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REVIEW

Human pancreas development

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ABSTRACT

A wealth of data and comprehensive reviews exist on pancreas development in mammals, primarily mice, and other vertebrates. By contrast, human pancreatic development has been less comprehensively reviewed. Here, we draw together those studies conducted directly in human embryonic and fetal tissue to provide an overview of what is known about human pancreatic development. We discuss the relevance of this work to manufacturing insulin-secreting β-cells from pluripotent stem cells and to different aspects of diabetes, especially permanent neonatal diabetes, and its underlying causes.

KEY WORDS: Human, Pancreas, Development, Embryo, Fetal, Gestation, Stem cells, Endoderm, Foregut, Liver, β-cells, Transcription factor

Introduction

The pancreas is a composite organ derived from two buds, dorsal and ventral, that arise from either side of the distal foregut endoderm. It contains a distinctive combination of cell lineages. The exocrine tissue comprises acinar cells that secrete digestive fluid and a duct system by which the fluid drains into the intestine. The endocrine portion is arranged as discrete islets of Langerhans, which comprise multiple distinct cell types secreting (at least) five different hormones into the circulation (α-cells, glucagon; β-cells, insulin; δ-cells, somatostatin; ε-cells, ghrelin; and γ [or PP]-cells, pancreatic polypeptide). That cells with such radically different adult functions develop from the same progenitors is a telling indication of a fascinating developmental pathway. Our understanding of how the organ forms in humans has relied very heavily upon extrapolation from data obtained in other species, particularly mouse, which have been periodically and comprehensively reviewed elsewhere (Jørgensen et al., 2007; Murtaugh, 2007; Pan and Wright, 2011; McCracken and Wells, 2012). These detailed articles and their underlying original research are not recycled here. Instead, we base this Review on data obtained directly from human development and limit extrapolation from other species.

Why focus a Review specifically on human pancreas development when knowledge is much greater in other species? There is particularly strong interest in understanding human pancreas development because it is relevant to different forms of

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and therapies for diabetes, a growing health problem worldwide. The advent of pluripotent stem cell (PSC) biology has provided a readily available human model in which to probe early developmental events and through which to generate insulin-secreting human β-cells *in vitro* (reviewed by Pagliuca and Melton, 2013). β-cells are lost by autoimmune destruction in type 1 diabetes mellitus (T1D) with the consequent failure to restrain blood glucose levels. PSCs differentiated to β-cells offer a great restorative hope for transplantation in T1D or for diabetes-related drug discovery (Hanley, 2014). Generating differentiated PSCs relies on an understanding of the developmental processes, and assessing their utility as potential therapeutics requires a detailed knowledge of the equivalent cells *in vivo*. In contrast to T1D, type 2 diabetes (T2D), the most prevalent form of the disorder, is typically characterized by a progressive failure of β-cells to meet the body's demands for insulin. Genome-wide association (GWA) studies have identified massive numbers of sequence variants across the human genome that associate with T2D (Mahajan et al., 2014). Important functional attributes have been ascribed to some of these variants in adult β-cells (Pasquali et al., 2014). However, for at least some loci, GWA signals may relate to developmental events, when sufficient functional β-cells must be generated for postnatal metabolic control (Travers et al., 2013). Indeed, some of the variants are located close to genes known to be monogenic causes of diabetes when mutated. These genes tend to encode transcription factors that regulate pancreas development in other species (Vaxillaire et al., 2012; Rubio-Cabezas and Ellard, 2013; Flanagan et al., 2014; Rubio-Cabezas et al., 2014; Schwitzgebel, 2014). Moreover, it is now apparent that developmental pathology can be associated with alterations in regulatory elements outside gene coding sequences (Weedon et al., 2014). Such elements are less well conserved than the coding sequences of the genes they regulate; thus, inference from other species is not straightforward and, in part, our knowledge has come through modeling pancreas development in differentiating human PSCs (Weedon et al., 2014). Taken together, these factors make it timely to review what we know directly about human pancreas development as well as the developmental disorders associated with its defective development or dysfunction.

Timeline and regulators of human pancreas development
Pancreas specification and morphogenesis

Human embryogenesis spans from fertilization to approximately 8 weeks post-conception (wpc), after which time, the embryo is typically referred to as a fetus. Embryonic development is classified into the 23 different Carnegie Stages (CS) (O'Rahilly and Müller, 2010) (Table 1). The continuum of development is (somewhat artificially) divided into individual stages by morphology. Post-implantation this includes early internal discriminators visible under light microscopy, such as numbers of paired somites. Thereafter, greater reliance is placed on external features, such as the detail of the developing limbs or the appearance of retinal pigmentation. Thus, human embryogenesis is staged by maturity and only by extension by time [e.g. 'days post-conception' (dpc)]; this is subtly

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Table 1. Stages of human embryonic development, key features and estimation of equivalent timeline of mouse development

Human embryonic stage	Approximate days post-conception (dpc)	Examples of morphological features	Key events in human embryonic pancreas development	Approximate equivalent stage of mouse development*
CS9	22-26	Up to 3 somite pairs, largely unfolded endoderm	Prior to formation of AIP	E7.5-E8
CS10	25-27	4-13 somite pairs, rostral and caudal neuropore	Folded endoderm with narrowed communication with yolk sac creating the AIP, notochord adjacent to foregut endoderm	E8-E8.5
CS11	27-29	13-20 somite pairs, rostral neuropore closing, looping of the heart tube		E8.5-E9
CS12	29-31	Lens and otic placodes, caudal neuropore closing, 1st-3rd pharyngeal arches	First detection of PDX1 in presumptive pancreatic endoderm	E9-E9.5
CS13	30-33	Early sign of upper limb bud	Clear dorsal and ventral pancreatic buds	E9.5-E10
CS14	33-35	Upper and lower limb buds clearly visible		E10-E11.5
CS15	35-37	Hand plate now visible		E11.5-E12.25
CS16	37-40	Clear retinal pigment, auricular hillocks, foot plate visible	Growth of organ and proliferation of multipotent pancreatic progenitors	E12.25-E12.75
CS17	39-42	Digital rays first visible in hand plate		E12.75-E13.25
CS18	42-45	Digital rays first visible in foot plate		E13.25-E14
CS19	45-47	Clearly notched hand plate	Distinction possible between central trunk cells and peripheral tip cells, e.g. GATA4 levels	E14-E14.5
CS20	47-50	Clearly notched foot plate, webbed fingers, scalp vascular plexus visible		E14.5-E15
CS21	49-52	Visible fingers, webbed toes, scalp vascular plexus halfway up head	Onset of detection of *NEUROG3* and first detection of insulin-positive cell (i.e. signs of endocrine commitment)	E15-E15.5
CS22	52-55	Scalp plexus two-thirds of the way up head, separated fingers	Ventral bud largely rotated around the gut and becomes opposed with the dorsal bud	E15.5-E16
CS23	53-58	Scalp vascular plexus at top of head, separated toes		E16-E16.5

Carnegie stages (CS) are shown with their estimates of corresponding days post-conception (dpc) adapted from O'Rahilly and Muller (2011) and the UNSW Human Embryo Resource (https://embryology.med.unsw.edu.au/embryology/index.php/Embryonic_Development). Human embryogenesis spans the first 8 weeks of development prior to the fetal period. Counting somite pairs by light microscopy becomes more difficult after CS11. AIP, anterior intestinal portal. *The estimation of mouse development relates broadly to the Carnegie stage, not the key event in human pancreas development.

different from model species such as mouse, for which staging classifications are prominent but embryogenesis is most commonly described as time measured 'post-coitus' or 'post-fertilization'. In some countries and under legislation, ethical review and codes of practice, human embryology can be studied during two phases: first, over a 14-day period following fertilization in culture *in vitro* (pre-implantation embryos), and second under informed consent from women who have chosen on clinical grounds to terminate their pregnancy (post-implantation embryos). This legal framework for pre-implantation study and the time that must elapse to notice deferred menstruation and to confirm pregnancy post-implantation carries two inevitable consequences. Direct study of human gastrulation is impossible and researching early organogenesis is exceptionally limited. Despite this, some insight into early human pancreas formation has been achieved.

In mouse, the flat sheet of endoderm folds to form the primitive gut tube, which is divided into foregut, midgut and hindgut (Jørgensen et al., 2007; Pan and Wright, 2011). The anterior endoderm invaginates to form the anterior intestinal portal (AIP), which marks the foregut-midgut boundary, and is the site of pancreas specification [which occurs at embryonic day (E) 7.5 in mouse]. Pancreas induction occurs at the equivalent place and is very similar, if perhaps slightly delayed, in human embryos (Fig. 1; Table 1). At the corresponding stage, CS9 in humans, the definitive endoderm still communicates with the visceral endoderm of the yolk

sac (Jennings et al., 2013). Endodermal folding (and thus formation of the AIP) is only apparent a stage later, at CS10 (corresponding to approximately E8-E8.5 in mouse). Nevertheless, as in other species, the resulting human foregut endoderm lies adjacent to the notochord (Sadler, 2000) (Fig. 1). From studies in other species, we know that the notochord patterns the adjacent foregut to develop into the dorsal pancreatic bud by excluding sonic hedgehog (SHH), which allows expression of the key transcription factor pancreatic and duodenal homeobox factor 1 (PDX1) (Jørgensen et al., 2007; Pan and Wright, 2011). In human embryos, SHH can be detected at CS10 and PDX1 at CS12. Thus, although the timing may be slightly later, these data suggest that this aspect of human pancreatic patterning is very similar to that in other mammalian species (Fig. 2). By contrast, there has been no detection of early pancreatic endocrine differentiation (the so-called 'primary transition' stage of mouse pancreatic development) in human embryos (Villasenor et al., 2008; Jennings et al., 2013), perhaps owing to a relative lack of proximity of the paired dorsal aortae to the early pancreatic endoderm, limiting the opportunity for the early pro-endocrine patterning that has been observed in mouse and chick (Lammert et al., 2001; Bonal and Herrera, 2008).

The ventral and dorsal pancreatic buds at CS13 are marked by the transcription factors SRY (sex-determining region Y)-box 9 (SOX9), PDX1 and GATA binding protein 4 (GATA4) (Piper et al., 2004; Jennings et al., 2013), all of which are required for
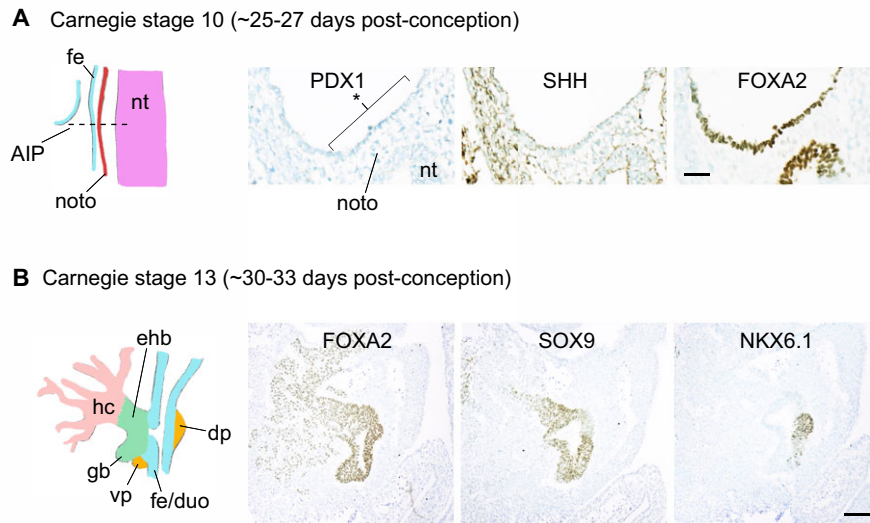
A Carnegie stage 10 (~25-27 days post-conception)

B Carnegie stage 13 (~30-33 days post-conception)

Fig. 1. Human embryonic pancreas specification. (A) Carnegie stage 10. The schematic (sagittal) shows the proximity between the dorsal wall of the foregut endoderm (fe; blue) and the notochord (noto; red) at the level of the anterior intestinal portal (AIP; dashed line), which allows patterning of that region of dorsal foregut endoderm to a future pancreatic fate. The bright-field immunohistochemistry images show transverse sections at this level of the AIP for PDX1, SHH and FOXA2. FOXA2 marks the foregut endoderm and anterior neural tube (nt). PDX1, the key pancreatic transcription factor, is not yet expressed; however, the asterisked area in the PDX1 panel shows the region of presumptive pancreatic endoderm where SHH is already excluded. (B) Early Carnegie stage 13. The schematic (sagittal) shows the first signs of budding of the dorsal pancreas (dp; yellow) from the foregut endoderm (fe)/early embryonic duodenum (duo). By this stage, the liver bud has already extended ventrally as hepatic cords (hc; pink), the extrahepatic biliary duct (ehb; green) and gall bladder (gb; green). The immunohistochemistry sections (sagittal) show staining for FOXA2 (marks all of the structures), SOX9 (most clearly marks the foregut/duodenum, gallbladder and part of the extrahepatic biliary duct) and NKX6.1 (marks endocrine competent endoderm of the dorsal pancreatic bud). The lack of clearly visualized ventral pancreatic bud (vp; yellow) in the immunohistochemistry sections is due to the plane of tissue sectioning. Counterstaining is with Toluidine Blue. Scale bars: 50 µm (A); 100 µm (B).

human pancreatic growth (Stoffers et al., 1997; Piper et al., 2002; Shaw-Smith et al., 2014). The dorsal bud at CS13 is also characterized by the appearance of microlumens (Jennings et al., 2013): the first sign of a developing luminal network by which acinar secretions will eventually drain into the intestine (Kesavan et al., 2009; Villasenor et al., 2010). Although the process of epithelial branching and polarity has not been elucidated fully during human development, data suggest that the interaction between regulators of neural migration (such as netrins) and components of the extracellular matrix (such as integrins) plays an important role in pancreatic endoderm cell adhesion and migration (Yebra et al., 2003, 2011). For the remainder of the embryonic period, the human pancreas undergoes a large expansion of proliferative progenitor cells. In human, unlike in mouse, Nirenberg and Kim homeobox factor (NKX) 2.2 (NKX2-2 – Human Gene Nomenclature Database) protein is not detected in these cells (Jennings et al., 2013). Differences in pancreatic progenitor cells become noticeable by CS19 when SOX9$^+$/NKX6.1$^+$ (NKX6-1 – Human Gene Nomenclature Database) central duct-like structures ('trunks') have less GATA4, whereas the more peripheral clustered cells ('tips') are SOX9$^+$/GATA4$^+$/NKX6.1$^+$ (Fig. 2). By 10 wpc, these pro-acinar 'tip' cells no longer contain NKX6.1 (Jennings et al., 2013). This division is reminiscent of the segregation of the acinar cell compartment in mouse (Esni et al., 2004; Solar et al., 2009; Schaffer et al., 2010) and is also similar in that it initiates prior to the major wave of endocrine differentiation. However, in human, complete resolution of the transcription factor profiles, for instance by the loss of SOX9 from the acinar cells, is delayed (between 10 and 14 wpc) (Jennings et al., 2013) compared with mouse, in which SOX9 is lost promptly from the peripheral tip cells (Schaffer et al., 2010).

Several groups have also shown that over time the developing human pancreas becomes less proliferative and that the remaining proliferation is more peripherally located away from the central trunk regions, as in other species (Polak et al., 2000; Piper et al., 2004; Sarkar et al., 2008). The developmental potential of human pancreatic progenitors from the end of embryogenesis/early fetal period was demonstrated clearly by Scharfmann and colleagues who transplanted the whole organ prior to significant endocrine differentiation (at ~8 wpc) under the kidney capsule of adult severe combined immunodeficiency (SCID) mice. They observed huge growth of pancreas containing all cell lineages, including islets that were capable of rescuing murine hyperglycemia (Castaing et al., 2001, 2005). Interestingly, through the use of bromodeoxyuridine (BrdU) labeling and *in vivo* incubation in the mice, they showed that acini seemed to develop clonally from acinar progenitors (i.e. cells that already contained carboxypeptidase A remained proliferative). By contrast, although the early PDX1$^+$ pancreatic progenitor cells were proliferative, once cells had undergone β-cell differentiation replication rates were greatly reduced (Castaing et al., 2005). More recently, Scharfmann and colleagues have used the same murine *in vivo* incubation technique of human fetal progenitor cells as part of a protocol to achieve the exciting development of a glucose-sensitive human β-cell line (Ravassard et al., 2011; Scharfmann et al., 2014).

Compared with research in other species, it has been challenging to gain insight into the signaling pathways regulating human pancreas development. In significant part this is due to the major challenge of establishing primary models of human pancreatic progenitors amenable to long-term culture and adaptation. In trying to delineate the precise signaling pathways that regulate, at least in part, the effects of peri-pancreatic mesenchyme on pancreatic growth, Scharfmann and colleagues used a combination of experiments in human and mouse to establish a role for fibroblast growth factor (FGF) signaling, most likely FGF1, FGF7 and FGF10 acting via the FGF receptor subtype 2B (which binds all three of these FGF isoforms) (Elghazi et al., 2002; Ye et al., 2005). These
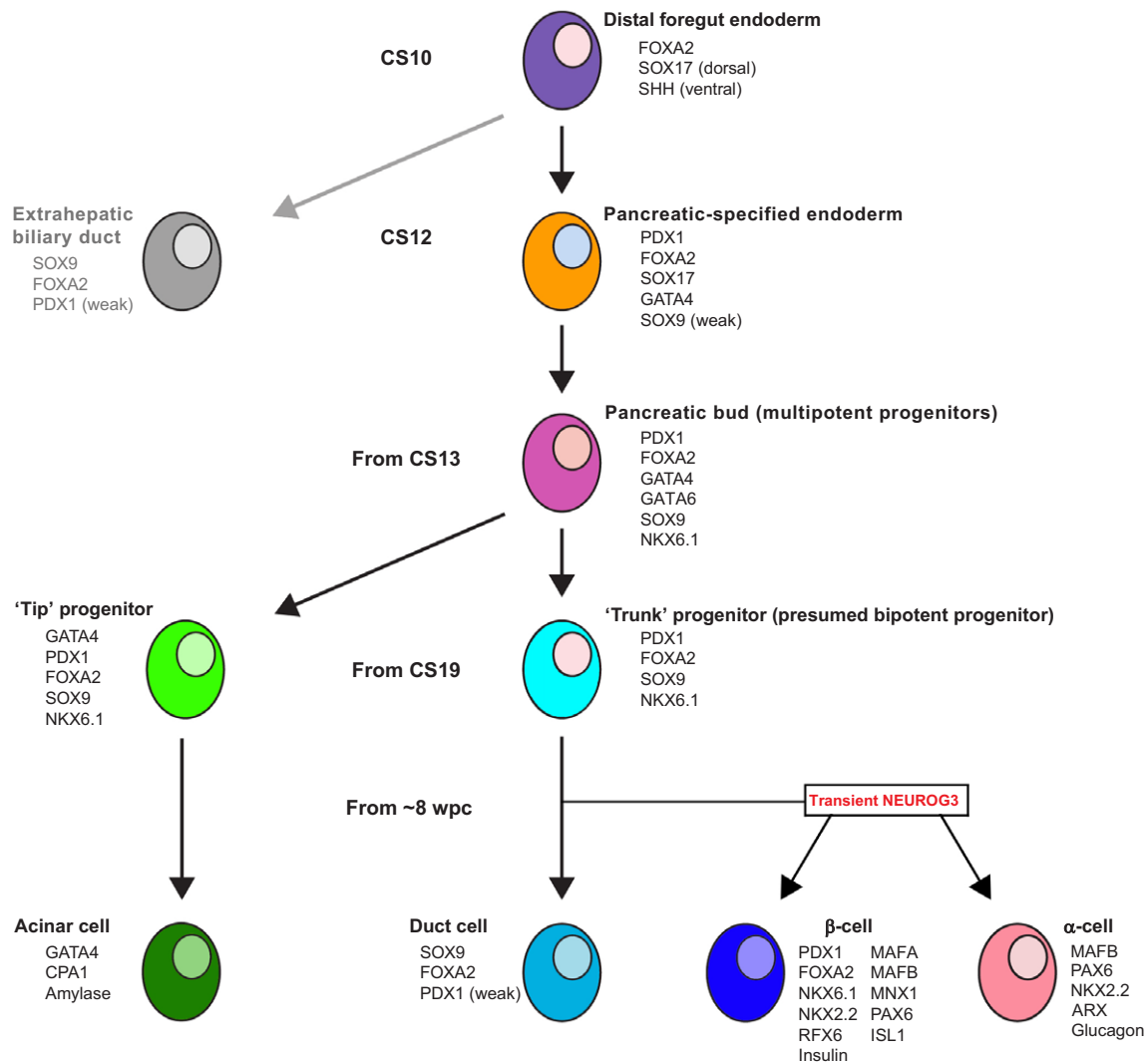
Fig. 2. Transcription factor network of human pancreas development. Transcription factors and other key markers that identify different cell types and stages during early pancreas specification and subsequent lineage commitment. Only dorsal pancreas specification is shown for simplicity (markers for the extrahepatic biliary duct are in gray). Factors highlighted are based on data from immunohistochemical studies of human pancreas development (see main text for details).

data are consistent with the concept that, in mouse, Fgf10 is not required for initial bud formation but is important for the proliferation of subsequent progenitor cells (Bhushan et al., 2001). By comparative transcriptomics, gene ontology analysis highlighted enrichment for components of WNT signaling in human pancreatic progenitors at CS16-CS18, prior to significant acinar cell differentiation and before noticeable endocrine differentiation (Cebola et al., 2015). These data align with those from mouse (Rodríguez-Seguel et al., 2013) and, most recently, with results from an exciting new methodology for long-term culture of human pancreatic progenitors showing positive effects of WNT, FGF10 and epidermal growth factor (EGF) signaling on cell proliferation (Bonfanti et al., 2015). Alongside this pro-proliferative maintenance of progenitor status, EGF also inhibited endocrine differentiation (Bonfanti et al., 2015). To date, the role of Notch and retinoic acid signaling in promoting progenitor cell proliferation, or bone morphogenetic protein (BMP) signaling in inhibiting it, has not been directly analyzed during human embryogenesis, although these factors are known to be important in the mouse (Jørgensen et al., 2007; Pan and Wright, 2011).

Endocrine differentiation

The transcription factor neurogenin 3 (Neurog3) is transiently required for the commitment of mouse progenitor cells within the central duct-like structures to an endocrine cell fate (Gradwohl et al., 2000; Schwitzgebel et al., 2000; Gu et al., 2002) (Fig. 2). During human development, NEUROG3 expression increases rapidly immediately after the embryonic period timed with the appearance of fetal β-cells, which are the first and most predominant islet cell-type to appear in human development (Piper et al., 2004; Lyttle et al., 2008; Piper Hanley et al., 2010; Jennings et al., 2013). SOX9 is absent in cells with robust NEUROG3 levels and is not detected in endocrine cells thereafter, although it persists in pancreatic duct cells (Jennings et al., 2013). There is some additional detection of both SOX9^{+}/ NEUROG3[+(weak)] cells and hormone[+]/NEUROG3[+(weak)] cells, consistent with the same transient role for NEUROG3 in human pancreas as in mouse (Lyttle et al., 2008; Jeon et al., 2009; Piper Hanley et al., 2010; Jennings et al., 2013). β-cell clusters are well vascularized by 10 wpc, and at 12-13 wpc islets are apparent that contain α-cells, β-cells, δ-cells and γ-cells (Piper et al., 2004;

Jennings et al., 2013). NEUROG3 detection peaks around the end of the first trimester and was not detected in human fetuses after 35 wpc (Salisbury et al., 2014). By additional inference from experiments transplanting human fetal pancreas into mouse, NEUROG3 is probably switched off at some point after 26-28 wpc (Capito et al., 2013). Along with data demonstrating minimal amplification of the pool of native human β-cell precursors post-NEUROG3 expression (Castaing et al., 2005), coupled to data in human PSCs implying that human endocrine differentiation relies completely on NEUROG3 (McGrath et al., 2015), this implies that human β-cell allocation *in utero* is completed at least 5 weeks, and more likely 3 months, prior to term. This is important because it implies that fetal β-cell mass thereafter is a reflection of β-cell proliferation versus apoptosis, rather than further specification of new β-cells.

The hypotheses connecting suboptimal development of β-cell mass as a fetus and the future risk of T2D have received much attention over the last two decades (Hales and Barker, 1992; Hattersley and Tooke, 1999). Studying the offspring from the Dutch winter famine of 1944-1945 demonstrated that later impairment in insulin secretion and glucose intolerance, but not resistance to insulin action, in non-diabetic individuals correlated to under-nutrition up to but not after 32 weeks gestation (de Rooij et al., 2006), a very similar time period to the profile of NEUROG3 expression (Salisbury et al., 2014). Therefore, one would predict that at least some GWA signals for T2D might relate to events during pancreas development, such as progenitor cell proliferation or β-cell differentiation, rather than to functional attributes of adult β-cells. One notable example is the *KCNQ1* locus. The two independent regions of association in this locus are unusual in lying in an imprinted region on 11p15.5 where risk is only apparent when maternally inherited (Kong et al., 2009). Gloyn, Travers and colleagues studied methylation patterns in human fetal pancreas during the period of endocrine differentiation and in adult human islets (Travers et al., 2013). Although numbers of fetal samples were relatively limited, they demonstrated that *KCNQ1* and its overlapping transcript were monoallelically expressed only in the fetal samples. In fact, the risk may relate to the function of the nearby gene *CDKN1C*, which was monoallelic in both fetal and adult samples, but this study opens up the possibility that at least some T2D risk variants relate specifically to events during human pancreatic development. This possibility warrants further exploration, especially if risk loci could be identified where associated genes are expressed in the fetal pancreas but not adult β-cells. At the moment, lead single nucleotide polymorphisms (SNPs) from comprehensive T2D meta-analyses fall close to a number of genes with key roles in pancreas development, such as *PDX1*, *HNF1B*, *HNF4A*, *GLIS3*, *HHEX*, *NOTCH2* and *PROX1* (Mahajan et al., 2014) (Table 2), or with broader regulatory roles in organ development, such as *HMGA2* (Ashar et al., 2010).

Mapping human PSC differentiation onto human pancreas development

There is great interest in generating functional human β-cells *in vitro* for potential transplantation or drug development in diabetes. Since the first experiments pursuing pancreatic differentiation with human PSCs ~15 years ago (Assady et al., 2001), there has been a convergence of differentiation protocols during the last decade around the pioneering publications of Baetge and colleagues (D'Amour et al., 2005, 2006; Kroon et al., 2008; reviewed by Docherty et al., 2007; Pagliuca and Melton, 2013). Although these protocols generated pancreatic progenitors, the differentiated

endocrine cells tended to produce both glucagon and insulin. This has been taken as a sign of immaturity, as has also been noted for other foregut endoderm derivatives (Baxter et al., 2015). Indeed, Melton and colleagues identified transcriptional similarities between PSC-derived β-cells and human fetal β-cells, which may explain a relative lack of glucose responsiveness (Hrvatin et al., 2014). Although the human fetal β-cell secretes insulin, and has shown some glucose responsiveness during the first trimester (Otonkoski, 1988; Otonkoski et al., 1988), fetal glucose levels are thought to be regulated normally by maternal glucose transfer. The relative immaturity of PSC-derived β-cells may also reflect the lack of an appropriate developmental niche containing the necessary signaling factors for pancreatic cell differentiation (Pagliuca and Melton, 2013), consistent with the greater maturity achieved following *in vivo* incubation in mouse compared with terminal differentiation in the dish (Kroon et al., 2008). This issue of immature β-cell differentiation has been at least partially redressed by two groups reporting improved protocols within the last year or so (Pagliuca et al., 2014; Rezania et al., 2014). The improved protocols are remarkably similar, with a particularly extensive analysis provided by Kieffer and colleagues (Rezania et al., 2014). These advances, allied to the comprehensive work of others, make it worthwhile trying to correlate their latest seven-stage protocol (Fig. 3) with what we know about human pancreas development.

Prior to the specification of pancreatic endoderm (stage 4 in the protocol), little can be inferred directly from studies of human embryogenesis. After this, expression profiles from human samples support the use of SHH inhibitors such as SANT to encourage pancreatic endoderm formation from its foregut precursor (D'Amour et al., 2005, 2006; Jennings et al., 2013; Pagliuca et al., 2014; Rezania et al., 2014). Use of FGFs, BMP inhibitors such as Noggin or LDN, and retinoic acid (RA) to direct foregut differentiation and pancreatic specification (stages 3 and 4) is untested in human embryos but consistent with studies in other species (Bhushan et al., 2001; Elghazi et al., 2002; Stafford and Prince, 2002; Ye et al., 2005; Wandzioch and Zaret, 2009; Guo et al., 2013; Pagliuca et al., 2014; Rezania et al., 2014). The pancreatic endoderm cells generated by Rezania et al. (2014), and those from a more recent report (Russ et al., 2015), were notable for their transcription factor profile of PDX1, NKX6.1 and SOX9 but relative lack of NKX2.2, mimicking human pancreas development (Jennings et al., 2013) (Fig. 3). The protocol from Rezania, Kieffer and colleagues also used three-dimensional culture, potentially allowing closer mimicry of *in vivo* development (Rezania et al., 2014). However, although it potentially accelerated the appearance of NEUROG3 (stage 5), it made no overall difference to the efficiency and eventual maturity of β-cell differentiation from human PSCs (McGrath et al., 2015). An interesting addition to mature endocrine progenitors into more functional β-cells (stages 6 and 7) was supraphysiological thyroid hormone, tri-iodothyronine (T3). Although T3 is known to advance liver maturity, there is no evidence that it affects pancreas development in human embryos; congenital hypothyroidism, a relatively common pediatric disorder that can exert hugely detrimental effects on neural development, does not overtly impact on glucose homeostasis.

The relevance of endocrine cells containing both insulin and glucagon in PSC protocols remains unclear. The latest methods have improved significantly to generate a much higher proportion of monohormonal cells – a key advance (Pagliuca et al., 2014; Rezania et al., 2014). This was most likely achieved by promoting the production of MAFA, a crucial β-cell transcription factor, in the nuclei of maturing β-cells (Rezania et al., 2014), which occurred

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Table 2. Factors affecting human pancreas development

Gene	Mutation	Phenotype/syndrome	Expression during human development	References
PDX1	Homozygous single nucleotide deletion/point mutations	PNDM; pancreatic agenesis/hypoplasia	Detected in presumptive pancreatic endoderm, multipotent pancreatic progenitors, β-cells (and duct cells)	Stoffers et al., 1997; Schwitzgebel et al., 2003; Piper et al., 2004; Thomas et al., 2009; Jennings et al., 2013
PTF1A	Homozygous nonsense, insertion and frameshift/alterations in a regulatory element 25 kb 3′ of the gene	PNDM; pancreatic and cerebellar agenesis/hypoplasia/isolated pancreatic agenesis	Detected as transcripts in multipotent pancreatic progenitors	Sellick et al., 2004; Weedon et al., 2014
GATA4	Deletion leading to haploinsufficiency; missense	PNDM or childhood-onset DM; variable exocrine insufficiency with congenital heart malformations and developmental delay	Detected in multipotent progenitors; pro-acinar 'tip' cells at CS19 and differentiated acinar cells	Jennings et al., 2013; Shaw-Smith et al., 2014
GATA6	Haploinsufficiency/heterozygous	PNDM; pancreatic agenesis; variable exocrine insufficiency with congenital heart, gastrointestinal and biliary tract malformations	Detected as transcripts in multipotent pancreatic progenitors	Decker et al., 2006; Lango Allen et al., 2011; Bonnefond et al., 2012; Cebola et al., 2015
NEUROG3	Homozygous or heterozygous point mutations	PNDM, malabsorptive diarrhea (absence of enteroendocrine cells); childhood-onset DM (hypomorphic)	Detected during late embryogenesis (CS20-CS21); peak expression at end of the first trimester; loss of detection in the third trimester	Wang et al., 2006; Pinney et al., 2011; Rubio-Cabezas et al., 2011; Salisbury et al., 2014
GLIS3	Homozygous frameshift; homozygous partial deletions	PNDM, congenital hypothyroidism, congenital glaucoma, polycystic kidneys	Detected as transcripts in multipotent pancreatic progenitors	Senee et al., 2006; Dimitri et al., 2011; Cebola et al., 2015
PAX6	Compound heterozygous	PNDM, microphthalmia, congenital hypopituitarism, cerebral malformation	Detected in hormone-positive cells from 10 wpc	Lyttle et al., 2008; Solomon et al., 2009
MNX1	Homozygous missense	PNDM, sacral agenesis, lung hypoplasia	Detected as transcripts in pancreas from 7 wpc; peak detection between 12 and 18 wpc	Jeon et al., 2009; Bonnefond et al., 2013; Flanagan et al., 2014
RFX6	Homozygous missense, frameshift and splicing; compound heterozygous	PNDM; pancreatic hypoplasia, intestinal atresia, gallbladder hypoplasia, diarrhea	Detected as transcripts in pancreas from 8 wpc; peak detection between 19 and 21 wpc	Mitchell et al., 2004; Smith et al., 2010
NEUROD1	Homozygous frameshift	PNDM, cerebellar hypoplasia, sensorineural deafness, myopia and retinal dystrophy	Detected in hormone-positive cells from 10 wpc	Rubio-Cabezas et al., 2010
NKX2.2	Homozygous nonsense, frameshift	PNDM, developmental delay, hypotonia, hearing impairment, cortical blindness	Not detected as protein in embryonic pancreas prior to endocrine differentiation; restricted to β-cells from 10 wpc	Lyttle et al., 2008; Jennings et al., 2013; Flanagan et al., 2014
HNF1B	Heterozygous frameshift	Spectrum from monogenic diabetes to pancreatic agenesis (head and tail), polycystic kidneys, dysgenetic gonads	Detected as transcript in multipotent pancreatic progenitors	Haumaitre et al., 2006
SOX9	Haploinsufficiency	Pancreatic hypoplasia with small, disordered islets; skeletal dysplasia, male-to-female sex reversal	Detected in multipotent pancreatic progenitors; excluded from robustly stained NEUROG3+ cells; becomes restricted to duct cells	Piper et al., 2002; Piper Hanley et al., 2010; Jennings et al., 2013
NEK8	Homozygous, nonsense	Absent pancreatic islets, reduced exocrine tissue and cystic ducts; cystic kidney and liver disease	Not yet examined in humans	Frank et al., 2013
NPHP3	Homozygous splice, nonsense	Variable pancreatic phenotype includes cystic dysplasia; cystic kidney disease, hepatobiliary ductal plate malformations	Not yet examined in humans	Bergmann et al., 2008; Frank et al., 2013
UBR1	Homozygous missense	Congenital exocrine insufficiency (due to exocrine destruction), nasal wing aplasia, developmental delay	Not yet examined in human development; detected in cytosol of postnatal exocrine cells	Zenker et al., 2005; Zenker et al., 2006

following the addition of an inhibitor to the AXL receptor tyrosine kinase, in combination with T3 and inhibition of TGFβ type 1 receptor kinase (ALK5; TGFBR1 – Human Gene Nomenclature Database). In addition, the antioxidant *N*-acetyl cysteine, in high doses, promoted MAFA nuclear localization (Fig. 3, stage 7) (Rezania et al., 2014). More recently, the generation of bihormonal cells *in vitro* has been associated with the use of BMP inhibitors during pancreatic specification (Russ et al., 2015). However, it is
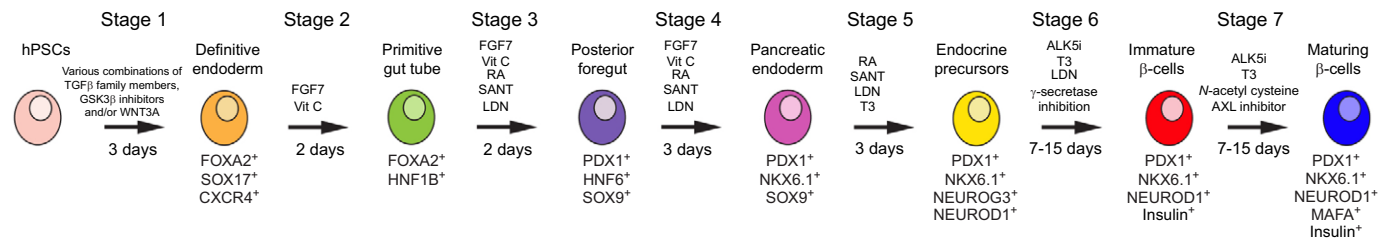
Fig. 3. *In vitro* differentiation of human PSCs (hPSCs) towards pancreatic β-cells. Schematic overview of the stages of *in vitro* pancreatic differentiation using recently published, improved protocols (Pagliuca et al., 2014; Rezania et al., 2014). Key molecular markers of each stage are highlighted, as are the factors used at each stage to direct differentiation along the appropriate route. SANT, Hedgehog signaling antagonist; LDN, BMP type 1 receptor inhibitor; T3, triiodothyronine; ALK5i, ALK5 inhibitor; Vit C, vitamin C.

perhaps an over-simplification to regard these insulin$^+$/glucagon$^+$ dual-stained cells as an *in vitro* artifact; they are a normal aspect of human fetal development (Polak et al., 2000; Piper et al., 2004; Sarkar et al., 2008; Jeon et al., 2009; Riedel et al., 2012; Riopel et al., 2014). The frequency of their detection has been strikingly variable, from ∼5% to 92% of endocrine cells, perhaps owing to differing sensitivity of immunohistochemistry protocols across different groups or variations in the age of material studied. Interestingly, Kieffer and colleagues have characterized these native fetal cells in greater detail (Riedel et al., 2012). Their abundance appeared to decline progressively from the onset of endocrine differentiation at 8 wpc and they were barely detected in adult pancreas. Although the dual-stained cells expressed the α-cell transcription factor ARX, they lacked β-cell ones, PDX1, NKX6.1 and MAFA, consistent with the *in vitro* PSC-derived bihormonal cells, which seem capable of forming monohormonal glucagon-positive cells upon transplantation (Kelly et al., 2011; Rezania et al., 2013). However, coupled with data from mouse, there is no evidence that the native bihormonal cells are the natural forerunner of any mature type of endocrine cell and, indeed, data imply the contrary (Herrera, 2000). Their eventual fate is unclear. Perhaps there is some redundancy in human fetal development, a natural tolerance to a few meaningless cells destined to die off; or perhaps these cells possess a hitherto unappreciated function, potentially secreting other factors or hormones. What is clear is that the best current PSC-derived monohormonal β-cells are PDX1$^+$, NKX6.1$^+$ and MAFA$^+$. However, they are not perfect insulin-secreting cells: it is not yet clear that insulin secretion stops in response to low blood glucose at the same thresholds as in normal β-cells, a vital requirement for clinical transplantation (Hanley, 2014), and only a minority of cells demonstrated appropriate calcium and incretin responses (Rezania et al., 2014). Thus, although considerable progress has been made towards *in vitro* generation of fully functional β-cells, this challenge has not yet been met and perhaps there are further lessons to be learned from human development to achieve this goal.

Developmental disorders of the human pancreas

Mutations, mostly homozygous, in a range of transcription factors are known to compromise human pancreas development and can cause permanent neonatal diabetes mellitus (PNDM) in a manner broadly consistent with findings from targeted disruption in mouse. PNDM tends to associate with low birth weight, presumably reflecting inadequate secretion of insulin, a major growth factor, from fetal β-cells. Known causes of PNDM are considered here as each of them almost certainly impacts on development from progenitor cells, not just the function of terminally differentiated β-cells, and at least some data are available directly from studies of human pancreas development (Table 2). A number of other conditions are also

highlighted in which pancreas development is affected. Other causes of monogenic diabetes, including transient neonatal diabetes, are covered expertly elsewhere (Flechtner et al., 2008; Vaxillaire et al., 2012; Rubio-Cabezas and Ellard, 2013; Flanagan et al., 2014; Rubio-Cabezas et al., 2014; Schwitzgebel, 2014).

Pancreas agenesis or hypoplasia

The first gene identified for which an inactivating mutation caused agenesis of the pancreas was *PDX1* (Stoffers et al., 1997). The affected neonate, with a homozygous point mutation causing a frame shift, showed signs of exocrine pancreas deficiency as well as hyperglycemia requiring insulin. The phenotype is entirely consistent with the complete pancreatic regression noticed after initial bud formation in *Pdx1*-null mice and with data in human embryos showing that PDX1 expression defines the earliest pancreatic endoderm and that PDX1 is expressed sequentially in the emergent buds and later multipotent progenitors (Piper et al., 2004; Lyttle et al., 2008; Pan and Wright, 2011; Jennings et al., 2013). Subsequently, other *PDX1* alterations have been identified (Schwitzgebel et al., 2003; Thomas et al., 2009), including one case with only a mild deficit in exocrine pancreas function alongside PNDM, presumably due to a hypomorphic mutation (Nicolino et al., 2010).

The second gene noted to cause clinical pancreatic agenesis was pancreas specific transcription factor 1a (*PTF1A*). PTF1A is a basic helix-loop-helix (bHLH) transcription factor that forms part of the PTF complex. Originally described for its role in murine exocrine differentiation (Krapp et al., 1996), Wright and colleagues demonstrated a more fundamental role of *Ptf1a* in early pancreas specification from foregut endoderm (Kawaguchi et al., 2002). In human, a mutation in *PTF1A* resulting in truncation of the C-terminal region was identified in a case of pancreatic and cerebellar agenesis (Sellick et al., 2004). Interestingly, pancreatic agenesis without cerebellar abnormalities has recently been attributed to alterations in a region 25 kb downstream of *PTF1A* (Weedon et al., 2014). This element was bound by the endoderm-restricted factor PDX1 *in vitro* in PSC-derived progenitor cells, presumably helping to account for the pancreas-specific phenotype and lack of a cerebellar defect. It will be interesting to study this element further in native human embryonic pancreas. Indeed, although transcripts have been detected, the profile of PTF1A protein has been difficult to define precisely in developing human pancreas owing to a lack of suitable antibodies.

GATA4 and GATA6 are important members of the GATA family of zinc-finger transcription factors that regulate cell differentiation and proliferation in a number of endodermal organs in mouse, including the pancreas (Ketola et al., 2004; Decker et al., 2006; Carrasco et al., 2012; Xuan et al., 2012). Investigation of GATA6 in human development has been limited, with its expression

throughout the pancreatic progenitor cell population having been described at a single time period, CS16-CS18 (Cebola et al., 2015). GATA4 is first detected within the foregut endoderm at CS12 (Jennings et al., 2013). It is expressed in early pancreatic progenitors, but at and after CS19 its detection becomes more restricted to the peripheral 'tip' cells (Jennings et al., 2013). It later localizes to the CPA1^{+} acinar cells, similar to findings in mouse (Decker et al., 2006). In 2011, haploinsufficiency of *GATA6* in humans was discovered to be the most common cause of pancreatic agenesis and hypoplasia (Lango Allen et al., 2011). Most of the cases showed cardiac malformations and other less frequent abnormalities of the gastrointestinal system. Interestingly, recent ChIP-seq data indicates that PDX1 and GATA6 are commonly found in close proximity at active enhancers, implying coordinated regulation of human pancreas development (Cebola et al., 2015). However, not all patients with GATA6-inactivating mutations causing congenital heart disease show pancreatic agenesis and diabetes, and not all mutations arise *de novo* in the affected individual, implying some variability in the clinical phenotype (Bonnefond et al., 2012). In the developing lung, *GATA6* expression is directly downstream of *HMGA2*, the nominated gene for a lead SNP in a T2D GWA study (Singh et al., 2014). Two manuscripts reported conditional inactivation of *Gata6* and *Gata4* in mouse and the data imply a degree of redundancy between the two factors (Carrasco et al., 2012; Xuan et al., 2012). Redundancy seems less apparent in human, although it could account for those cases of *GATA6* inactivation without diabetes mellitus (DM). Indeed, for a while it seemed surprising that *GATA4* had not been identified in association with human pancreas agenesis or hypoplasia. However, several cases of *GATA4* mutation resulting in PNDM and at least some degree of exocrine insufficiency have more recently been described (Shaw-Smith et al., 2014).

Heterozygous mutations in hepatocyte nuclear factor 1B (HNF1B) cause early-onset diabetes, polycystic kidneys and a range of other genitourinary abnormalities. The pancreas is characteristically hypoplastic (Haumaitre et al., 2006), consistent with findings in mouse (Haumaitre et al., 2005). However, recently, complete pancreatic agenesis has been observed in some cases at post-mortem examination (Body-Bechou et al., 2014), demonstrating a spectrum between hypoplasia and complete agenesis, similar to that observed for GATA6 (Bonnefond et al., 2012). Ivemark syndrome features cystic dysplasia of the pancreas, kidney and liver amongst other abnormalities. The disorder is recognized as a ciliopathy with homozygous mutations identified in *NEK8* and nephrocystin-3 (*NPHP3*; also known as nephronophthisis 3) (Bergmann et al., 2008; Frank et al., 2013). Indicative of variability, in some patients with *NPHP3* mutations the pancreas was normal on post-mortem examination and/or showed normal endocrine or exocrine function (Bergmann et al., 2008). By contrast, affected fetuses from a consanguineous kindred with *NEK8* mutations showed more severe pancreas disorganization when examined post-termination in the second trimester of pregnancy (Frank et al., 2013). The pancreatic acini were under-developed with loss of the normal branched appearance and islets were not detected (Frank et al., 2013). In one case, the ducts were cystic. Molecularly, NPHP has been postulated to control the switch between canonical and non-canonical WNT signaling (Bergmann et al., 2008). It is also known to interact with NEK8, which has been linked with both WNT signaling and also in regulating the activity of TAZ, coactivator along with YAP of TEA domain (TEAD) transcription factors, the nuclear effectors of the Hippo signaling pathway (Frank et al., 2013). As TEAD has recently been revealed as a core regulator of human pancreas development,

this provides an exciting putative link to human pathology (Cebola et al., 2015).

Alagille syndrome is caused by defects in the Notch signaling pathway. Amongst foregut endoderm derivatives, biliary abnormalities predominate, but rare cases have been described that also showed exocrine pancreas insufficiency (Krantz et al., 1997). Recently, this proportion has been revised upwards to include perhaps 40% of cases, based on an observable benefit from pancreatic supplementation; cases of DM have also been noted (Turnpenny and Ellard, 2012). One effector of Notch signaling is SOX9 (Pritchett et al., 2011). The first indication that SOX9 is required for pancreas development and growth came from studies in human embryonic and fetal material, and from post-mortem material in campomelic dysplasia (a skeletal disorder), caused by haploinsufficiency of *SOX9* (Piper et al., 2002). This leads to death soon after birth, as well as male-to-female sex reversal (46,XY disorder of sex differentiation). The early lethality has precluded insights into whether permanent neonatal diabetes might ensue. Consistent with the profile of SOX9 expression in human pancreatic progenitors, in cases of campomelic dysplasia the entire pancreas was hypoplastic with disordered, small islets (Piper et al., 2002), very similar to the consequences of homozygous inactivation in mouse (Seymour et al., 2007).

Hypoplasia of the exocrine pancreas is also observed in Johanson-Blizzard syndrome owing to inactivating mutations in *UBR1* (Zenker et al., 2005, 2006). However, rather than being a problem of developmental biology, the pathology relates to severe pancreatitis that has a remarkably early onset during the intrauterine period.

Other causes of permanent neonatal diabetes reflecting abnormal differentiation of β-cells and other islet cell types

The link between cystic kidney disease and neonatal diabetes is extended by *GLIS3*, a downstream target of HNF1B that encodes a Kruppel-like zinc-finger transcription factor. Inactivating mutations in *GLIS3* cause neonatal diabetes, cystic renal disease, liver fibrosis, congenital hypothyroidism and other abnormalities (Senee et al., 2006). In mouse, GLIS3 is required for pancreatic duct morphogenesis and the generation of NEUROG3^{+} cells, and also functions in differentiated β-cells to regulate insulin transcription (De Vas et al., 2015). Indicating a more restricted phenotype compared with mutations in *HNF1B*, exocrine pancreas development appeared normal in both patients and mice with *GLIS3* inactivation (Senee et al., 2006; De Vas et al., 2015).

Homozygous inactivating mutations in *NEUROG3* can cause neonatal diabetes, which has been attributed to a lack of β-cell differentiation (Wang et al., 2006; Pinney et al., 2011; Rubio-Cabezas et al., 2011). This phenotype is also accompanied by a striking absence of intestinal endocrine differentiation resulting in congenital diarrhea. Although clinical endoscopic biopsy has allowed immunohistochemical analyses of the intestinal phenotype, the pancreatic phenotype has been debated. The first patients identified showed a complete lack of enteroendocrine cells on intestinal biopsy but diabetes did not develop until the age of 8 years, implying the differentiation and function of at least some β-cells (Wang et al., 2006). Functional modeling of the R93L and R107S *NEUROG3* mutations in chick endoderm provided evidence that the affected allele was hypomorphic rather than fully inactivated (Jensen et al., 2007). Interestingly, this implies that intestinal endocrine differentiation in human is more sensitive to loss of NEUROG3 function than the corresponding pancreatic process. NEUROG3-independent routes of pancreatic endocrine differentiation seem unlikely based on recent differentiation

studies of human PSCs in which the *NEUROG3* gene had been excised by recombination (McGrath et al., 2015). Moreover, inactivating mutations of *NEUROG3* have resulted in permanent diabetes at 5 months of age in an affected infant born small for gestational age (implying insulin deficiency *in utero*) (Pinney et al., 2011), and biallelic inactivation of *NEUROG3* resulted in permanent neonatal diabetes (Rubio-Cabezas et al., 2011). Surprisingly for an endocrine commitment factor, some signs of exocrine inadequacy were observed in one case (low serum amylase and low fecal elastase levels) (Pinney et al., 2011).

Inactivating mutations in *RFX6* underlie the syndrome of PNDM, small bowel atresia and gall bladder hypoplasia (Smith et al., 2010). Despite the widespread disruption in differentiation from foregut endoderm, pancreatic exocrine function was normal, albeit with a slight reduction in pancreatic size. In addition to a failure of β-cell differentiation, α-cells and δ-cells were also missing. *RFX6* transcripts were detected progressively in normal human fetal pancreas from the end of embryogenesis, similar to the onset of *NEUROG3* and insulin transcription (Lyttle et al., 2008; Smith et al., 2010; Jennings et al., 2013). More recently, RFX6 has been shown to regulate components in the insulin secretory pathway in differentiated β-cells (Chandra et al., 2014; Piccand et al., 2014).

Downstream of NEUROG3, four other transcription factors have been identified as causative for PNDM when mutated: NEUROD1 (Rubio-Cabezas et al., 2010), NKX2.2 (Flanagan et al., 2014), PAX6 (Solomon et al., 2009) and MNX1 (Bonnefond et al., 2013; Flanagan et al., 2014). The first three factors are expressed in human β-cells from early fetal development onwards (Lyttle et al., 2008; Jennings et al., 2013), with *NEUROD1* and *NKX2.2* considered to be direct targets of NEUROG3 action (Sussel et al., 1998; Gradwohl et al., 2000). Interestingly, inactivation of *Mnx1* in mouse causes dorsal pancreas agenesis because of deficiencies in the peri-pancreatic mesenchyme (Harrison et al., 1999; Li et al., 1999). However, in human, inactivating mutations in *MNX1* cause permanent neonatal diabetes, to date without any evidence of exocrine pancreas failure, implying the primary site of dysfunction is more restricted to the β-cell (Bonnefond et al., 2013; Flanagan et al., 2014).

Conclusion: towards comprehensive, dynamic maps of how human pancreas development is regulated

Until now, studies of human development have tended to be small scale and user defined; attempts at higher throughput analysis have been most likely confounded by cell heterogeneity. With new technologies based on large-scale parallel sequencing, this is changing rapidly. Not only have studies on PSC-derived pancreatic progenitors emerged, but the first transcriptome of native human embryonic pancreatic progenitors is now available (Cebola et al., 2015). Coupled with computational analysis, this has already given strong clues that at least one signaling pathway, WNT signaling, important for pancreas development in other species, is similarly active in human pancreatic progenitors, and has directly demonstrated a new pathway regulating pancreatic growth centered on the function of TEAD and YAP. We can anticipate more comprehensive 'regulome' data defining active and repressed enhancers (based on histone marks) and DNA methylation status, both compared with other human embryonic organs and also over time to produce a truly comprehensive, dynamic genomic atlas of human pancreas development to complement what is already known in adult islet cells (Morán et al., 2012; Bramswig et al., 2013; Pasquali et al., 2014). Such data should set the scene for determining the relevance of human pancreas development and

β-cell differentiation to the wider array of GWA sequence variants associated with T2D, complementing the functional annotation available for adult β-cells (Pasquali et al., 2014). It is possible that research over the next few years will prove that several aspects of T2D risk relate firmly to molecular events during *in utero* development. With the increasing ability to perform analyses at the level of single cells, we can also expect to gain insight into the degree of heterogeneity among progenitors and, for instance, whether this influences their subsequent differentiation. All of this will highlight far more accurately where we are at with the latest human PSC differentiation protocols (Pagliuca et al., 2014; Rezania et al., 2014) . In turn, the protocols can then be used for the validated interrogation of precise aspects of human pancreas development and, using new genome-editing technologies, the roles of particular factors (McGrath et al., 2015). Collectively, it is an exciting period for studies of human developmental biology.

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