestational ages (20–23 weeks; ESM Fig. 2). *PTF1A*, a major transcriptional regulator of exocrine cell gene expression and development, was not present on the Affymetrix chip. The expression of other exocrine transcription factors, such as *BHLHB8/MIST1* and *GATA4*, increased with time (Fig. 6b).

Expression of genes for ductal cells Pancreatic ductal cell functional markers such as carbonic anhydrase (*CA1*), mucin1 (*MUC1*), *CFTR* and several aquaporins (*AQP1*, *AQP8*, *AQP12B*) were detected at all gestational ages and increased from 11 weeks onwards (Fig. 6c). Genes encoding keratins (*KRT*; 7, 8, 10, 18 and 19) were expressed at 9–10 weeks but declined gradually by 15–23 weeks (Fig. 6c).

Expression of genes for mesenchymal cells The genes encoding the mesenchymal markers smooth muscle actin (*ACTG2*), collagen type V1-alpha 2 (*COL6A2*), alpha 3 (*COL6A3*) and vimentin (*VIM*), were highly expressed in 9–10 week fetal tissue and subsequently declined from 11–15 weeks (Fig. 6d). A similar profile was observed for other mesenchymal-specific genes in cluster 8 and presumably reflect the relative decrease in mesenchymal to epithelial mass as development proceeds (Fig. 1).

Developmental transcription factors Transcriptional regulators of the NOTCH signalling pathway (*HES1*, *HES4*, *JAG1*, *JAG2*, *NEUROD1* and *NOTCH* (*1*, *2*, *3*, *4*) that are involved in the early specification of the endocrine cell lineage were observed at all embryonic ages (Fig. 8a). The genes for a number of factors with an established role in pancreatic endocrine gene transcription, *IRX2*, *ISL1*, *MAFB*, *NKX2-2*, *NEUROD1*, *OCT1*, *PAX6*, *PBX1*, *PKMYT1* and *FOXO3*, were also expressed at all times as were the *SOX* family members, *SOX9* and *SOX11*. The

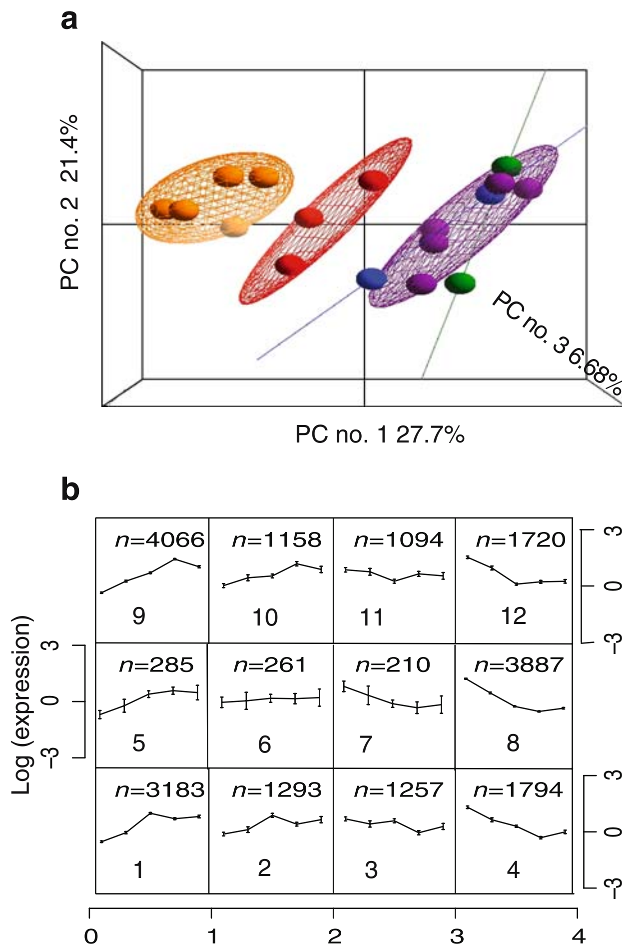

Fig. 5 **a** PCA of human fetal pancreas microarray data. Orthogonal projection of the data with ellipsoids drawn around groups of samples show that the distribution of 9–10 week samples are distinct from the 11 week samples, which form a separate cluster. Considerable overlap is seen among fetal pancreatic samples of higher gestational ages (15–23 weeks). **b** Temporal profile of gene expression was mapped using SOM analyses. A 4×3 cluster revealed a majority of endocrine and exocrine genes aggregated in cluster 1

latter have been implicated in human [22] and mouse development (Fig. 8a–c). The observation that the expression levels of most factors did not change by more than a few fold from 9–23 weeks is consistent with there being multiple foci of endocrine development within the pancreas each initiated at different times.

The key pancreatic transcription factors in this context, namely *PDX1*, *NGN3*, *NKX6.1*, *PAX4* and *MAFA*, were not detected on microarrays but could be quantified by RT-PCR using Taqman probes (Fig. 9a). *NGN3* was expressed at all developmental ages from 9–23 weeks, although undetectable in adult islets as expected (Fig. 9a). *PDX1* expression was evident at all gestational ages and increased from 9–19 weeks before declining. Similarly, expression of *MAFA*, *NKX6.1* and small amount of *PAX4* were confirmed from 9–23 weeks (Fig. 9a).

Discussion

Our current knowledge of transcriptional regulation of mammalian pancreatic development is largely derived from studies on mice, in which gene regulation can be manipulated in a conditional and tissue-specific manner to provide information on signalling pathways and cell lineage [23]. The evagination of the human foregut to form the ventral and dorsal pancreas takes place around 4–5 weeks post coitum (Carnegie stage 12-15) corresponding to mouse embryonic day (E)9.5 (six to ten somites) [24, 25]. Significant insulin production would be anticipated in the human at this time but is not observed until 2 weeks later [2]. Conversely the emergence of the four major endocrine cell types in the mouse near term (E18) first occurs at 8.5 weeks in man [2]. The human equivalent of the secondary transition in the mouse (E12.5–13.5) when a wave of NGN3-positive cells differentiate into clusters of insulin and glucagon cells has not been described in man. Such a transition conceivably occurs prior to the earliest time-point in our study (9 weeks); however, our observation of NGN3-positive from 9–23 weeks and in a previous study at 14 weeks [26] argues against this. Nevertheless, many structural features of human endocrine pancreatic development and accompanying patterns of gene expression are reminiscent of processes described in the mouse and we hypothesise that essentially similar transitional events do occur but at multiple foci and without the same temporal coincidence as the mouse. The epithelial thickenings and evaginations that appear to precede the appearance of endocrine cells thus appear to be similar to the multilayer stratified cells previously described in the mouse in the progenitor epithelium that exists prior to the onset of the secondary transition (mouse: E13.5) and which subsequently disappears following terminal differentiation (mouse: E15.5). The cells display a high nuclear/cytoplasm ratio, are β-catenin-positive and diffusely positive for ductal marker CK19 and transcription factor PDX1. Scattered insulin or glucagon cells are evident in the non-luminal areas of these structures [27, 28]. PDX1 production was cytoplasmic at all stages of development in our study except at a later stage (>19 weeks), where nuclear production of PDX1 was observed in endocrine cells (beta cells). In this respect our histochemical results were similar to CK19 and somewhat at variance for PDX1 [2], which the authors localised at 12–13 weeks in non-endocrine epithelial cells.

The abundance of endocrine cells from 9–23 weeks that were Ki67-negative suggested that the endocrine cells are formed by terminal differentiation of actively replicating epithelium. Peak proliferation of glucagon cells (6.5%) occurred at 20 weeks, for insulin (5.4%) and somatostatin cells (5.5%) at 23 weeks. This is in contrast to previous findings [29] where peak proliferation of alpha/beta cells

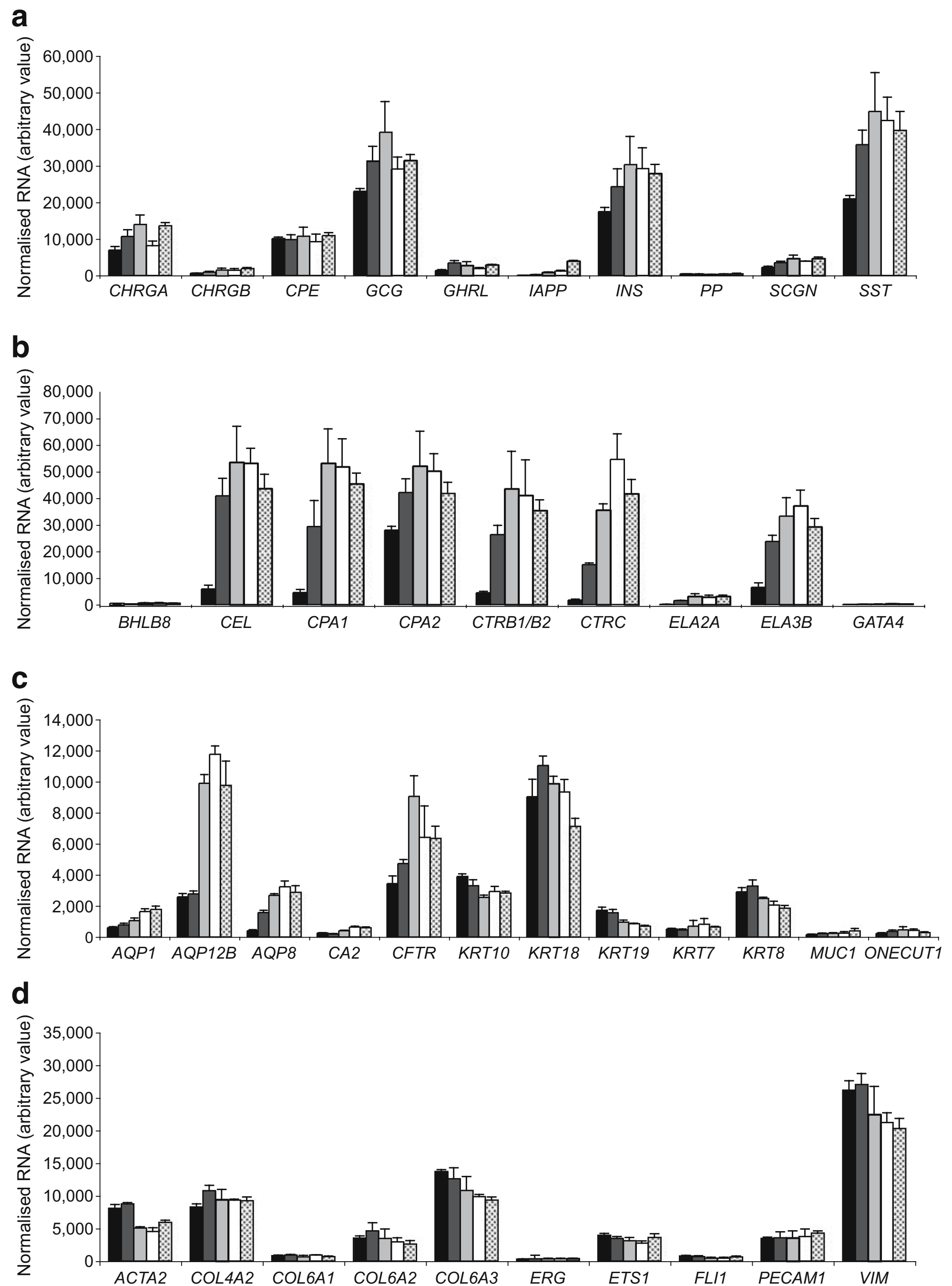

Fig. 6 The normalised intensity of mRNA expression (MAS5.0) from data obtained on an HG U133 Plus 2.0 Affymetrix chip is grouped according to: endocrine (**a**), exocrine (**b**), ductal (**c**) and mesenchymal (**d**) compartments. Each data-point is the mean±SEM of three or four observations. Black bars, 9–10 weeks; dark grey bars, 11 weeks; light grey bars, 15 weeks; white bars, 19 weeks; grey dotted bars, 23 weeks

(3%) was noted earlier at 14–16 weeks. The continued expression of endocrine regulatory genes such as *IRX2*, *ISL1*, *MAFB*, *NKX2-2*, *PAX6*, *PBX1* and *PKMYT1* throughout the early to late gestational period is consistent with the focal endocrine developmental hypothesis, as is the maintenance of expression of the NOTCH signalling pathway of *HES1*, *HES4*, *JAG1*, *JAG2* and *NOTCH* (*1*, *2*, *3*, *4*). High transcript levels of *SOX9* were notable in this context as this factor was recently described as pancreatic progenitor-specific factor acting in conjunction with NOTCH to maintain the progenitor state [30]. The persistent expression of *NGN3* is likewise consistent with the appearance of foci of progenitor cells at different time-points resulting in the continuous differentiation of endocrine cells in the 9–23 week developmental window. NGN3 has a non-redundant role in mouse endocrine development [31, 32] and further studies aimed at systematic characterisation and isolation these of these putative endocrine precursors are warranted.

Islet formation occurs just prior to birth in the mouse [1] but in the human samples islet-like clusters appeared as early as 11–15 weeks. The continuing genesis of islets over time

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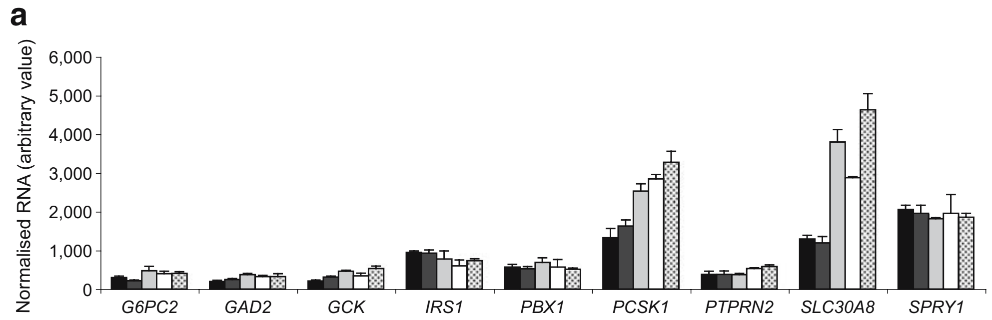


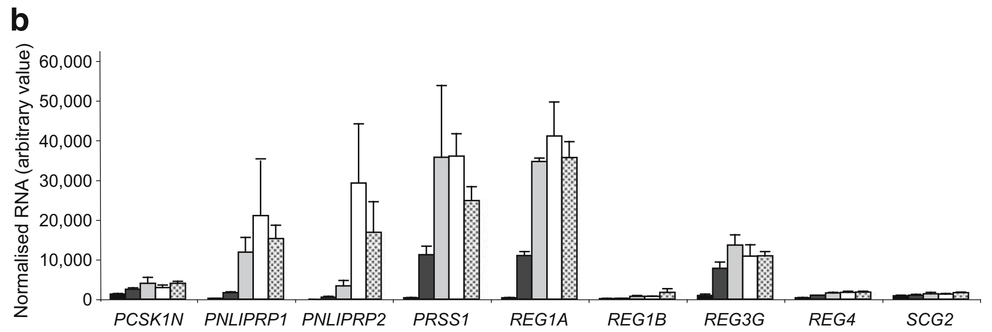
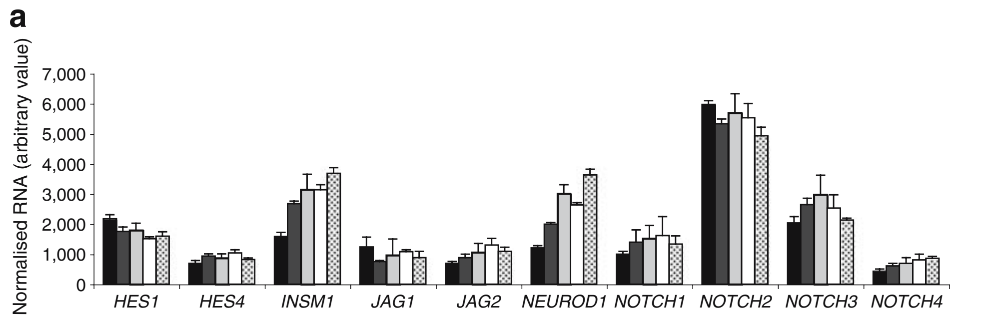
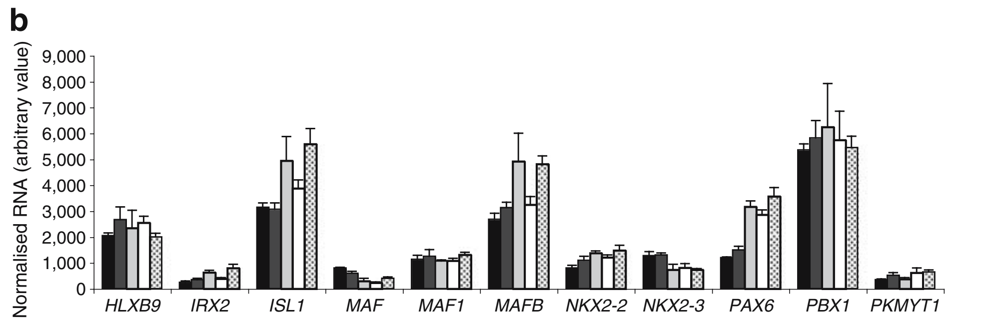


Fig. 7 Normalised expression of the genes encoding selected markers of endocrine and exocrine tissue function. Each data-point is the mean±SEM of three or four observations. Black bars, 9–10 weeks; dark grey bars, 11 weeks; light grey bars, 15 weeks; white bars, 19 weeks; grey dotted bars, 23 weeks





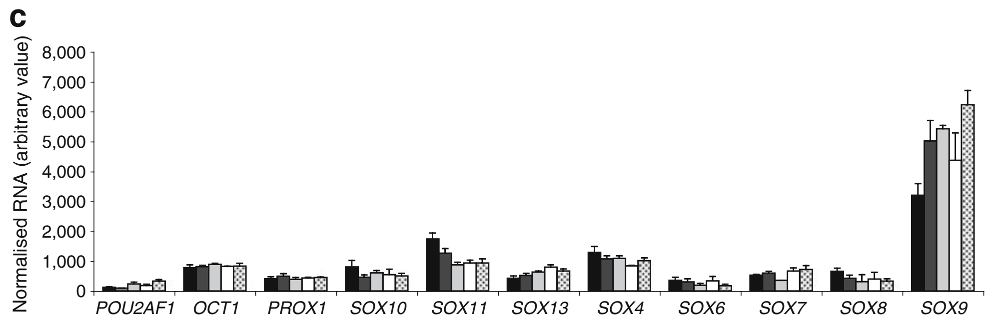


Fig. 8 Normalised expression of the genes encoding selected transcription factors in the developing human pancreas. Each data-point is the mean±SEM of three or four observations. Black bars, 9–10 weeks; dark grey bars, 11 weeks; light grey bars, 15 weeks; white bars, 19 weeks; grey dotted bars, 23 weeks

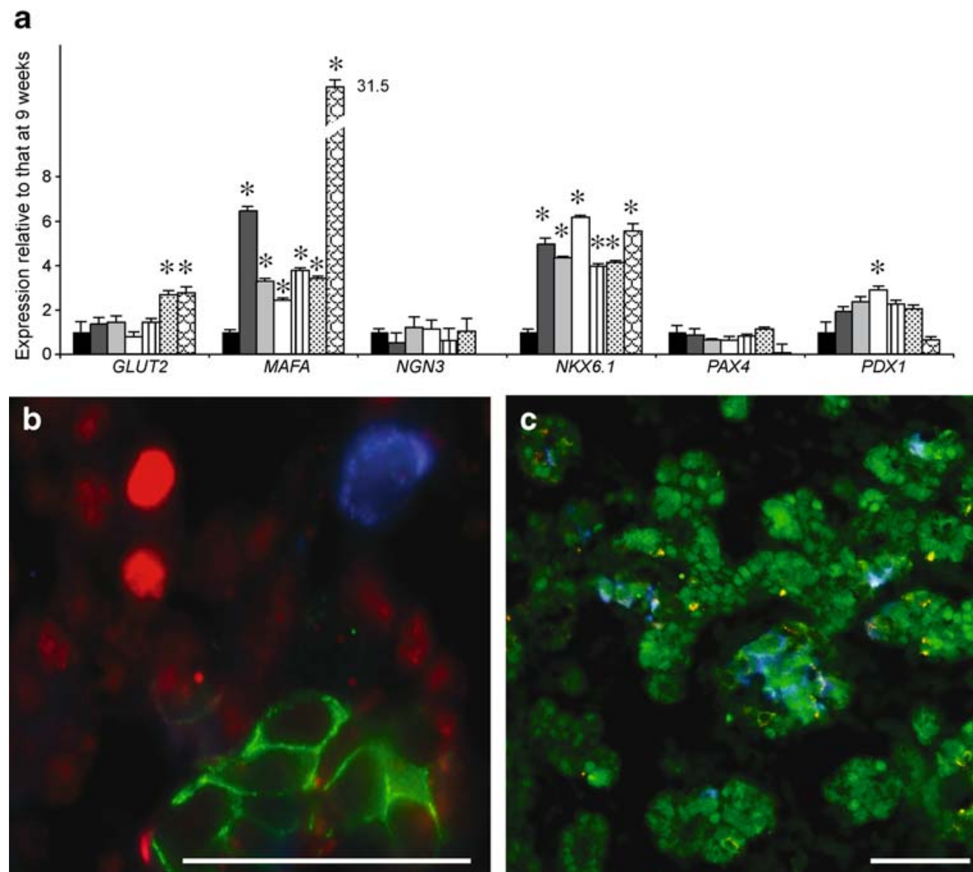


Fig. 9 **a** Expression of *GLUT2*, *MAFA*, *NGN3*, *NKX6.1*, *PAX4* and *PDX1* mRNA by quantitative PCR. Data in triplicate (means±SEM) are normalised to endogenous *GAPDH* and expressed relative to 9 week fetal samples. *NGN3* was undetectable in the adult islets. Data were analysed by ANOVA followed by a *t* test assuming equal variances between groups. *Significant difference compared with 9 weeks ($p<0.05$). Black bars, 9 weeks; dark grey bars, 11 weeks; light grey bars, 15 weeks; white bars, 19 weeks; vertically lined bars, 20 weeks; grey dotted bars, 23 weeks; fish scale-patterned bars, adult human islets. **b** Immunofluorescent detection of NGN3 (Alexa 546, red), β-catenin (Alexa 488, green) and insulin (cyanine 5, pseudocoloured blue) in 13 week fetal pancreas (cryosection). **c** Immunofluorescent detection of PDX1 (cyanine 2, green), insulin (AMCA, blue) and chromogranin A (cyanine 3, red) at 19 weeks. Both nuclear and cytoplasmic staining of the pancreatic epithelium and islet-like clusters was seen. Scale bars, 50 μm

would account for the cumulative increase in islet number with fetal age. Since abnormal glycaemic control is a typical complication of very early pre-term infants it seems likely that by 25 weeks the pancreatic endocrine cells are not capable of controlling blood glucose if put to the test. This implies that they are developmentally immature as suggested [33] and that although unresponsive to glucose in vitro, they are eventually able in vivo to respond to environmental cues by induction of genes required for glucose sensing and granule exocytosis. After all, at 23 weeks the islets were vascularised and expressed mRNA for *GLUT2* and genes that control prohormone conversion and insulin storage such as *INS*, *PCSK1*, *IAPP* and *PTPRN*.

The development of the fetal acinar compartment in humans also is not synchronised with that of endocrine differentiation following the secondary transition as it is in the mouse [34, 35]. Rather it appeared to be initiated abruptly around week 11 as evidenced by the appearance of transcripts for *CEL*, *CPA1*, *CTRB1*, *RNASE2*, *PRSS1* and *ELA3B*. The concomitant appearance of transcriptional regulators of exocrine cell formation such as *BHLB8*, *GATA4* and *RBPJL* suggests a common mechanism of gene regulation in humans as in the mouse. However, only low levels of transcripts for *ELA2A* and pancreatic lipase were evident at 11 weeks and amylase could only be detected histologically at 20–23 weeks, indicating that the cells may still be immature in terms of the molecular composition of their secretory product.

As in the mouse, the human mesenchyme probably plays an important role in the differentiation of the pancreatic epithelium into endocrine and exocrine components as evidenced by the presence of transcripts for fibroblast growth factor-10 [36] and integrins that coordinate epithelial cell adhesion and movement [37]. Considering

the overall architecture of the pancreas at 9–10 weeks and the large volume of the mesenchyme relative to epithelium, it seems unlikely that the mesenchyme would provide strict spatial gradients of extracellular growth factors or morphogens.

The general conclusion that the endocrine precursors are generated continually throughout the second trimester and remain competent in terms of islet production has important implications for the use of fetuses as a source of tissue for therapeutic transplantation in type 1 diabetes. Animal studies have demonstrated that fetal islet-like cell clusters comprise a mixture of hormone-containing cells and undifferentiated epithelial cells may be a better source of functional islet tissue than isolated adult islet tissue [38–40], possibly because they contain endocrine precursors. This raises the prospect of maintaining such tissue ex vivo to extend the progenitor state and expand the precursor cell population prior to differentiation. The present study provides a framework for further study of the intra-organ environment and discovery of the genes that might orchestrate this process.

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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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