Generation of a human foetal pancreatic organoid model of pancreas development

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Author: Michael R Elsy

School of Medical Sciences

Division of Diabetes, Endocrinology and Gastroenterology

Contents

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Preface:

All research was conducted by Michael Elsy as part of the MPhil in Regenerative Medicine programme. Primary supervision was provided by Professor Neil Hanley and Dr Rachel Jennings, as well as secondary supervision by Dr Karen Piper Hanley and Professor Alberto Saiani. The report will follow a traditional Master's thesis style.

Declaration:

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Abstract:

Introduction: Diabetes mellitus (DM) represents a significant global health and economic burden, and its incidence is increasing. Although injectable insulin is the gold standard for Type 1 DM management, it confers life-long dependence and hypoglycaemia may instead occur. Therefore, restoration or regeneration of damaged insulin-producing β-cells could provide a cure. Despite their immense potential, human pluripotent stem cell (PSC)-derived β-cells currently do not have the appropriate functionality or maturation capability compared to *bona fide* β-cells. A greater understanding of β-cell specification during early human development is required for safe transplantation of PSC-derived β-cells. Organoid systems refer to clusters of multiple cell types in 3-dimensions (3D) that can self-organise, allowing mimicry of the cell-cell contacts and signalling events of the native tissue environment. Human foetal pancreatic organoids are emerging as exciting models for pancreas development, enhancing our understanding of hPSC-derived β-cell differentiation protocols, and providing opportunities for disease modelling.

Aims and Objectives: This project aims to establish and maintain a 3D organoid model of human pancreas development using foetal tissue. Light sheet fluorescence microscopy (LSFM) will be optimised as a method of imaging key pancreatic transcription factors (TFs) throughout pancreas morphogenesis.

Methods: Human foetal pancreata were dissected and dissociated into single-cell suspension before incubation with Matrigel. Organoids were cultured for up to 30 days before analysis. Following incubation, organoids were assessed by immunofluorescence. For comparison, immunohistochemistry and LSFM were performed on native embryonic or foetal pancreata.

Results: Immunohistochemistry reveals the spatial and temporal expression profiles of key pancreatic TFs in embryonic and foetal pancreata. Distinct 3D ductal and islet structures were observed in foetal samples using LSFM. The expression of these TFs characterises our human foetal pancreatic organoids. Ductal, PPC and endocrine markers are detected in the organoid system, as represented by CK19, SOX9 and NKX2.2 expression, respectively.

Discussion and conclusions: Pancreas forms when foregut endoderm is specified to pancreatic endoderm, followed by PPCs and then acinar or bi-potent ductal and endocrine populations, including β-cells. 3D organoid culture of foetal pancreas permits expansion of multipotent PPCs, which can be used as a platform for modelling downstream endocrine differentiation. LSFM enables the rapid imaging of whole foetal pancreata and organoids; the localisation and expression of candidate PPC regulators could be investigated using LSFM. The surrounding niche and growth factor environment could also be manipulated, enabling study of their effects on endocrine (and β-cell) differentiation in both foetal pancreas organoids and PSC-derived pancreatic organoids. In summary, the establishment of this pancreas organoid system is a valuable asset for furthering our knowledge of pancreas development and endocrine commitment.

Introduction:

What is the impact of diabetes mellitus?

Diabetes mellitus is a chronic condition that affects the production of insulin from β-islet cells in the pancreas, an organ located adjacent the stomach with endocrine and exocrine function (Gerich *et al.*, 1976; Pfeifer *et al.*, 1981). Insulin is the main anabolic hormone in the body, regulating glucose and fatty acid uptake into cells to maintain blood sugar homeostasis (Balcerczyk *et al.*, 2019). There are two main categories of diabetes: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM); gestational diabetes also occurs during pregnancy, increasing both maternal and offspring susceptibility to obesity and T2DM (Reece, 2010). The dogma for T1DM is that pancreatic β-cells are destroyed by the body's own immune cells, resulting in an absolute insulin deficiency and prevention of glucose absorption into cells (Daneman, 2006). Although there is also β-cell dysfunction in end-stage T2DM, T2DM has traditionally been characterised by peripheral insulin resistance (Holman *et al.*, 2008; Stumvoll *et al.*, 2005). Repeated exposure to high blood glucose concentrations decreases insulin receptor sensitivity in adipose, muscle and liver cells (Mackenzie and Elliott, 2014; Stumvoll *et al.,* 2005).

Both types of diabetes are metabolic diseases that cause elevated blood glucose concentrations, otherwise known as hyperglycaemia. These abnormally high glucose levels are inducive to increased formation of toxic advanced glycated end products, which promote dysfunctional growth factor (GF) signalling, aberrant protein kinase C (PKC) activation and the production of harmful reactive oxygen species (ROS) (Koya and King, 1998; Negre-Salvayre *et al*., 2009; Vlassaria, 1992). Therefore, chronic hyperglycaemia can lead to damage of blood vessels, nerves and almost all cell types (Long and Dagogo-Jack, 2011). This damage can ultimately result in vision loss via retinopathy (Stitt *et al.*, 2016), cardiovascular disease (CVD) and cardiac failure (Laakso, 1999; Singh *et al.*, 2018), kidney dysfunction (Middleton *et al.*, 2006), strokes (Ergul *et al.*, 2009), and even lower limb amputation due to infected foot ulcers (Noor *et al*., 2015).

Recent years have seen diabetes be described as a "global epidemic", with incidences increasing drastically (Kolb and Mandrup-Poulsen, 2010; Oputa and Chinenye, 2012). For example, there was a stark increase in diabetes cases from 108 million in 1980 to 422 million in 2014 (NCD Risk Factor Collaboration, 2016). Currently 463 million people have diabetes worldwide; this number is estimated to increase to 700 million by 2045 (Saeedi *et al*., 2019). The majority of cases (90%)

are accounted for by T2DM, with T1DM occurring in the remaining 5-10% of cases (Tuomilehto, 2013). T2DM usually develops in adults over the age of 45, whereas T1DM commonly affects children and younger adults, albeit both diseases can manifest in any age group (American Diabetes Association, 2019). The global economic cost of the two combined diseases was approximately US$1·31 trillion in 2015 (Bommer *et al.*, 2017); the immense socioeconomic burden of diabetes highlights the need to develop new treatments.

T1DM management – β-cell replacement therapy:

Although many genetic and environmental factors are thought to play a role in T1DM progression, there are no current preventative treatments (Atkinson *et al.*, 2014; Pociot and Lernmark, 2016; Rewers and Ludvigsson, 2016). Injectable insulin remains the gold standard for T1DM management, offering a life-saving method of restoring blood glucose concentrations to healthy levels (euglycaemia). However, insulin administration confers life-long dependence and some patients experience hypoglycaemia instead (Cryer, 2009; International Hypoglycaemia Study Group, 2015). In these patients, daily insulin injections heighten the risk of excessive glucose uptake into cells, decreasing blood glucose concentrations to dangerous levels. These hypoglycaemia-associated autonomic failure (HAAF) events can then result in seizures, loss of consciousness or even hypoglycaemia-induced comas (Bolo *et al.*, 2011; Cryer, 2012; Cryer, 2013; McAulay *et al.*, 2001; Seaquist *et al.*, 2013). Insulin analogues, insulin pumps or continuous glucose monitoring (CGM) can somewhat alleviate iatrogenic hypoglycaemic events, but HAAF may still occur and patients remain insulin dependent for life (Mathieu *et al*., 2017; Pickup, 2018; Scheiner *et al.*, 2009; Vashist, 2013). Therefore, the transplantation or regeneration of β-cells has emerged as one of the best options for the treatment of T1DM absolute insulin deficiency over the past decade. Recent advances have made the prospect of a cure for T1DM ever closer (Ellis *et al.*, 2017; Tan *et al.*, 2019).

Cadaver-derived islet transplantation:

Islet transplantation using cadaver-derived islets has developed considerably since the first implants were performed in rodents to successfully reverse diabetes, and in dogs to improve immunosuppressive regimens throughout the late 1970s and 1980s (Ballinger and Lacy, 1972; Horaguchi and Merrell, 1981; Rajotte *et al.*, 1983). After the initial success in animal models, there were complications in the translation of islet cell therapies to in-man studies (Bottino *et al.*, 2018; Najarian, 1977; Sutherland *et al*., 1980). Some improvements in glucose control were observed following

implantation in humans, with increased C-peptide indicating elevated insulin secretion (Sutherland *et al*., 1980). However, there were issues of immune rejection and poor glycaemic management (Bottino *et al.*, 2018; Sutherland *et al.*, 1980). Shapiro *et al.* (2000) pioneered the proof-of-concept Edmonton Protocol for harvesting islet cells, increasing efficacy, and making the procedure feasible in humans. Donor islet cells are implanted into the liver through a catheter leading from the stomach and into the portal vein, with the patient only requiring local anaesthetic (Shapiro *et al*., 2000).

Cadaver-derived islet transplantation has produced promising results using the Edmonton protocol. The implanted islet cells remove patients' dependence on insulin injections to an extent. There are multiple reports of reduced HbA1c, increased insulin secretion represented by higher C-peptide levels and reduction in diabetic retinopathy progression (Barton *et al.*, 2012; Emamaullee *et al.*, 2019; Hering *et al.*, 2016; Posselt *et al*., 2010; Ricordi *et al*., 2016; Shapiro *et al.*, 2017; Warnock *et al*., 2008). All of these factors contribute to a reduction in iatrogenic hypoglycaemic events, which continues despite islet graft survival failure (Warnock *et al*., 2008). Between 2000 and 2017, more than 1500 patients were successfully treated by cadaver derived islet transplants, highlighting this method as a routine, safe treatment for patients with unstable T1DM or poor hypoglycaemic awareness (Shapiro *et al.*, 2017). Although some patients require a return to insulin therapy in between additional islet cell infusions, the number and rate of these infusions decreased considerably between 2007-2010, compared to 1999-2006 (Barton *et al.*, 2012). Shapiro *et al.* (2017) also reported complete insulin independence in up to 50% of patients 5 years post-transplantation. 73% experience euglycemia following insulin re-administration after 10 years. Therefore, clinical islet transplantation has been suggested as a better long-term glycaemic control method, compared to current insulin administration techniques, for patients predisposed to hypoglycaemic events (Bellin *et al.*, 2012; Berney *et al.*, 2009; Shapiro *et al.*, 2017).

Disadvantages of Cadaver-derived islet transplantation:

Nevertheless, islet cell transplantation is not without shortcomings. Islet function will vary considerably between patients, due to a variety of factors: genetic variance, age and health of the donor, time between extraction and transplantation, and spoiling during transport (Lyon *et al.*, 2016; Russ *et al*., 2015). Moreover, life-long immunosuppressive routines are required that are accompanied with many side effects (Colegio and Billingsley, 2011; Hirshberg *et al.*, 2003; Kuschel *et al*., 2006; Ojo *et al*., 2003). In addition, the procedure of obtaining β-cells is itself complex. For

instance, some patients still experience renal dysfunction and the yield, and potency, of β-cells is markedly lower than living cells. Only 50% of extracted β cells are appropriate for clinical use (Shapiro, 2011). Finally, two pancreas donors are often needed to generate the approximate 10,000 islet cells/kg body weight ratio required for graft survival and efficacy (Warnock *et al.*, 2008); there may be long delays for treatment availability due to ever-worsening organ donor shortages.

PSC-derived β-cells emerging as new T1DM therapies:

Resultantly, pluripotent stem cell (PSC)-derived β-cells have become a very appealing alternative that may revolutionise T1DM treatment, improving patient welfare drastically. Protocols for differentiating pluripotent stem cells (PSCs) into β-cells have improved substantially in recent years, but challenges for generating fully functional β-cells with correct glucose responsiveness and mono-hormonal insulin production remain (Hanley, 2014; Hrvatin *et al.*, 2014). Therefore, human foetal development can be turned to as a means of revealing the main transcription factors (TFs) and signalling pathways active during key pancreas developmental checkpoints, facilitating the improvement of protocols (Jennings *et al.*, 2015). The effect of the extracellular matrix (ECM) and three-dimensional (3D) microenvironment on stem cell and foetal tissue differentiation has been emerging as an exciting area of research, which could further aid generation of functional β-cells (Bonnans *et al.*, 2014; Greggio *et al.*, 2013). Organoid culture of stem cells or foetal tissue may provide the correct mechanical and chemical stimuli to enable natural pancreas development, β-cell maturation and drug modelling of new therapeutics (Grapin-Botton, 2016).

Human development provides clues for PSC endocrine commitment:

Pancreas structure and function:

The pancreas is not limited to controlling blood glucose levels: exocrine and endocrine cells have distinct functions. The exocrine pancreas consists of ductal cells assisting fluid drainage and acinar cells secreting a range of enzymes, including lipase, protease and amylase, and bicarbonate into the duodenum to aid micronutrient digestion (Figure 1B) (Domínguez-Muñoz and Cigarrán, 2018). Enzyme-mediated digestion of nutrients subsequently stimulates gut-incretin hormone production by enteroendocrine L-cells, which increases insulin sensitivity (Tasyurek *et al*., 2014). The endocrine system maintains blood glucose homeostasis through 5 specific subsets of cells within the islets of Langerhans (Laguesse, 1893), releasing hormones to either positively or negatively regulate glucose metabolism (Da Silva Xavier, 2018). When blood glucose concentrations are low, α-cells produce glucagon to promote the release of glucose from liver cells following glycogenolysis and gluconeogenesis, as well as the inhibition of insulin production (Gustavson *et al*., 2003; Ramnanan *et al*., 2011). Conversely, β-cells secrete insulin to decrease blood glucose levels when they are above a healthy threshold (Rutter *et al.*, 2015). Other islet cells include: δ-cells, producing somatostatin to inhibit insulin and glucagon production (Huising *et al.*, 2018; Rorsman and Huising, 2018); ε-cells, secreting ghrelin (Heller *et al.*, 2005); and γ cells, which release pancreatic polypeptide (PP) to increase satiety signals, prevent insulin in response to glucose and protect β-cells against apoptosis (Goldstone, 2006; Khan *et al.*, 2017). Ghrelin-producing ε-cells are mainly present during foetal development (Arnes *et al.*, 2012; Suissa *et al.*, 2013). There is a dramatic decrease in ε-cells postnatally in mice and humans (Andralojc *et al.*, 2009; Heller *et al.*, 2005). This is likely due to them functioning as multipotent pancreas endocrine progenitors that give rise to α, γ and even β-cells (Arnes *et al*., 2012).

The importance of human foetal pancreas research:

Most of our understanding of pancreas morphogenesis and development arises from the easily accessible and more simple vertebrate models, primarily rodents, *Xenopus* (African clawed frog) and zebrafish (Pearl *et al.*, 2009; Singh *et al*., 2017; Slack, 1995; Zorn and Wells, 2009). Pancreas research in large animals, such as dogs and non-human primate models, or humans have been more limited due to high maintenance fees, low sample availability and ethical concerns (Bakhti *et al.*, 2019). Despite some similarities and usefulness as models for T1DM pathology, fundamental differences between murine and human pancreas development have

been identified (Jennings *et al.*, 2013; Jennings *et al*., 2017; Jennings *et al*., 2020). Other models do not fully recapitulate the complex processes in humans (Pan and Brissova, 2014). Therefore, it is imperative to conduct research with human tissue. Embryos are categorised by Carnegie stages (CS) up to CS23, relating to 8 weeks post conception (wpc), at which point the embryo is classified as a foetus (O'Rahilly and Müller, 2010). Early human pancreas development is reviewed in detail by Jennings *et al*. (2015). Knowledge of the key signalling molecules and transcription factors (TFs) active during these processes is essential for developing PSC differentiation and organoid culture protocols, potentially aiding the generation of functional β-cells ready for transplantation. This information will also further our understanding of monogenic diabetes aetiology.

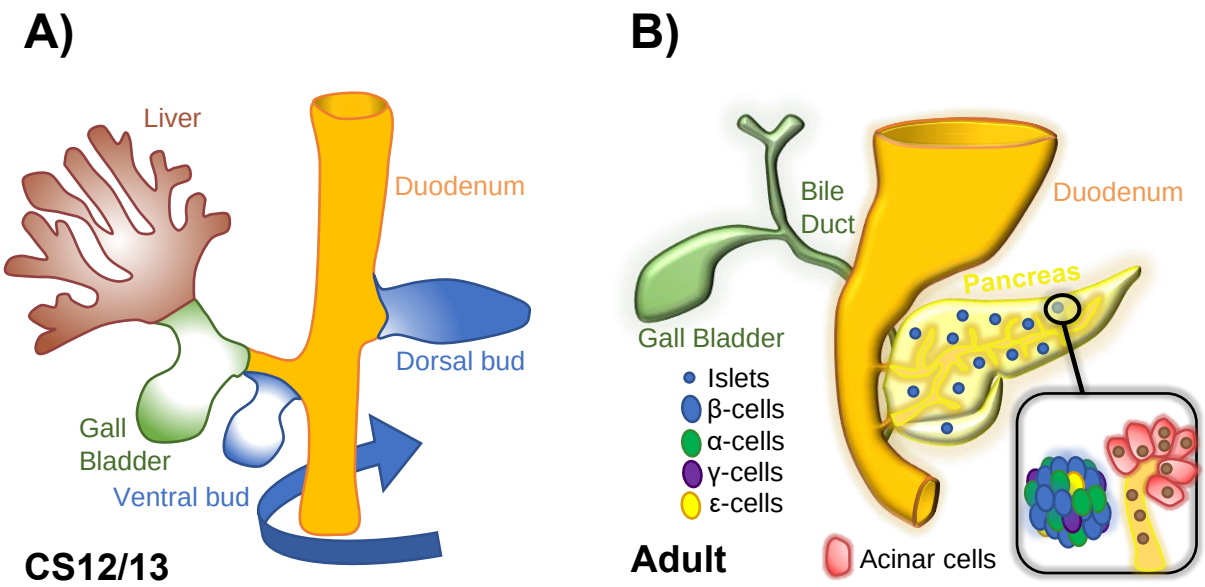


Figure 1. Simplified schematic representing the process for human pancreas formation, from ventral and dorsal bud initiation to fusion. Adapted from Suckale and Solimena (2008)**. A) CS12/13:** Dorsal and ventral buds have emerged from foregut endoderm specified to pancreatic endoderm (PE). Dorsal aorta and notochord, as well as other neighbouring signalling events, commit the dorsal bud; the ventral bud is committed by the vitelline veins and signals from the neighbouring gall bladder and liver region. Blue arrow indicates rotation event that occurs at CS15/16 for the fusion of the ventral and dorsal buds. **B) Adult pancreas:** dorsal and ventral buds have fused. Pancreas head sits in between the C-shape of the duodenum, with the hooked uncinate process apparent. Two ducts are present, with the major duct connecting to the bile duct (green). Islets (α/β/δ/γ/ε endocrine cells)

are represented as blue dots. Exocrine acinar cells located at the end of duct branches secrete digestive enzymes and bicarbonate (represented as brown dots).

Pancreas development summary:

Gastrulation results in the formation of three germ layers: ectoderm, mesoderm, and endoderm. This period of development from 14 dpc was deemed to be a “black box”, with ethical guidelines restricting the study of human gastrulation using *in vitro*-fertilised human embryos (Pera, 2017; Taniguchi *et al.*, 2019). Therefore, alternative vertebrate systems have been used to study human gastrulation; the process is highly conserved across species despite very different embryo structures (Hyun *et al.*, 2016; Petersen *et al.*, 2018). The recent development of human gastruloids, defined as ESCs differentiated into cell aggregates comprising all three germ layers, may also enable further study of human gastrulation (Moris *et al.*, 2020). These *in* vitro systems can now be compared against the *in vivo* single-cell transcriptomic data generated from a CS7 (16-19 dpc) human embryo (Tyser *et al*., 2020).

The germ layers provide the body plan for subsequent organ development (Solnica-Krezel, 2005; Stern, 2004). The ectoderm forms the epidermis and central nervous system, mesoderm forms the notochord, dermis, skeleton, muscle, kidney, heart and blood, and endoderm compromises the gut, liver, stomach, pancreas and lungs (Solnica-krezel and Sepich, 2012). Epiboly describes this process of rearranging cells within the blastocyst into endoderm (Sui *et al.*, 2013). Definitive endodermal (DE) cells form a 2D contiguous sheet, before folding into the primitive gut tube (PGT) and developing into organs. Foregut becomes thyroid, liver, pancreas, stomach and lungs, whilst small-intestine, colon and rectum arise from mid-hindgut endoderm (Zorn and Wells, 2009).

Endoderm is committed to pancreatic tissue, followed by the evagination of dorsal and ventral pancreatic buds in both mice and humans (Figure 1A) (Jennings *et al.*, 2015; Mccracken and Wells, 2012; Pan and Wright, 2011). These buds form at Embryonic day (E) 9 and E9.5 respectively for dorsal and ventral tissue in mice (Pictet *et al*., 1972; Slack, 1995); the dorsal bud predates ventral bud development at approximately 28 days post conception (dpc) in humans (Jennings *et al*., 2013). Pancreatic progenitor populations then drive cells down exocrine (acinar and ductal) or endocrine lineages (islet cells). During this process, the pancreas is formed by the dorsal and ventral buds, located adjacent to the distal foregut endoderm, fusing next to the duodenum (Pan and Wright, 2011). This gut rotation and fusion event happens between E12 and E13 in mice, or CS15 to CS16 in humans (Gittes, 2009;

Jørgensen *et al.*, 2007). The ventral bud forms the uncinate pancreas, a small "hooked" region, and both buds contribute to the pancreas head. The body and tail arise solely from the dorsal bud (Pandol, 2011). Lineage tracing experiments in mice initially allowed detailed tracking of pancreatic cells throughout differentiation and endocrine or exocrine commitment (Gu *et al.*, 2003). Early human developmental cues were not well studied, due to low tissue availability, before Jennings *et al*. (2013) performed a comprehensive review of the key transcription factors active during embryonic and foetal pancreas development (outlined in Figure 2).

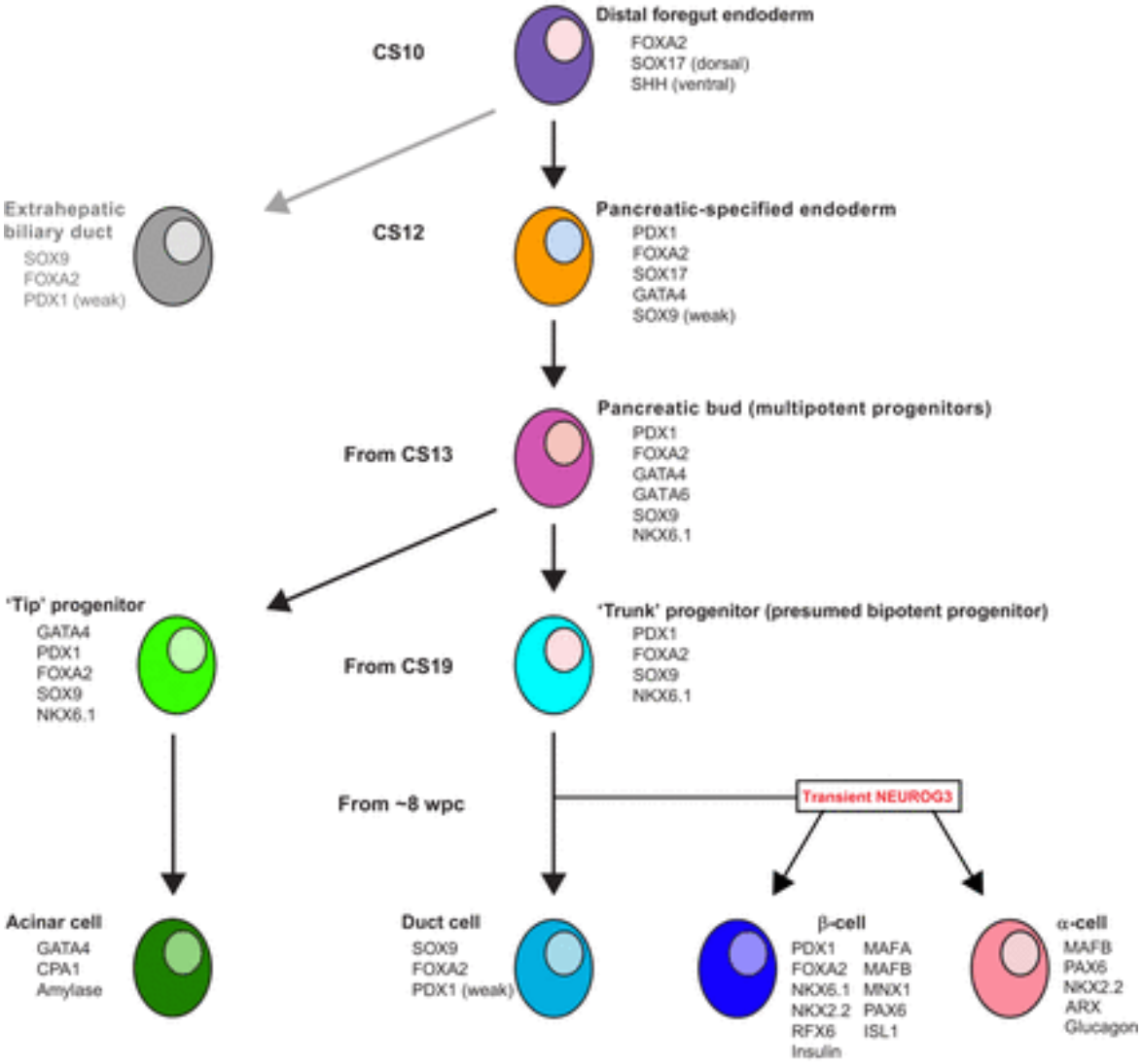


Figure 2. Human pancreas development requires a complex interaction of TFs, as replicated from Jennings *et al*., (2015). This schematic focuses on pancreatic bud progression for simplicity and the relevance to PSC differentiation protocols (Jennings *et al*., 2017). TF temporal and spatial expression are based

upon immunohistochemistry staining (Jennings *et al*., 2013). Foregut cells are specified into definitive endoderm (DE), primitive gut tube (PGT), pancreatic endoderm (PE) and pancreas progenitor cells (PPC), before splitting into ductal or endocrine progenitor cells (EPC), depicted here as “tip” or “trunk” progenitors, respectively. A range of TFs regulate this process. EPCs then differentiate into immature β-cells, or other islet cells, via increased NEUROG3 activity and eventually mature when MAFA is expressed.

Lineage specification in the human embryonic pancreas:

Jennings *et al.*, (2013) provided a crucial insight into how the early human pancreas develops from distal foregut tissue, through to buds and eventually into committed endocrine cells. The report also highlighted how murine and human pancreas development progress via these same spatial stages, but that temporal regulation is different between the two species. Study of the earlier human pancreatic developmental stages had been limited, with only pancreatic and duodenal homeobox 1 TF (PDX1, also referred to as IPF1) and SRY (sex-determining region Y)-box 9 (SOX9) expression being recorded from ~4 wpc by *in situ* hybridisation (Piper *et al.*, 2002; Piper *et al*., 2004). A range of immunohistochemistry (IHC) experiments clearly mapped early TF expression patterns (Jennings *et al.*, 2013).

The initial specification of foregut endoderm occurs due to signals received from the notochord (Hebrok *et al*., 1998; Kim *et al*., 1997), as well as from neighbouring vasculature and the dorsal aorta (DA) (Cleaver and Dor, 2012; Lammert *et al.*, 2001). Foregut endoderm specification is outlined in Figure 3. The formation of the anterior intestinal portal (AIP), or PGT, begins from E8.5 in mice or CS10 (28–30 dpc) in humans (Gittes, 2009; Jørgensen *et al.*, 2007). At CS9 (25 to 27 dpc) the notochord produces sonic hedgehog ventrally (shh), inhibiting PDX1 transcription, along with SOX17 dorsally and FOXA2. Between CS10 and CS12 (29-31 dpc), shh is suppressed by FGF2 and Activinβ2 released from the notochord, as well as increased retinoic acid (RA) activity (Hebrok *et al.*, 1998; Jennings *et al*., 2013; Pan and Wright, 2011). Release of vascular endothelial growth factor (VEGF) by the DA also induces PDX1 expression dorsally in mice (Pan and Wright, 2011). Therefore, there is a loss of PDX1 inhibition from CS11 (Piper *et al*., 2004); PE is specified by PDX1, FOXA2, SOX17, GATA4 and low SOX9 expression (Figure 2). PE specification appears conserved between mice and humans (Jennings *et al.*, 2015). From CS13 (30–33 dpc), Nirenberg and Kim homeobox factor 6.1 (*NKX6.1*) is induced, determining multipotent pancreatic progenitor cells (PPCs). *GATA6* is also now expressed and SOX9 activity is increased, whilst *SOX17* expression is lost

(Jennings *et al.*, 2013). Microlumens are first detectable, predating the formation of the branched ductal-acinar network for fluid drainage (Villasenor *et al.*, 2010). During the bud fusion event between CS15 (35–37 dpc) and CS16 (37–40 dpc), there is wide expression of *PDX1*, *NKX6.1*, *SOX9* and *FOXA2* (Jennings *et al*., 2013). Figure 1A represents the fusion of ventral and dorsal pancreas buds. In contrast to humans, a “primary transition” driven by *Neurogenin3* occurs in mice between E9.5 and E12.5 (Gu *et al.*, 2003; Villasenor *et al.*, 2008). This is reviewed in detail (Pan and Wright, 2011).

PPCs are then split into bi-potent progenitor populations capable of becoming endocrine or ductal cells from CS19 (45–47 dpc). The expression profile for human development reported in Jennings *et al*. (2013) mirrored that seen in mice (Cleveland *et al.*, 2012; Schaffer *et al*., 2010; Solar *et al.*, 2009; Zhou *et al*., 2007); cells still expressing GATA4 differentiate into “tip” PPCs located on the periphery of the branched pancreas, or those without GATA4 become central “trunk” PPCs. Both “tip” and “trunk” progenitors retain PDX1, FOXA2, SOX9 and NKX6.1 production in humans. Instead, SOX9 is lost immediately from mouse “tip” PPCs (Schaffer *et al*., 2010). The same pattern of TF expression is observed at CS21 but carboxypeptidase A1 (CPA1) is also produced in “tip” cells, indicating the start of differentiation into acinar cells (Jennings *et al.*, 2013). Approximately 20 days later at 10 wpc, CPA1+/GATA4+ acinar cells now completely lack NKX6.1 expression, whilst retaining some SOX9 expression (Jennings *et al.*, 2013). The same expression pattern is observed in mice too (Decker *et al.*, 2006). By 14 wpc, terminal differentiation has occurred with a mutual expression between GATA4 and SOX9 becoming apparent. For example, ductal and centroacinar cells producing SOX9 are located in the middle, whereas GATA4-positive acinar cells are at the periphery (Jennings *et al*., 2013). Peripheral “tip” cells have a greater proliferative capacity compared to the central trunks in both mice and humans, which is lost as the pancreas develops (Piper *et al.*, 2004; Sarkar *et al.*, 2008). This is consistent with SOX9/PTF1a co-localising in pancreas progenitor cells at the periphery from the second trimester (Villani *et al.*, 2019).

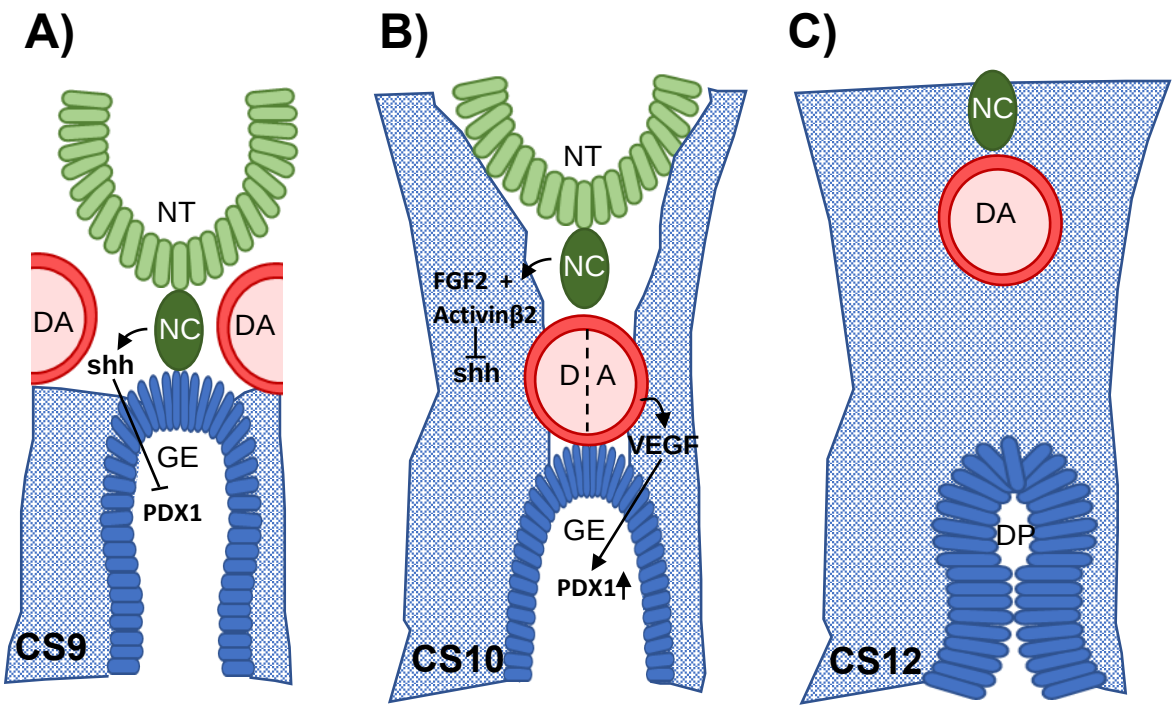


Figure 3. Simplified schematic representing human foregut initiation at the dorsal pancreatic bud, as adapted from (Slack, 1995)**. A) Between CS9 and 10:** PDX1 expression is inhibited in the gut endoderm (GE) by sonic hedgehog (shh) produced by the notochord (NC) adjacent to the neural tube (NT). The dorsal aorta remains separate. Hashed blue indicates mesenchyme. **B) CS10:** FGF2 and activinβ2 production by the NC inhibits shh, enabling PDX1 induction. Dorsal aorta (DA) fusion is complete, as represented by the dotted line, separating the GE from NC spatially. Vascular endothelial growth factor (VEGF) is released, promoting PDX1 expression. **C) CS12:** dorsal pancreatic bud (DP) begins to form due to increased PDX1 expression. Increased mesoderm formation distances the DP from the DA and NC.

<u>Endocrine commitment – formation of β-cells:</u>

NEUROG3 has been identified as the driver of endocrine differentiation and islet cell formation in both mice and humans (Gradwohl *et al.*, 2000; Gu *et al.*, 2002; Jennings *et al.*, 2013; Piper Hanley *et al.*, 2010; Schwitzgebel *et al.*, 2000; Villasenor *et al.*, 2008). NEUROG3 is expressed from ~8 wpc in humans and is accompanied by a large increase in *INS*, *NKX2.*2, *NKX6.*1, *PDX1* and *ISL1* expression, as well as a complete removal of SOX*9*, in β-cells (Jennings *et al.*, 2013). Resultantly, neonatal diabetes can be caused by homozygous inactivating mutations in NEUROG3 dramatically reducing β-cell number (Pinney *et al.*, 2011; Wang *et al.*, 2006). Therefore, endocrine differentiation of PSCs *in vitro* or PPCs *in*

vivo does not occur unless NEUROG3 is active (McGrath *et al*., 2015; Wang *et al.*, 2006). NEUROG3 regulates formation of all endocrine cells across the population, but individual NEUROG3+ cells are restricted to becoming one endocrine cell type (Desgraz and Herrera, 2009). The exact mechanisms of NEUROG3-mediated endocrine commitment are not well known, although lineage-tracing experiments have started to elucidate the TF network involved (Helman and Melton, 2020).

β-cells are the first islet cell to develop (Lyttle *et al*., 2008). Glucagon, somatostatin and PP production indicate the presence of other islet cells from 10-14 wpc (Jennings *et al*., 2013; Lyttle *et al.*, 2008). As ductal cells do not produce NEUROG3, they maintain *SOX9* expression, along with *FOXA2* and *PDX1* (Jennings *et al.,* 2013). Endocrine cells form in mice following a secondary transition with *Neurog3* between E13 and E13.5 (Gradwohl *et al*., 2000; Gu *et al.*, 2002; Gu *et al*., 2003; Schwitzgebel *et al.*, 2000). Endocrine islet clusters form when cells delaminate from the ductal progenitor epithelial layer and migrate via epithelial-mesenchymal transition (EMT) into surrounding mesenchyme, as initiated by Neurog3 (Bechard *et al.*, 2016; Gouzi *et al*., 2011). *Snail2* is upregulated in *Neurog3*$^{+}$-endocrine cells, accompanied by downregulation of *E-cadherin* (*ECAD*). Increased *N-cadherin* expression is not observed (Rukstalis and Habener, 2007). This is unlike other organ development, suggesting regulation by alternative mechanisms (Bakhti *et al.*, 2019).

Differences between human and mouse pancreas development:

Although mice function as a cheap, simple model with high conservation to the human genome (70.1% amino acid homology) (Hedrich, 2004; Mouse Genome Sequencing Consortium, 2002), there are also large discrepancies in pancreas development between the two species (Jennings *et al*., 2015). This is evident by the different proportion of β-cells to other islet cells in humans and mice, altered architectures, and mice having two insulin genes, *Ins1* and *Ins2*, in comparison to the one in humans, *INS* (Brissova *et al*., 2005; Mellou*l et al*., 2002). These changes may account for the different insulin release kinetics in humans and mice (Dai *et al*., 2012). There are also key developmental disparities during pancreas progenitor differentiation and endocrine commitment (Jennings *et al.*, 2013; Jennings *et al*., 2015). For example, NUEROG3 induction is monophasic in humans, as opposed to following two temporal waves in mice (Jennings *et al*., 2013; Villasenor *et al.,* 2008). This may be due to the slight differences between human and mouse PE positioning reducing stimulatory signals, such as VEGF, received from the dorsal aorta (Jennings *et al*., 2015; Lammert *et al*., 2001; Pan and Wright, 2011). Nirenberg and

Kim homeobox factor (NKX) 2.2 (NKX2.2) is also observed in mouse PPC populations, but not in humans (Jennings *et al*., 2013). This indicates that NKX2.2 functions downstream of NEUROG3 in humans, but is located upstream in mice (Jennings *et al.,* 2013). Furthermore, there is a delayed reduction in SOX9 expression in human foetal acinar cells, as opposed to a drastic loss in mice (Jennings *et al.*, 2013; Schaffer *et al*., 2010). Moreover, insulin+ endocrine cells first appear in humans, whereas glucagon+ cells develop first in mice (Jeon *et al*., 2009; Pan and Wright, 2011; Piper *et al*., 2004).

Another difference is the roles of GATA4 and GATA6 in pancreas development. Mutations of GATA4 (D'Amato *et al*., 2010) and GATA6 (Allen *et al*., 2012) in humans result in pancreas agenesis (failure of organ development), with GATA6 being an important regulator of pancreas specification and β-cell function (Tiyaboonchai *et al*., 2017). Although GATA4 and GATA6 are essential for PPC maintenance in mice (Xuan *et al.*, 2012), they are more redundant during early mouse pancreas development and GATA6 is more important for acinar differentiation (Villamayor *et al*., 2020). Therefore, future work using human embryonic and foetal tissue is essential for furthering our understanding of pancreas development and endocrine commitment, allowing PSC-derived β-cell differentiation protocols to be improved.

Islet cell architecture:

Acinar and ductal cells of the exocrine system comprise 95% of the pancreas parenchyma, with only 5% filled by clusters of islet cells assorted throughout the tissue (Paris *et al*., 2004). This distribution of islets was widely considered to be random in both mice and humans (Aguayo-Mazzucato *et al.*, 2006; Wang *et al*., 2013), although "uniform scattering" of spherical islets was recently observed in the human pancreas body (Ionescu-tirgoviste *et al.*, 2015). There are approximately 1-3 million islets in the adult human pancreas (Hellman, 1959; Ionescu-tirgoviste *et al*., 2015), albeit some studies have reported greater numbers (Da Silva Xavier, 2018). These disparities are potentially due to methodology alterations. On average 1000 cells form an individual islet, meaning over a billion islet cells are in the pancreas (Cabrera *et al*., 2006; Zhou and Melton, 2018). The human pancreas islet population is primarily comprised of β-cells (~60%), ~30% α-cells, ~5% δ-cells and the remainder consist of ε- and γ-cells (Brissova *et al.*, 2005).

Islet composition and architecture surprisingly varies significantly between human and murine pancreases (Steiner *et al*., 2010); human β-cells are often located on the outside of islet clusters, whereas murine β-cells form a core surrounded by the

remaining islet cell types on the periphery (Brissova *et al*., 2005; Cabrera *et al.*, 2006; Gannon *et al*., 2000; Jeon *et al*., 2009). Human β-cells favour “heterologous cell-cell contacts”, enabling close positing to blood vessels (Bosco *et al*., 2010). This is likely reflected by discrepancies in β-cell electrical activity and insulin function between mice and humans (Rorsman and Ashcroft, 2017). Nevertheless, other reports have suggested that mice and human islet architecture is not as distinct as previously thought (Bonner-Weir *et al*., 2015). The large variety of islet cell types makes developing well defined differentiation protocols capable of committing pancreas progenitor cells specifically to β-cells essential.

A key paper using bulk-RNA-seq and deep sequencing was the Jennings *et al.* (2017) laser-capture article, which revealed the main active genes during early pancreas and liver organogenesis. 35 of these genes identified were TFs that had previously not been linked to pancreas development. The paper also identified that PSC-derived pancreatic tissue differentiation protocols mimic signalling events relative to formation of the dorsal pancreatic bud, as opposed to the ventral bud (Jennings *et al*., 2017). This is likely due to BMP inhibitors restricting the ventral pancreas regime (Petersen *et al*., 2018). Mimicking dorsal pancreas development may influence islet structure and function. For example, there is a greater proportion of larger, less uniform PP cells in pancreas derived from the ventral bud in both mice and humans, compared to the dorsal bud (Brereton *et al.*, 2015; Fiocca *et al.*, 1983). Moreover, ventral pancreas-derived PP-rich islets were deficient in α-cells (Brereton *et al.*, 2015).

β-cell heterogeneity:

The advent of single-cell RNA-Sequencing (scRNA-seq) has furthered our knowledge of islet subpopulation structure, development and signalling pathways (Dominguez-Gutierrez *et al*., 2019; Yu and Xu, 2020). Bulk RNA-seq data only takes an average snapshot of the total gene expression across a group of cells. ScRNA-Seq can instead identify differences in thousands of genes between individual cells within the same, or different, clusters with high-throughput and precision (Tritschler *et al*., 2017). This has revealed that β-cells can be separated into distinct cellular state groups and that there is heterogeneity within these groups in humans and mice (Aguayo-mazzucato *et al*., 2017; Baron *et al*., 2016; Enge *et al.*, 2017; Fang *et al.*, 2019; Muraro *et al.*, 2016; Petersen *et al*., 2017; Roscioni *et al*., 2016; Spitzer and Nolan, 2016; Wang *et al*., 2016). Further regulation between ventral and dorsal programmes in mouse was revealed by scRNA-seq (Li *et al*., 2018). Mouse ventral *Pdx1*^{low} cells represent intermediate progenitor cells that differentiate into

hepatoblasts or extrahepatic bile ducts, and PDX1^{high} are PPCs (Li *et al*., 2018; Rodríguez-Seguel *et al*., 2013). Dorsal *Pdx1*^{low} cells occur during the first wave of endocrinogenesis, with PDX1^{high} cells likely directly transitioning from endoderm (as reviewed in Yu and Xu (2020)). ScRNA-seq has also been especially useful for lineage tracing, identifying “branch points” for PPC fate changes (Yu and Xu, 2020).

The varying expression of the 18,000 different genes in β-cells results in sub-populations (Xin *et al.*, 2018). For example, 4 β-cell subpopulations have been identified in humans through specific cell surface markers, which are disrupted in T2DM patients (Dorrell *et al.*, 2016; Wang *et al*., 2016). This β-cell heterogeneity is thought to affect insulin secretion, maturity and vulnerability to auto-immune responses (Liu and Hebrok, 2017; Nasteska and Hodson, 2018; Wojtusciszyn *et al.*, 2008). Heterogeneity is evident by a small proportion of β-cells (1-10%) being responsible as “hubs” for metabolic control, regulating most glycaemic changes through controlling the Ca^{2+} response and insulin release (Johnston *et al*., 2016; Lei *et al*., 2018). The presence of these “hubs” for basal secretion is supported by single-molecule fluorescence in situ hybridization (smFISH) of mouse pancreas tissue (Farack *et al.*, 2019). “Hub” cells are more susceptible to targeting by pro-inflammatory cytokines and glucolipotoxicity. They may therefore experience reduced hub survival, connectivity and insulin secretion during T2DM pathogenesis (Johnston *et al*., 2016).

“Dynamic interchangeable states” within β-cell populations displays varying levels of unfolded protein response (UPR) genes and *Insulin* activity when grouped using pseudotime ordering (Dominguez-Gutierrez *et al.*, 2019; Xin e*t al.*, 2018). Pseudotime ordering, or trajectory inference, refers to tracing cells along a continuous route as they evolve, rather than clustering cells into subgroups (Trapnell *et al*., 2014). This can be particularly useful for grouping cells during dynamic processes, such as differentiation or the cell cycle. A period of high insulin secretion is followed by UPR stress release, as proinsulin is highly susceptible to misfolding during synthesis, resulting in high endoplasmic reticulum (ER) stress (Liu *et al.*, 2005). If these fluctuations are mis-regulated, diabetes may develop (Dominguez-Gutierrez *et al*., 2019).

Discrepancies in Wnt-planar cell polarity (PCP) pathway activity also affects β-cell heterogeneity, due to Wnt-PCP-mediated β-cell polarity being responsible for complete β-cell maturation and function (Bader *et al*., 2016; Cortijo *et al*., 2012; Roscioni *et al*., 2016; Sharon *et al.*, 2019). Multiple other pathways mediating β-cell proliferation and maturation have been identified in mice by scRNA-seq (Qiu *et al*.,

2017; Zeng *et al.*, 2017), but it remains to be elucidated whether the mechanisms are evolutionary conserved or biologically relevant in humans. Further study of human development will identify which β-cell populations should be selected for and are preferable for clinical translation. The application of emerging technologies, such as multiplex mass spectrometry imaging, fluctuation localization imaging-based fluorescence *in situ* hybridization (fliFISH) and single-cell western blot, may enable this (Cui *et al*., 2018; Kang *et al*., 2016; Thiery *et al.*, 2007).

Developmental defects and monogenic diabetes:

Multifarious genes can contribute to T1DM and T2DM progression. However, in 1-5% of cases single-gene (monogenic) mutations are responsible and they primarily characterise T2DM (Yang and Chan, 2016). Meta-analysis of genome-wide association studies (GWAS) have revealed a vast range of single nucleotide polymorphisms (SNPs) which may cause T2DM progression (Langenberg and Lotta, 2018; Mahajan *et al.*, 2018). Many of these variants are located proximally to monogenic diabetes genes, most commonly β-cell insulin secretion genes and key pancreas developmental TFs in mice and humans (Dimas *et al*., 2014; Jennings *et al.*, 2015). Greater comprehension of the interactions between these genes and TFs may reduce developmental defects, such as permanent neonatal diabetes mellitus (PNDM) or pancreas agenesis (De León and Stanley, 2016; Schnedl *et al.*, 2009). For example, a single nucleotide deletion in *PDX1* was the first mutated gene associated with pancreas agenesis and is implicated in PNDM (Nicolino *et al.*, 2010; Stoffers *et al*., 1997). *PDX1*-null mice lack PPCs and hence the pancreas does not develop (Jonsson *et al*., 1994).

Since then, mutations in the other main pancreas developmental TFs have been connected to pancreas agenesis, including: GATA4 (D'Amato *et al.*, 2010; Shaw-smith *et al.*, 2014), GATA6 (Allen *et al*., 2012), pancreas specific transcription factor 1a (*PTF1A* – the pancreas progenitor marker) (Kawaguchi *et al.*, 2002; Sellick *et al.*, 2004; Weedon *et al*., 2014), hepatocyte nuclear factor 1B (HNF1B) (Body-bechou *et al*., 2014), motor neuron and pancreas homeobox 1 (MNX1) (Harrison *et al.*, 1999), and NEK8 (Frank *et al.*, 2013). GATA6 mutations are the most common cause of pancreas agenesis (Allen *et al*., 2012). A missense mutation in CNOT1, a transcriptional repressor that promotes PSC stemness, was most recently shown to result in pancreas agenesis (De Franco *et al.*, 2019). SOX9 mutations also result in underdeveloped pancreases and abnormal islet structure (Piper *et al*., 2002). Human pancreas development, the common genetic mutations and the emerging role of PSC-derived β-cells are reviewed in detail (Petersen *et al*., 2018).

Signalling pathways active in human and mouse pancreas development:

As well as identifying many of the TFs essential for pancreas formation, upstream signalling molecules have been investigated. For example, fibroblast growth factor (FGF) molecules produced by the mesenchyme, primarily FGF7 and FGF10, were found to promote pancreas proliferation in rat and human embryonic pancreas epithelial cells (Elghazi *et al*., 2002; Ye *et al.*, 2005). This supported observations of FGF10 regulating pancreas proliferation, but not being involved in initial bud development, in FGF10-/- mouse embryos (Bhushan *et al*., 2001). Wnt signalling was identified as a key regulator of SOX17 expression during mouse definitive endoderm formation (Haegel *et al*., 1995), as well as more recently as a driver of endocrine commitment in a global transcriptomic analysis of human pancreas (Cebola *et al.*, 2015). Cells were harvested from CS16-18 pancreatic buds and gene expression compared to hPSC-derived multipotent PPCs in parallel via RNA-seq and chromatin immunoprecipitation (ChiP)-seq; hPSC-derived PPCs largely recapitulate native global transcriptional profiles despite their artificial origins (Cebola *et al.*, 2015). The functions of BMP, notch or retinoic acid (RA) signalling have not been documented during human *in vivo* pancreas progenitor maintenance (Jennings *et al*., 2015), although these pathways have defined roles in endoderm and pancreas lineage commitment in *Xenopus*, zebrafish and mice (Pan and Wright, 2011; Pearl *et al.*, 2009; Tiso *et al*., 2009). Knowledge of the impact of signals from neighbouring tissue on pancreas development continues to guide the development of PSC differentiation protocols towards committed endocrine lineages, and specifically towards functional β-cells (Petersen *et al*., 2018).

PSC-derived β-cells for transplantation:

Why use Pluripotent stem cells?

Pluripotent stem cells (PSCs) are obtained from the inner cell mass (ICM) of the blastocyst (Martin, 1981). Human PSCs address the issue of tissue availability; stem cells have the ability to self-renew and to differentiate into almost any of the approximate 200 cell types in the body (Watt *et al.*, 2010). Embryonic stem cells are pluripotent, meaning they can form all three germ layers (Smith, 2006). For this report, it can be assumed that PSCs refer to embryonic stem cells. Growth conditions can be controlled easily and PSCs can be genetically engineered to enhance proliferation or differentiation capabilities, as well as to investigate the importance of certain genes during these processes (Balboa *et al*., 2019). Multipotent stem cells (MSCs) have been used to generate β-cells (Pavathuparambil *et al.*, 2019). However, MSCs are more limited in their expansion capabilities, differentiation potentials and are derived from adult tissue, perhaps making them less useful for studying foetal development (Nardi and da Silva Meirelles, 2008). Hence, PSCs are ideal candidates for producing the large amounts of islet cells required for β-cell transplantation and also to enable the screening of new diabetic drugs (Kieffer, 2016; Millman and Pagliuca, 2017).

Progress of PSC-β-cell differentiation protocols:

A seminal proof-of-concept study for stem-cell derived β-cells was Assady *et al.* (2001). This paper utilised an embryoid body (EB) system and spontaneous differentiation to generate β-like cells which could produce insulin. Despite some success, β-like cells were not glucose responsive, differentiation was not regulated through addition of key developmental TFs or GFs, and cell purity remained an issue. Over the next few years, improvements in PSC differentiation into β-cells were made (D'Amour *et al.*, 2005; D'Amour *et al*., 2006; Shim *et al.*, 2007). Nodal and β-catenin signalling pathways were activated through combined addition of activin a (AA) and WNT3. β-like cells were now capable of producing Chromogranin A (CHGA), a secretory proprotein located within β-cells, but retained poor glucose sensitivity (D'Amour *et al.*, 2006; Winkler and Fischer-Colbrie, 1992). Kroon *et al.* (2008) expanded on these preliminary studies to establish a four-stage PSC differentiation protocol towards endocrine progenitor cells (EPCs). Implantation of these progenitors in immune-deficient mice achieved increased maturity and functionality compared to *in vitro* differentiation (Kroon *et al*., 2008). This protocol was further developed by other studies via the addition of GFs or small molecules, allowing generation of β-cells capable of producing insulin and displaying some

glucose responsiveness within approximately two weeks (Kieffer, 2016). Issues of poorly matured poly-hormonal β-like cells remained until large advancements were made in 2014 (Pagliuca *et al*., 2014; Rezania *et al.*, 2014). The prominent PSC differentiation protocols are outlined in Figure 4.

Pagliuca *et al*. (2014) and Rezania *et al*. (2014) improve differentiation considerably:

A major improvement of both protocols was the addition of CHIR99021, a glycogen synthase kinase 3β (GSK3-β) inhibitor that specifically activates the canonical Wnt/β-catenin pathway, in combination with AA to drive differentiation to DE. PDX1+ progenitor populations were also derived through the introduction of protein kinase C (PKC) inhibitors, such as (2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam (TPB) and Phorbol 12,13-dibutyrate (PDBu) (Pagliuca *et al*., 2014; Rezania *et al.*, 2014). Pagliuca *et al.* (2014) focussed on the long term scalability of β-cell production, efficiently generating partially functional PSC-derived β-like cells (referred to as SC-β cells) by adapting the existing differentiation protocols (Kroon *et al.*, 2008; Rezania *et al*., 2012). SC-β cells transplanted in mice displayed glucose-stimulated insulin secretion (GSIS) and reversed diabetes within 15 days (Pagliuca *et al.*, 2014). Although mono-hormonal populations could be produced, insulin^{+}-glucagon^{+} cells were also generated that lacked a functional GSIS response. Therefore the differentiation protocol has been optimised to enable culturing of aggregates, or organoids, which generate β-cells with heightened insulin production and function (Velazco-cruz *et al.*, 2019).

A seven-stage differentiation protocol was developed by the Kiefer group to convert PSCs into mono-hormonal β-like cells (Rezania *et al.*, 2014). The stage (S) 7 cells representing mature β-like cells expressed elevated levels of key markers for maturity, such as MAFA, and had functional GSIS responses. β-cell maturity was promoted through the induction of MAFA, a basic leucine zipper TF essential for the regulation of insulin secretion and a regulator of mature β-cell function (Hang *et al.*, 2014; Wang *et al.*, 2007; Zhang *et al*., 2005), via the addition of a GF cocktail (Rezania *et al*., 2014). This protocol therefore addressed the issue of bi-hormonal insulin-glucagon production by PSC-derived β-cells (Rezania *et al*., 2014). For instance, approximately 50% of the cells obtained were insulin positive, displaying yields much higher than previous protocols (Bruin *et al.*, 2013; Rezania *et al.*, 2011; Rezania *et al.*, 2012; Rezania *et al*., 2013). Streptozotocin (STZ)-induced diabetes was reversed in mice after 40 days, showing the effectiveness of these S7 cells (Rezania *et al*., 2014).

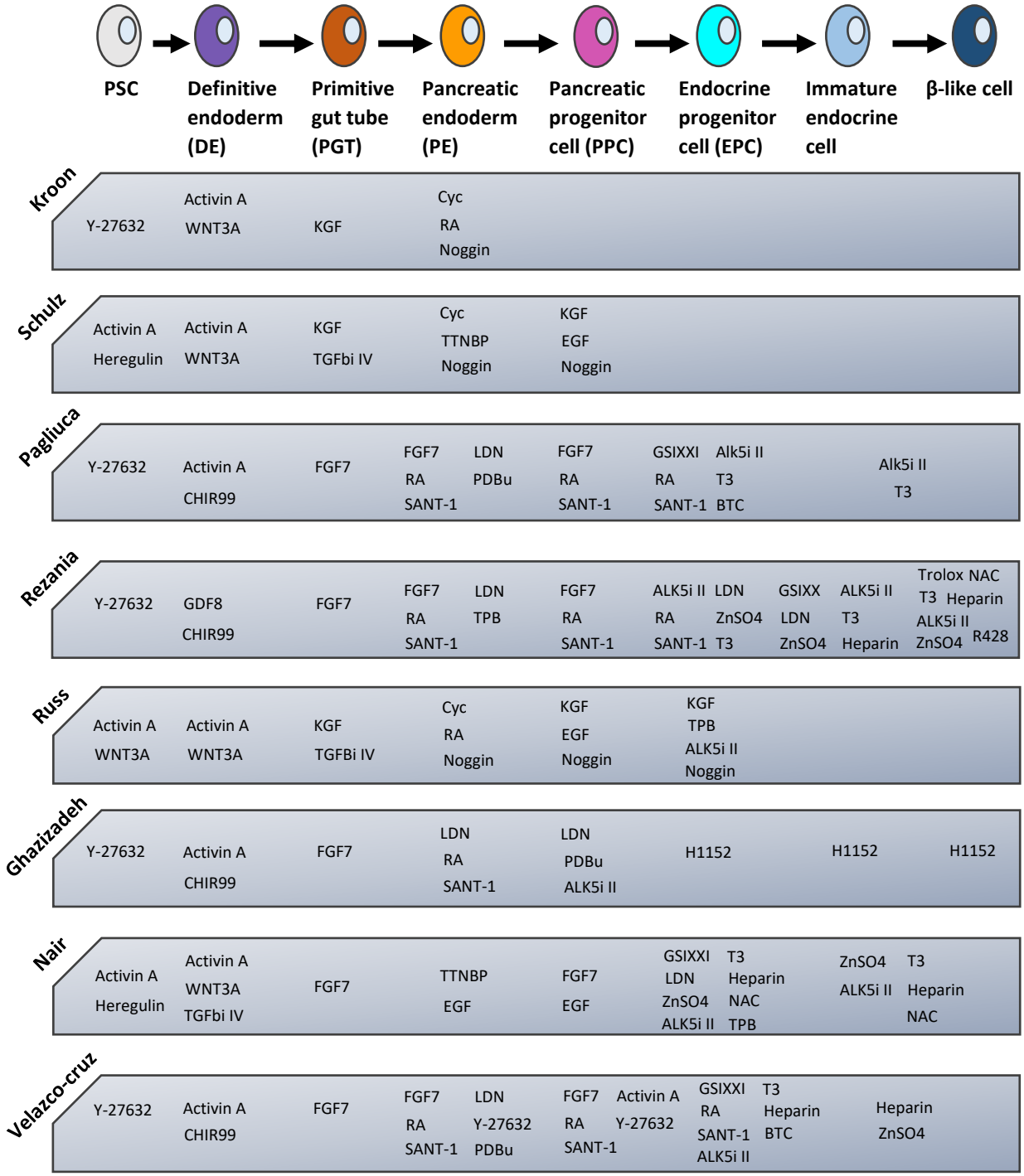


Figure 4. The improvement of PSC-derived β-cell differentiation protocols, as adapted from Tremmel *et al*. (2019). The four-stage protocol of Kroon *et al*. (2008) generated poly-hormonal insulin+/glucagon+ cells. The growth factor regime was adapted and large advances in mono-hormonal β-like cell generation were seen (Pagliuca *et al.*, 2014; Rezania *et al*., 2014). Russ *et al*. (2015) identified the optimal BMP inhibition period, inducing PPCs expressing NKX6.1. ROCK II inhibition (H1152) promoted β-cell maturity (Ghazizadeh *et al*., (2017). Schulz *et al.* (2012) pioneered a 3D culture system, which were adopted by later protocols with organoids. Dramatic improvements in β-cell function and calcium signalling were achieved using these PSC-organoid cultures, although full maturity has remained elusive (Nair *et al*, 2019; Velazco-cruz *et al*., 2019). Abbreviations refer to: Cyc, cyclopamine; ALK5i II, TGF-β RI kinase inhibitor; TGFbi IV, β-secretase inhibitor IV; GSI XX(I), γ-secretase inhibitor; PDBu, phorbol 12,13-dibutyrate; RA, retinoic acid; T3, 3,3'5-Triodo-L-thryonine, NAC, N-acetylcysteine; BTC, betacellulin; GDF8, growth differentiation factor 8 (TGF-β family); TPB, α-amyloid precursor protein modulator; TTNBP, arotinoid acid (RA analogue).

Limitations to the landmark Rezania *et al.* (2014) and Pagliuca *et al*. (2014) papers:

Both papers reported similar results of approximately 50% insulin$^+$/NKX6.1$^+$β-like cells, which could restore euglycemia within weeks of transplantation in mice (Pagliuca *et al.*, 2014; Rezania *et al.*, 2014). Therefore, these two prominent papers have been vital for understanding β-cell development and have made the prospect of readily available transplantable β-cells much more attainable. However, the papers are not without their caveats (Hanley, 2014; Johnson, 2016; Kushner *et al.*, 2014). For example, PSC-derived β-cells may have different glucose thresholds for insulin secretion compared to *bona fide* β-cells, potentially resulting in patients still experiencing hypoglycaemia (Hanley, 2014; Pagliuca *et al.,* 2014; Rezania *et al*., 2014). Furthermore, glucose release kinetics of PSC-derived S7 β-cells did not match those of native β-cells. This was evident from a much slower GSIS in smaller quantities for S7 cells, compared to a rapid burst of insulin release that more closely mimics fluctuating glucose concentrations from adult β-cells (Rezania *et al.*, 2014). Calcium release kinetics did not resemble human islets either (Johnson, 2016; Kindmark *et al.*, 1991). Both protocols boldly claimed to have generated “functional” β-cells, due to the approximate 2-fold increase of insulin secretion following glucose stimulation, but the GSIS responses observed remain minimal compared to the up to 10 times increase in insulin production in cadaver-derived islets (Shapiro *et al.*, 2000). Finally, mice were only reported to respond to fluctuating blood glucose levels 2-6 weeks post-implantation, demonstrating that the β-cells are still not fully mature following culture *in vitro* (Pagliuca *et al.*, 2014; Rezania *et al*., 2014). Pagliuca *et al.* (2014) could not achieve the MAFA-mediated β-cell maturation adopted in Rezania *et al*. (2014), making Rezania *et al.* (2014) the preferable differentiation protocol of the two.

BMP signalling duration regulates β-cell hormonal fate via NKX6.1 induction:

Enrichment of PDX1$^+$ PPCs for NKX6.1 was also found to promote β-cell maturation, following implantation *in vivo* (Kelly *et al.*, 2011; Kroon *et al.*, 2008; Rezania *et al.*, 2013). Nostro *et al*. (2015) demonstrated that activation of both epidermal growth factor (EGF) and nicotinamide signalling pathways, as well as BMP inhibition via NOGGIN, stimulate *NKX6.1* expression in progenitor cells. The size of these *NKX6.*1$^+$-progenitor populations is controlled by the GF regimen time period; a short 24-hour treatment results in mono-hormonal β-cells, whereas 72-96 hours treatment β-cells have poly-hormonal characteristics. They revealed that this

early induction of *NKX6.1* is essential for producing mono-hormonal cells across numerous hPSC cell lines (Nostro *et al*., 2015).

The findings of Nostro *et al.* (2015) were supported by another prominent paper for PSC-β-cell differentiation (Russ *et al.*, 2015). They also showed that premature BMP-mediated inhibition of *PDX1*$^{+}$ PPC populations actually increases the proportion of poly-hormonal cells, with short-term BMP stimulation required for mono-hormonal β cell formation (Russ *et al.*, 2015). Pagliuca *et al*. (2014) and Rezania *et al*. (2014) had focussed on improving end-stage β-cell differentiation, whilst employing NOGGIN-mediated BMP inhibition, but this will have been detrimental for deriving mono-hormonal β-cells (Russ *et al.*, 2015). Russ *et al.* (2015) utilised the recently developed INS$^{GFP/W}$ reporter cell line as an elegant system for the simple selection of β-cell populations via fluorescence-activated cell sorting (FACS) (Micallef *et al*., 2012). 31-45% of cells expressed GFP, with MAFA expression elevated in the β-cell population (Russ *et al.*, 2015). Nonetheless, implantation of β-like cells had limited effectiveness *in vivo*. STZ mice still developed diabetes, albeit at a reduced rate, and no dynamic insulin secretion assays were performed (Johnson, 2016; Russ *et al.*, 2015).

Differences in maturity between neonatal and mature β-cells:

There remain issues of β-cell maturity in these stem cell differentiation protocols. For example, PSC-derived cells more closely resemble foetal phenotypes, as opposed to functional adult cells, in a range of tissue types (Baxter *et al.*, 2015; Hrvatin *et al*., 2014; Karakikes *et al*., 2015; Playne and Connor, 2017). Although β-cells first develop in the foetus from 8-9 wpc, neonatal β-cells still do not display the hallmarks of fully mature β-cells in both rodents and humans (Aguayo-Mazzucato *et al.*, 2011; Arda *et al*., 2016; Blum *et al.*, 2012; Dhawan *et al*., 2015; Gregg *et al*., 2012; Jermendy *et al.*, 2011). Between 9 and 16 wpc, 20-40% of human foetal endocrine cells are polyhormonal, and only become mainly monohormonal by 21 wpc (Jeon *et al*., 2009; Riedel *et al.*, 2012).

Mature β-cells have a very complex mechanism for nutrient sensing and subsequent rapid GSIS, which may not be fully developed in PSC-derived β-cells (MacDonald *et al.*, 2005; Rutter *et al.*, 2015). Immediately after birth, β-cells are classified as “having high basal insulin output”, due to responding to a lower glucose threshold than mature β-cells (Kieffer, 2016). This may be due to neonatal β-cells requiring feeding and hunger signals received postnatally to trigger a more mature β-cell state during the transition from glycolysis to oxidative phosphorylation, which can be mimicked by addition of certain factors during differentiation (Aguayo-

mazzucato *et al.*, 2013; Stolovich-Rain *et al.*, 2015). For example, estrogen-related receptor γ (ERRγ) has been shown to be essential for the maturation of β-cells into fully glucose-responsive cells through upregulating mitochondrial oxidative metabolism (Yoshihara *et al*., 2016). This is supported by high oxygen concentrations promoting induced pluripotent stem cell (iPSC) differentiation into *Neurog3* positive β-like cells, with enhanced insulin secretion compared to lower oxygen tensions (Hakim *et al.*, 2014). WNT4 signalling regulates ERRγ-mediated induction of β-cell maturation markers (Yoshihara *et al*., 2020).

Paracrine signalling between β-cells and other islet cells also promotes insulin secretion and maturation. For example, α-cells produce the incretin hormone, glucagon-like peptide-1 (GLP1), for improved β-cell function and proliferation (Chambers *et al*., 2017; Drucker, 2018). α-cell function is also greatly reduced in T1DM patients, indicating that they could also be targeted to aid β-cell function post-implantation (Brissova *et al*., 2018). Therefore, GLP1 or analogues, such as exendin-4, can be incorporated into the GF differentiation cocktail to ensure β-cell maturation (List and Habener, 2004; Ohmine *et al*., 2012). Inclusion of ROCKII inhibitor from the EPC stage was shown to inaugurally promote upregulation of the maturity marker, *UROCORTIN-3* (*UCN3*) (Ghazizadeh *et al*., 2017). Somatostatin release and expression of shared β-cell surface markers by δ-cells additionally mediates β-cell function (Huising *et al.*, 2018; Van Der Meulen *et al.*, 2015). Other factors or small molecules used include: forskolin, to activate adenylate cyclase, with dexamethasone (Kunisada *et al.*, 2012), insulin growth factor-1 (IGF1) and hepatocyte growth factor (HGF) (Ohmine *et al*., 2012). However, they have not achieved full β-cell maturity (Al-Khawaga *et al*., 2018).

Furthermore, there is a large difference in the gene expression profile between neonatal and mature β-cells. The transition between states is regulated by activation or repression of key genes to maintain a Yin-Yang homeostasis, wherein blood glucose levels do not reach hypo- or hyper-glycaemic levels (Schuit *et al.*, 2012). For instance, key pancreas-defining TFs such as: *Neuorod*, *Pdx1*, *Pax6* and both *Mafa* and *Mafb* are upregulated to promote β-cell maturity, with the downregulation of *Ldha*, *Hk1* and *Mct1* (Dhawan *et al.*, 2015; Lemaire *et al.*, 2016). This results in a “visible face” of gene expression for neonatal β-cells as they mature, increasing eventual production of proteins required for controlling insulin secretion. The “hidden face” represses genes to prevent elevated β-cell GSIS in response to exercise (Lemaire *et al.*, 2016). PSC-derived β-cells are still in a neonatal state due to diminished expression of maturity-associated genes, such as *MAFA, UCN3,* and

NEUROG3, compared to *bona fide* human β-cells (Hrvatin *et al*., 2014; Nair *et al*., 2019; Pagliuca *et al.*, 2014; Rezania *et al*., 2014; Velazco-cruz *et al.*, 2019; Veres *et al*., 2019). SIX2, a homeobox TF, was recently identified as a β-cell differentiation and maturation regulator (Velazco-Cruz *et al.*, 2020). Further elucidation of the signalling mechanisms and interacting pathways may address the issue of maturity. Although it would be preferable to have fully mature β-cells for transplantation, mature β-cells do have reduced proliferation rate (Yu and Xu, 2020). Hence, alternative strategies need to be investigated to aid mature β-cell expansion.

PSCs and organoid systems - the means to fully functional β-cells:

Two recent protocols utilising PSC-derived β-cell aggregates have helped to address the issue of β-cell maturity and function (Nair *et al.*, 2019; Velazco-cruz *et al.*, 2019). These papers report the first functionally relevant dynamic GSIS response, almost matching those reported for cadaver-derived islet cells (Nair *et al.*, 2019; Velazco-cruz *et al.*, 2019). Enriched β-cell clusters (eBCs) generated by Nair *et al*. (2019) can mimic the endocrine cell clustering that occurs *in vivo* to produce more mature adult β-cells and additional islet cells (Borden *et al*., 2013; Jeon *et al*., 2009; Kim *et al.*, 2016; Nair and Hebrok, 2015). Velazco-cruz *et al*. (2019) adjusted their growth factor-small molecule treatment regime during the pancreatic endocrine commitment step, yielding the largest increase in dynamic GSIS response. The large improvements of β-cell function obtained in Russ *et al*. (2015) and Nair *et al*. (2019) were partially due to the application of the transgenic $INS^{GFP/W}$ reporter cell line to increase cell purity (Micallef *et al.*, 2012). This has also been performed through the sorting of cluster populations via magnetic assisted cell sorting (MACS) for specific β-cell markers, such as CD49a (otherwise referred to as ITGA1) (Veres *et al.*, 2019). ScRNA-Seq was used on over 100,000 cells to identify multiple cell types within differentiated clusters (Klein *et al*., 2015). These clusters included β-/α-cells, non-endocrine cells and an enterochromaffin (ECh) cell population for the first time (Veres *et al*., 2019). ECh-cells have similar expression profiles to β-cells, expressing *NKX6.1* and *CHGA*, whilst demonstrating no glucagon activity (Veres *et al*., 2019). ScRNA-Seq has additionally identified that CD9 expression is absent in glucose-responsive INS+ populations (Li *et al.*, 2020). These results represent the large heterogeneity within islet populations and offer a sorting method to isolate β-cells with greater purity.

Organoids as a model for human pancreas development:

The benefits of 3D cell culture:

The extracellular matrix (ECM) is immensely complex and controls myriad processes; the ECM and microenvironment must be considered during culture of PSCs or foetal tissue into β-cells (Aamodt and Powers, 2017; Grapin-Botton, 2016). Cells are often grown on 2D surfaces, such as glass or plastic, with purified mixtures of ECM components (Abbott, 2003; Kuschel *et al.*, 2006). For example, initial PSC pancreas differentiation protocols utilised monolayer cultures after sequential addition of GFs and small molecules (Pagliuca *et al*., 2014; Rezania *et al.*, 2014). However, these conditions do not mimic the natural ECM as morphogen gradients are absent and adhesions are only in the planar direction (Lv *et al*., 2015). For instance, correct PCP and apico-basal polarity are required for functional β-cell differentiation and maturity (Bader *et al.*, 2016; Cortijo *et al*., 2012; Kesavan *et al*., 2009). Gap-junction mediated cell-cell contacts are also essential for β-cell survival and insulin secretion (Klee *et al.*, 2011; Ravier *et al.*, 2005; Santos-silva *et al.*, 2012; Wojtusciszyn *et al.*, 2008). The ECM in the epithelial plexus niche, where endocrine progenitor cells are clustered during pancreas development, additionally regulates endocrine lineage commitment via notch-dependent signalling (Bankaitis *et al.*, 2015). Therefore, there has been a shift towards 3D culture (Abbott, 2003; Johnson *et al*., 2007), which has recently been adopted in PSC-β-cell differentiation protocols (Kim *et al.*, 2016; Nair *et al.*, 2019; Russ *et al.*, 2015; Schulz *et al*., 2012; Shim *et al*., 2015; Velazco-cruz *et al*., 2019).

Primary cell lines have been generated for acinar, ductal and islet cells (Bläuer *et al*., 2011; Houbracken *et al.*, 2011; Paraskevas *et al*., 2000), but production of PPC lines has been challenging (Furuyama *et al.*, 2011; Sugiyama *et al.*, 2007). Nevertheless, none of these cell lines can be maintained for a long period of time without inclusion of mesenchyme or the essential intercellular contacts provide by the 3D environment (Burke *et al.*, 2010; Duvillié *et al*., 2006; Johansson *et al*., 2007). Although a genetically engineered “β-cell” line was produced via lentiviral transfection, cells are aneuploid and do not represent *bona fide* β-cell signalling mechanisms or function (Tsonkova *et al*., 2018). Organoids are clusters of self-organising epithelial cells grown *in* vitro with correct polarity and cell-cell adhesions (Rossi *et al*., 2018). They ideally have the same *in vivo* function, genetic signature, and structure of the native tissue. Organoid systems are an important tool for drug screening, development of regenerative therapies and genome-wide gene function studies, allowing treatment of cancer and neurodegenerative diseases (Clevers,

2016; Dutta *et al*., 2017; Fatehullah *et al*., 2016). Organoid culture with a 3D environment more akin to the native pancreas environment will provide the closest representation of natural foetal human pancreas development, permitting *in vivo* signalling mechanisms to be recapitulated (Balak *et al*., 2019; Dayem *et al.*, 2019; Grapin-Botton, 2016). This will enable the study of cell-cell interactions, the importance of the ECM in pancreas differentiation and endocrine commitment to mature β-cells, as well as gene editing techniques to investigate pancreatic developmental disorders or drug modelling for T1DM (Bakhti *et al*., 2019; Koo *et al.*, 2012; Koo *et al.*, 2013; Sugiyama *et al*., 2013).

Pancreatospheres as stepping-stones to pancreas organoids:

Before the adoption of fully complex 3D organoid systems, spheroids were utilised for 3D culture with less advanced un-branched structures forming. For example, a neuronal spheroid system was first established to enable study of neurogenesis (Reynolds and Weiss, 1992). Spheroid systems for pancreas, referred to as pancreatospheres (Grapin-Botton, 2016), initially enabled the study of mouse early embryonic pancreas development. For instance, Sugiyama *et al*. (2013) were able to select for $SOX9^{+}$ pancreas progenitors from E11.5 mouse tissue, using a $Ngn3^{-}$ tdTomato transgenic cell line to exclude endocrine progenitor cells. Pancreas progenitors were then cultured as spheres on Matrigel, the ECM supplement produced by mouse sarcoma cells (Kleinman and Martin, 2005), which could be differentiated into $insulin^{+}$ cells with static GSIS. Similar to PSC-pancreas progenitor differentiation protocols, the main media supplements added were FGF10, RA, insulin, IGF1 and R-spondin, to promote the WNT/β-catenin signalling pathway (Kim *et al.*, 2006), as well as mesenchyme (Sugiyama *et al*., 2013). However, these PPC only retained potency for a few passages; T1DM and T2DM progress over a long time period, meaning near unlimited expansion potential is a necessity to study the effects of aging on differentiation and function. Another prominent paper for pancreas development used a similar spheroid system to differentiate mouse pancreas epithelia on Matrigel or synthetic polyethylene glycol (PEG) hydrogels (Greggio *et al*., 2013). These PPCS maintained potency after multiple passages over two weeks (Greggio *et al*., 2013). The importance of cell interactions was also clear; isolated single progenitors could still form spheroids but had drastically reduced efficiency (2% vs. 90% with other cells present).

Mouse pancreas organoids:

PPCs and mature β-cell organoids can be created using isolated foetal or adult pancreas tissue, and by the defined PSC differentiation protocols (Tremmel *et al.*, 2019). Foetal organoids are simpler to culture, due to their increased regenerative capabilities and plasticity compared to adult tissue (Huch and Koo, 2015). As well as developing pancreatospheres, Sugiyama *et al.* (2013) and Greggio *et al*. (2013) established more complex pancreas organoids using mouse foetal tissue. A genetic screen was performed in $SOX9^{+}$ progenitor cells, identifying how deficiency of key genes affects islet cell development (Sugiyama *et al*., 2013). As expected, they showed *Neuorg3* to be essential for endocrine commitment, with both *islet amyloid polypeptide* (IAPP) and *Arx* or *Pax4* required for α- or β-cell development, respectively. *PR domain zinc-finger protein 16* (PRDM16), a methyl transferase, was also implicated in regulating islet fate and function (Sugiyama *et al.*, 2013). The Grapin-Botton group generated pancreas progenitor organoids capable of differentiating into a full range of ductal, acinar or endocrine cells, whilst lacking endothelial, neuronal or mesenchymal lineages (Greggio *et al.*, 2013). The organoid system also displayed branching (Dahl-jensen *et al*., 2016; Greggio *et al*., 2013). Due to the ability to form all pancreatic cells, the system was particular useful for investigating signalling pathways present during lineage commitment (Hindley *et al*., 2016). Similar developmental cues were found to be required as compared to *in* vivo; FGF and Notch signalling were essential for differentiation (Greggio *et al.*, 2013). The main finding was the importance of Matrigel for expansion of pancreas progenitors, indicating the necessity of including *ex vivo* cell-cell contacts and signalling that mimics the *in vivo* microenvironment. However, long-term expansion of pancreas progenitors was not capable.

Noggin was incorporated in pancreas organoid protocols to inhibit BMP signalling. This enabled repeated expansion of embryonic and adult organoids for up to 5 months (Huch *et al*., 2013a), as was utilised in PSC differentiation procedures (Nair *et al*., 2019; Pagliuca *et al.*, 2014; Rezania *et al.*, 2014; Velazco-cruz *et al.*, 2019). As with the Grapin-Botton group, other factors added included FGF10 potentiated by heparin, R-spondin and EGF (Greggio *et al*., 2013; Huch *et al.*, 2013a). Resultant pancreatic organoid cells all displayed ductal phenotypes, in addition to expressing the pancreatic marker, *Pdx1,* and stem cell markers, *Lgr5* and CD133 (Ahlgren *et al*., 1996; Huch *et al*., 2013a; Oshima *et al.*, 2007). It has proven challenging to commit these ductal cells towards endocrine lineages, such as β-cells, perhaps due to a loss of bi-potency (Huch and Koo, 2015). Despite this, ductal cells can be

reprogrammed to form islet cells, indicating that the capability remains (Sancho *et al*., 2014; Zhou and Melton, 2018). These ductal cells also form mono-hormonal insulin^{+}β-like cells upon implantation into SCID mice (Huch *et al.*, 2013a). Improvements to the culture conditions, including growth factors and substrate composition, and addition of other cell types found within the pancreas niche may enable reliable endocrine differentiation (Aamodt and Powers, 2017; Grapin-Botton, 2016; Huch and Koo, 2015).

Human pancreas organoids:

A comparison of mouse and human foetal pancreas pancreatospheres and organoids was performed (Bonfanti *et al.*, 2015). 8-11 wpc human foetal tissue was isolated and cultured on Matrigel by combinations of FGF10, EGF, Y-27362 and R-spondin. PPCs were maintained under these culture conditions, as represented by high PDX1, NKX6.1, SOX9 and HNF1β, but did not differentiate readily, due to low NKX2.2, NEUROG3 and hormone production (Bonfanti *et al*., 2015). EGF was included to inhibit differentiation, allowing isolation of pancreas progenitor populations (Bonfanti *et al*., 2015). Organoid systems for ductal pancreatic cancer using adult human tissue have also been developed (Boj *et al*., 2015; Huang *et al*., 2015). Adult and foetal human pancreas tissue was only recently used to generate PPC organoids capable of differentiating into endocrine cells (Loomans *et al*., 2018). Similar morphologies and transcriptional profiles were observed for organoids from either tissue type. Organoids were able to differentiate into partially functional β-like cells following transplantation into SCID STZ mice (Loomans *et al.*, 2018). Nevertheless, only 1.5% of cells became insulin^{+}, GSIS or calcium response were not recorded, and hyperglycaemic events were not ameliorated significantly. Interestingly, the authors confirmed findings by Zhou *et al.* (2008) that ALDH expression can be used as an indirect measure of endocrine differentiation; high levels of ALDH correspond to elevated PPC gene expression, including PDX1, carboxypeptidase A1 (CPA1), PTF1A, and MYC, whereas insulin^{+} committed β-like cells have low ALDH (Loomans *et al.*, 2018). ALDH is localised to branching buds in the tips and is a defining trait for PPC populations, which also localise there (Jennings *et al*., 2013; Loomans *et al*., 2018; Zhou *et al*., 2008). Importantly, cryopreserved pancreatic tissue was shown to maintain the same genetic profile and morphology upon organoid culture, potentially increasing feasibility for clinical translation (Loomans *et al*., 2018).

PSC-derived β-cell and organoids summary:

Although fundamental advancements in PSC-to-β-cell differentiation have been made in recent years, there still remain issues to be addressed before clinically viable therapies for diabetes are available. Halban *et al.* (2001) clearly outlined the need for fully mature glucose-responsive β-cells that only produce insulin, prevent hyperinsulinemia and hypoglycaemia, and still respond to calcium signals. Nearly two decades on we are much closer to this goal of readily available β-cells that remove insulin dependence and drastically improve patient welfare, but the thorough and high standards for islet transplantations are not currently met. Organoid culture techniques have improved β-cell function and calcium signalling considerably, but issues of maturity remain (Kim *et* E*al.*, 2016; Nair *et al.*, 2019; Velazco-cruz *et al.*, 2019). Overall, transplanted PSC-derived β-cells remain inferior in insulin production and responsiveness compared to cadaver islet sources (Nair *et al.*, 2019; Pagliuca *et al.*, 2014; Rezania *et al*., 2014; Velazco-cruz *et al*., 2019). We are also yet to determine what GSIS response magnitude is therapeutically effective, with β-cell functionality poorly defined between groups. Deeper phenotyping has recently been performed through the use of scRNA-Seq, allowing analysis of the transcriptome active during β-cell differentiation in more detail (Dominguez-Gutierrez *et al.*, 2019; Veres *et al.*, 2019). The large variation in cell transplantation number and differentiation lengths across different cell lines make comparisons of protocol clinical efficacy challenging. Therefore, the concept of a universal differentiation protocol using good manufacturing procedure (GMP), that can be applied to multiple cell lines, is vital for successful clinical translation of PSC-derived β-cells (Tremmel *et al.*, 2019). Further development of organoid culture systems for foetal tissue and PSCs will improve our knowledge of human development and facilitate generation of fully functional mature β-cells. Likewise, the effect of substrate stiffness on endocrine commitment should be investigated in more detail (Greggio *et al*., 2013). They will allow the identification of markers for islet cell populations, reducing β-cell heterogeneity, and may improve insulin secretory capacity (Tremmel *et al*., 2019).

Project aims:

1) Establish and maintain an organoid culture system with foetal pancreas tissue, enabling the characterisation of transcriptional regulators of pancreatic progenitor maintenance and endocrine commitment towards islet cells.
2) Investigate light sheet fluorescence microscopy (LSFM) as a method for analysing pancreas and organoid morphogenesis.

Research design and methods:

Human tissue collection:

All human tissue research and storage was conducted under the ethical approval of the North West Research Ethics Committee, as regulated by the Human Tissue Authority (HTA) and legislation of the U.K. Human Tissue Act 2008. Human embryonic and foetal tissues were collected from medical or surgical pregnancy terminations, following informed consent. Measurements for embryonic and foetal gestational age (GA) are shown in Figure 5. Table 1 provides a guide to CS classification based upon crown rump length (CRL) and the identification of visual events. Tissue handling and dissection were performed as follows (Piper *et al.*, 2004; Jennings *et al.*, 2013). Pancreases were dissected under light microscope (ZEISS SteREO Discovery.V8), and mesenchyme removed using forceps. Pancreases for organoid formation were stored in DMEM/F12 basal media (Thermo Fisher Scientific, Waltham, MA), often overnight before processing in the morning. Samples to be processed for histology or light sheet fluorescence (LSFM) microscopy were fixed for 2-3 hours using 4% paraformaldehyde (PFA; Alfa Aesar, Haverhill, MA). Samples for histology were embedded in paraffin wax and sectioned at 5µm intervals using a Leica RM2235 microtome (Leica Microsystems, Wetzlar, Germany). Four sections were placed per slide. Every eight section or slide was used for haematoxylin and eosin (H and E) staining of embryonic or foetal tissue respectively, providing a reference for anatomical landmarks and morphology.

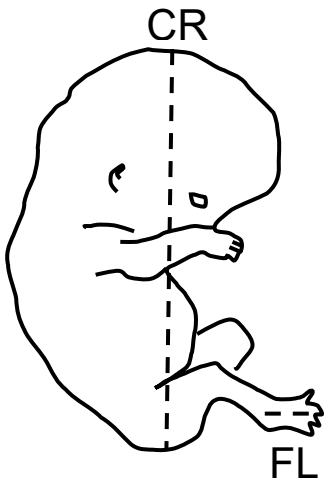
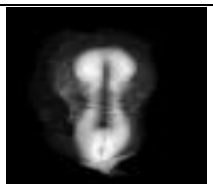
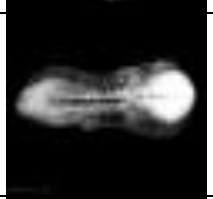
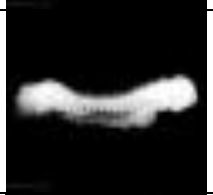
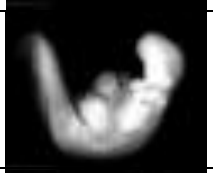
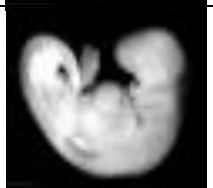
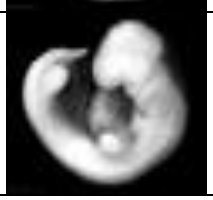
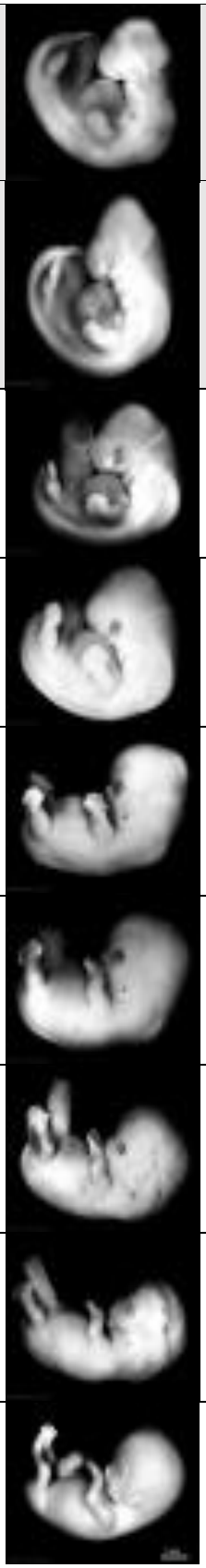


Figure 5. A representative embryo-foetus stage outlining the methods for age determination, as adapted from O'Rahilly and Müller (2010)**.** Embryos are staged based upon crown rump length (CRL), representing crown-heel distance. Foetuses are staged by foot length (FL), as measured from the heel to the longest toe.

Table 1. Comparison of the crown rump length (CRL) and developmental characteristics of Carnegie stage (CS) embryos throughout human development. Approximate gestational age and CRL data are obtained from O'Rahilly and Müller (2010). Embryo images are acquired from the Kyoto and Carnegie collections, both available as digital references for human embryology and histology (Hill, 2018; Hill, 2020). Key developmental events are outlined from CS9 (Hill, 2007; Larsen, 2001). At CS9, gastrulation has occurred and somites have begun to form; the CS9 embryo is more developed and is the earliest stage the Hanley-Piper laboratory uses for research. Grey shading indicates CS15-16, where dorsal and ventral pancreatic buds fuse.

Carnegie Stage (CS)	Gestational age (days)	Image (not to scale)	Mean CRL (mm)	Key developmental events
9	25-27		1.4	Somitogensis begins (1-3 total), cardiac primordium emerges and head folds
10	~28-30		2.1	4-12 somites, fusion of neural fold and formation of the first pharyngeal arch
11	28-30		3.2	13-20 somites, closure of rostral neuropore, sinus venosus and mesonephric duct formation
12	29-31		3.9	21-29 somites, closure of caudal neuropore, first 3 pharyngeal arches are visible
13	30-33		4.9	30 Somites total, leg buds emerge and lens placode forms
14	33-35		6.5	Optic cup and lens pit formation

15	35-37		7.8	Formation of lens vesicle, nasal pit and hand plate, herniated midgut and intermediate kidney are detectable under heart
16	37-40		9.6	Retinal pigment development, formation of early ears on first two pharyngeal arches, rotation of limbs begins, and vertebrae become visible
17	39-42		12.2	Distinct finger digits and face begin to from
18	42-45		14.9	Ossification starts
19	45-47		18.2	Trunk begins to straighten, upper and lower eyelids are clearly apparent, external ears are visible
20	47-50		20.7	Face fuses with lower jaw, upper limbs begin to bend at elbow and increase in length
21	49-52		22.9	Prominent upper jaw forms, hands and feet begin to turn inwards
22	52-55		25.5	Formation of eyelids and external ears
23	53-58		28.8	Head, body and limbs become more rounded

Generation of human foetal pancreatic organoids:

Human foetal organoids were generated using an adapted protocol, as outlined in Figure 7 (Greggio *et al*., 2013). Following removal of mesenchyme in cold PBS (Sigma-Aldrich, St. Louis, MO), pancreases were dissected into small pieces using sterile blades (Swann-Morton, Sheffield, UK). Pieces were then transferred to MAX recovery Eppendorf tubes; one quarter of pancreas tissue was used for RNA bulk collection and the remainder for organoid culture. Samples were centrifuged for 5 minutes at 200g at 4°C, as for each spin step. RNA samples were stored in 300µl TRIzol and snap-frozen using dry ice, prior to storage at -80°C. Organoid samples were washed once in cold PBS and incubated for 40-50 minutes in filtered collagenase IV solution at 37°C. Every 10-20 minutes, tissue was mechanically dissociated using a pipette. Collagenase-mediated dissociation was inhibited by addition of heat-inactivated FBS. Two further spin steps were required, with the pellet washed once in cold pancreatic epithelial medium (PEm). PEm composition is outlined in Table 2. After this final wash and removal of media (ensuring a dry pellet), 30µl Matrigel (Corning, New York, NY) was added and the sample placed on ice for 5 minutes. Following gentle resuspension of the pellet, 30µl Matrigel cell suspension was plated in a pre-heated 4-well Nunclon Delta Surface plate (Thermo Fisher Scientific) and incubated at 37°C for at least 30 minutes. Adequate incubation time is essential for Matrigel dome formation. Warm PEm media was added to each well and changed every 2-3 days.

Organoid passage:

Organoids were passaged every 7-10 days after dissociation with collagenase IV for 10 minutes at 37°C. Matrigel was broken down by rapid mechanical dissociation with wide-bore pipette tips (Axygen, New York, NY), after inactivation with FBS. Organoid populations were cleaned by 2-3 spin cycles of 30 seconds at 13,000 RPM with 5% FBS/PBS, to ensure pellet formation and removal of debris. Before immunofluorescence imaging, Matrigel was broken down completely by addition of Corning Cell Recovery solution (Corning) for 20 minutes on ice.

Table 2. Pancreas epithelia media (PEm) for maintenance of pancreatic organoids.

Reagent	Provider	Catalogue number	Stock Concentration	Final Concentration
DMEM/F-12 (+ 15µM HEPES + L-Glutamine)	Thermo Fisher Scientific	31330-038	100%	97%
Penicillin-Streptomycin	-	-	100%	1%
B27 x50	-	-	100%	2%
Recombinant human FGF2 (bFGF)	Peprotech EC Ltd	100-18B-100UG	100µg/ml	64ng/ml
Y-27632 (Rock inhibitor)	EMD Millipore	688000-1MG	50mM	10µM

Immunohistochemistry:

Immunohistochemistry was performed on sectioned embryonic or foetal tissue using the previously described method (Piper *et al*., 2004). Slides were de-waxed and rehydrated by washing twice in xylene, followed by single washes in 100% and 90% ethanol, as well as rinsing in water. Sections were treated with 3% (v/v) H_20_2 for 20 mins at RT, enabling endogenous peroxidase removal, washed, and boiled in 10mM sodium citrate for antigen retrieval. Each washing step involves three 5-minute PBS washes. Boiling time was adapted depending on the primary antibody used and tissue fixation time. Primary antibodies (Table 3) were diluted in PBS supplemented with 0.1% Triton-X 100 and blocked with 3% serum, from which the secondary antibody was derived (Table 4). Slides were incubated overnight at 4°C with primary antibody solution in a humidified container, washed and secondary antibody solution (PBS with 0.1% Triton-X 100) applied for 2 hours at RT. Secondary antibodies were biotinylated, allowing detection by streptavidin-horse radish peroxidase (HRP) binding and labelling. Following washing, slides were incubated for 1 hour in 0.5% Strepatavidin-HRP (Vector laboratories, Burlingame, CA) diluted in PBS/0.1% Triton-X 100 at RT. Colour detection was achieved via DAB solution (Thermo Fisher Scientific), and counterstaining performed with a 2-minute wash in toluidine blue. Slides were dehydrated as follows: rinse in water, 10 seconds in 70% and 90% ethanol, 3 minutes in 100% ethanol, 2 minutes in xylene repeated twice. Coverslips were mounted using Entalan (Merck, Darmstadt, Germany).

Immunofluorescence:

For confocal imaging, organoids were split onto uncoated 6-well plates (Corning) and cultured in 2D for 7 days, ensuring a more rounded morphology. Organoids were then split onto 96-well glass-bottomed high content imaging plates (Corning) pre-coated with 6µg collagen I per cm^2 for 2 hours at RT. Organoids were fixed in 4% PFA for 15 minutes on ice, washed twice with PBS and stored for up to a week before imaging. Organoids were permeabilised with PBS/0.1% Triton-X 100 for 10 minutes and blocked in PBS/0.1% Triton-X 100 with 10% serum, both for 1 hour. Organoids were incubated overnight at 4°C in primary antibody solutions of PBS/0.1% Triton-X 100 with 1% serum. Organoids were washed and incubated for 1 hour at RT in secondary antibody solution (same as primary). After washing, DAPI was applied for 10-15 mins at RT, organoids briefly washed and transferred to PBS before imaging. Images were acquired using the Leica TCS SP8 AOBS inverted confocal microscope (Leica Camera AG) with the 20x objective lens and 0.75 confocal zoom. The Leica Application Suite X (LAS X) software was used to adjust

the settings as follows: pinhole (1 airy unit), scan speed (600Hz unidirectional), format (1024 x 1024). Images were collected using 3 hybrid detectors with the following detection mirror settings; FITC 494-530nm (Alexa Fluor green 488nm); Texas red 602-665nm (Alexa fluor Orange 555nm); Cy5 640-690nm (Alexa Fluor Far-red 647nm). White light laser lines were as follows: 488nm (20%), 594nm (10%) and 633nm (10%). DAPI was detected using the photon multiplying tube (PMT). Each channel gain was manually adjusted to have optimum fluorescence signal without saturation. Frame accumulation by line was manually set to 3, reducing noise. The “Sequential imaging” function was used to generate maximum intensity Z-stacks, preventing bleed-through between similar wavelength fluorophores. Images were saved as .lif files and processed using Fiji is just imageJ (FIJI) (Schindelin *et al.*, 2012).

Table 3. List of primary antibodies used for immunohistochemistry, immunofluorescence, light sheet fluorescence microscopy (LSFM) and flow cytometry.

Antibody	Species	Provider	Catalogue number	Dilution
CPA1	-	-	-	-
Cytokeratin 19 (CK19)	Rabbit	Abcam	Ab52625	1:500
FOXA2 (HNF3β)	Goat	R&D systems	AF2400	1:100
GATA4	Rabbit	ProteinTech	19530-1-AP	1:1000
Glucagon	Rabbit	Zymed	-	1:100
Insulin	Rabbit	Abcam	ab63820	1:50
PDX1	Guinea pig	Abcam	47308	1:500
NKX2.1	Mouse	DSHB	74.5A5	1:50
NKX6.1	Mouse	DSHB	F64A6B4	1:50
SOX9	Rabbit	Millipore	AB5535	1:5000

Table 4. List of secondary antibodies used for immunohistochemistry, immunofluorescence, light sheet fluorescence microscopy (LSFM) and flow cytometry.

	Antibody	Host	Provider	Catalogue number	Dilution
Biotinylated	α-goat	Horse	-	BA-9000	1:200
	α-guinea pig	Goat	-	BA-7000	1:100
	α-mouse	Horse	-	BA-2000	1:100
	α-rabbit	Goat	-	BA-1000	1:800
Alexa Fluor	α-goat (488)	Donkey	-	-	1:1000
	α-guinea pig (488)	Goat	-	-	1:1000
	α-mouse (594)	Donkey	-	-	1:1000
	α-rabbit Far-red (647)	Donkey	Invitrogen	A21207	1:1000 (1:1000 and 1:1500 for LSFM)

Light sheet fluorescence microscopy (LSFM):

Tissue preparation:

Following fixation in 4% PFA, some samples were treated with 1% sodium borohydride ($NaBH_4$; Sigma-Aldrich)/PBS solution for 15 minutes at RT to eliminate Schiff's base reactions (Clancy and Cauller, 1998). These samples were then washed thoroughly in PBS three times for 15 minutes each. All steps were performed on shakers and sterile PBS is supplemented with 0.02% sodium azide (NaN_3; Sigma-Aldrich) to prevent microbial growth. Samples without $NaBH_4$ treatment were transferred to PBS at 4°C, if processed immediately, or stored in 70% ethanol for later use up to months later. All samples were then dehydrated by washing in 50% methanol/PBS solution, 80% methanol/PBS, 100% methanol/PBS sequentially for 1 hour each at RT. Dehydration removes water and lipid content, resulting in a protein rich sample with a more homogenous refractive index (RI) (Richardson and Lichtman, 2015). Samples were then bleached to remove pigment by overnight incubation in 6% hydrogen peroxide (H_2O_2)/methanol (100%) at 4°C. Bleaching reduces protein autofluorescence (Tainaka *et al*., 2014). Following bleaching, samples were re-hydrated with two 1-hour washes in 100% methanol, followed by 1-hour incubations in 80% methanol/PBS and 50% methanol/PBS. Samples were then stored overnight in PBS at 4°C.

Tissue clearing and immunostaining:

Clear, unobstructed brain imaging cocktails and computational analysis (CUBIC) (Susaki *et al*., 2014; Susaki *et al.*, 2015) was used for tissue clearing with an adapted protocol from (Nojima *et al.*, 2017). Samples were washed briefly in PBS prior to incubation in 50% CUBIC1/PBS overnight (compositions outlined in Table 5). All incubation steps were performed at 37°C in a HB-1000 Hybridization oven with a rotating motor. Samples were then incubated in 100% CUBIC1 for 2-7 days. Sufficient clearance was achieved when most pigment was removed, and tissue was nearly fully transparent. Samples were washed once in PBS for 2 hours, followed by a PBS wash overnight and final 2 hour wash the next day. Primary and secondary antibodies were diluted to the desired concentration in PBS supplemented with 0.1% Triton X-100 and 0.5% blocking agent (serum the secondary antibody was raised in). Alternatively, SuperBlock PBS (Thermo Fisher Scientific) with 0.1% Triton-X 100 was used as a replacement blocking agent. Samples were incubated for 3 days in primary or secondary antibody solution, with three 2-hour PBS washes in between and after immunostaining steps. The final clearing step was performed by incubation in 30% sucrose/PBS overnight, a further

night incubating in 50% CUBIC2/PBS and two nights in 100% CUBIC2. Final incubation in CUBIC2 was performed at different dilutions to achieve optimum clearing.

Table 5. CUBIC1 and CUBIC2 compositions for tissue clearing, as described in Nojima *et al*. (2017). CUBIC1 removes lipids and CUBIC2 matches the refractive index (RI) of the sample to the reagent. Urea/Tetrakis solution was incubated in a heated shaker or water bath (50°C) to aid dissolution of urea. Triton X-100 was added at RT and mixed thoroughly before incubating samples.

	Reagent	Provider	Cat. number	Concentration (w/w%)
CUBIC1	Urea	Sigma-Aldrich	GE17-1319-01	25
	N,N,N′,N′-Tetrakis(2-hydroxypropyl)ethylenediamine	Sigma-Aldrich	BCBZ7575	25
	Triton X-100 (100%)	Sigma-Aldrich	-	15
	Distilled H_2O (dH_2O)	-	-	35
CUBIC2	Sucrose	Sigma-Aldrich	BCBX4074	50
	Urea	Sigma-Aldrich	GE17-319-01	25
	2,2′,2″-Nitrilotriethanol (triethanolamine)	Sigma-Aldrich	1168857	10
	Triton X-100 (10%)	Sigma-Aldrich	-	1
	dH_20	-	-	14

Imaging:

Foetal pancreases were imaged using the Zeiss Z1 LSFM microscope (Carl Zeiss AG, Oberkochen, Germany). Samples were mounted onto the stage inside the 5x/0.16 or 20x/1.0 chambers and immersed in CUBIC2 at the desired concentration for RI matching to the respective objective lenses. Zeiss' ZEN Microscope Software was used for all LSFM imaging. Orientation of samples was achieved using the manual motor controls and bright field light detection. Zoom was adjusted to ensure the whole sample was in the field of view. The Z1 light sheet has 5 different laser channels that can illuminate the same number of fluorophores, allowing a wide range of staining to be performed. The camera was manually focussed, and laser power altered for the optimum contrast. The light sheet has two lasers; samples were imaged using either the left or right lasers, based upon proximity to the sample and resulting focus, for preliminary imaging. Later the settings “dual side when experiment” and “online dual side fusion” were used, in order to merge left and right

laser images. “Z-stacks” were created by hundreds of alternating images being taken from the left or right side along the Z-axis of the sample, allowing a full 3D image to be created when Z-stacks were combined. Images were saved as .czi and .ims file type, the latter being required to enable image processing in IMARIS.

Image processing:

IMARIS (Bitplane AG, Andor, Zurich, Switzerland) was used for the 3D reconstruction of Z-stacks, allowing visualisation of pancreas structures. High intensity fluorescent residues were then removed. Surfaces were created, and threshold background subtraction manually adjusted to include the region of interest (ROI), but also to exclude the artefacts. Voxel size was set by trial and error (for example 10µm) and masks created to only contain the ROI surface, whilst removing the artefact surface. To do this, voxels were either set outside or inside the surface to zero, respectively. Slices of the model could be viewed at any point, allowing study of internal structures. The Snapshot feature was used for screenshots. The default 360° animation feature was used to create 150 frames per second (fps) movies (Appendix 1). Arivis 4D (Arivis AG, Munich, Germany) was also used for stitching together image sets from 0°, 90°, 180°, and 270°, creating a more complete 3D image.

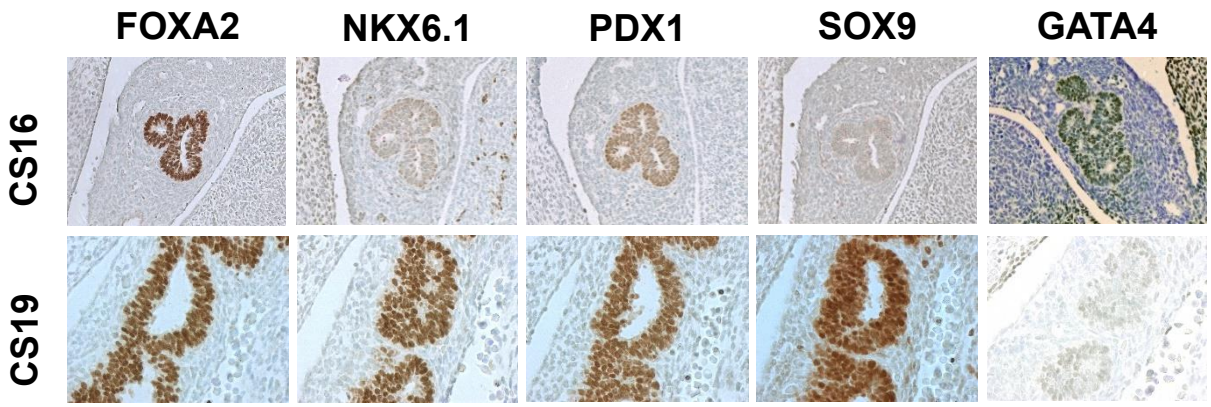
Results:

Key pancreatic TF expression profiles during embryonic and foetal development:

From CS16 (37-40 dpc), fusion of the ventral and dorsal buds has occurred, with epithelial cells forming branched structures one to two cells thick. Definitive endoderm marker, FOXA2, is still strongly expressed at this stage (Figure 7), persisting in CS19 (45-47 dpc) embryos. NKX6.1 and PDX1 expression increases from CS16 to CS19, with expression of another PPC marker, SOX9, also drastically increasing from CS19. Throughout foetal development, PDX1 is expressed in the ducts in a similar expression pattern to SOX9. The sagittal CS19 sections also demonstrate strong expression of SOX9 and PDX1 in the duodenum.

Distinction between peripheral and central cells starts to be observed from CS16 (Figure 6). For example, GATA4 appears localised to the “tip” cells and is not strongly expressed elsewhere in the pancreas at CS16. Although staining is very weak for GATA4 at CS19, detectable GATA4 expression remains constrained to the peripheral “tip” cells. The spatial expression pattern of GATA4 is maintained at 10 wpc, with the nuclear expression of NKX6.1, PDX1 and SOX9 in the central trunk region. Expression of CPA1 as an acinar cell marker is not detectable for 10 wpc or 14 wpc pancreases. Nevertheless, CPA1 is detected from 18 wpc in the acinar “tip” regions. NKX6.1 expression is mainly ductal from 14 wpc, but also appears outside the main branched structures in β-cell islets. Therefore, NKX6.1 localises with PDX1. NKX2.2 expression is observed outside of the “trunk” or “tip” cells from 8 wpc in a similar expression pattern to insulin, indicating its presence in β-cells as previously described (Jennings *et al.*, 2013).

Expression of insulin and glucagon are both detectable from week 8 of foetal development, with slightly higher levels of insulin detected (Figure 6). There is a subsequent large expansion in β-cell and α-cell islet populations between 8 and 10 wpc, with insulin and glucagon production respectively increasing throughout foetal development. Arrows indicate the genuine glucagon expression for 8-10 wpc, with a large amount of background observed. When specific staining is compared between insulin and glucagon, expression appears largely distinct.



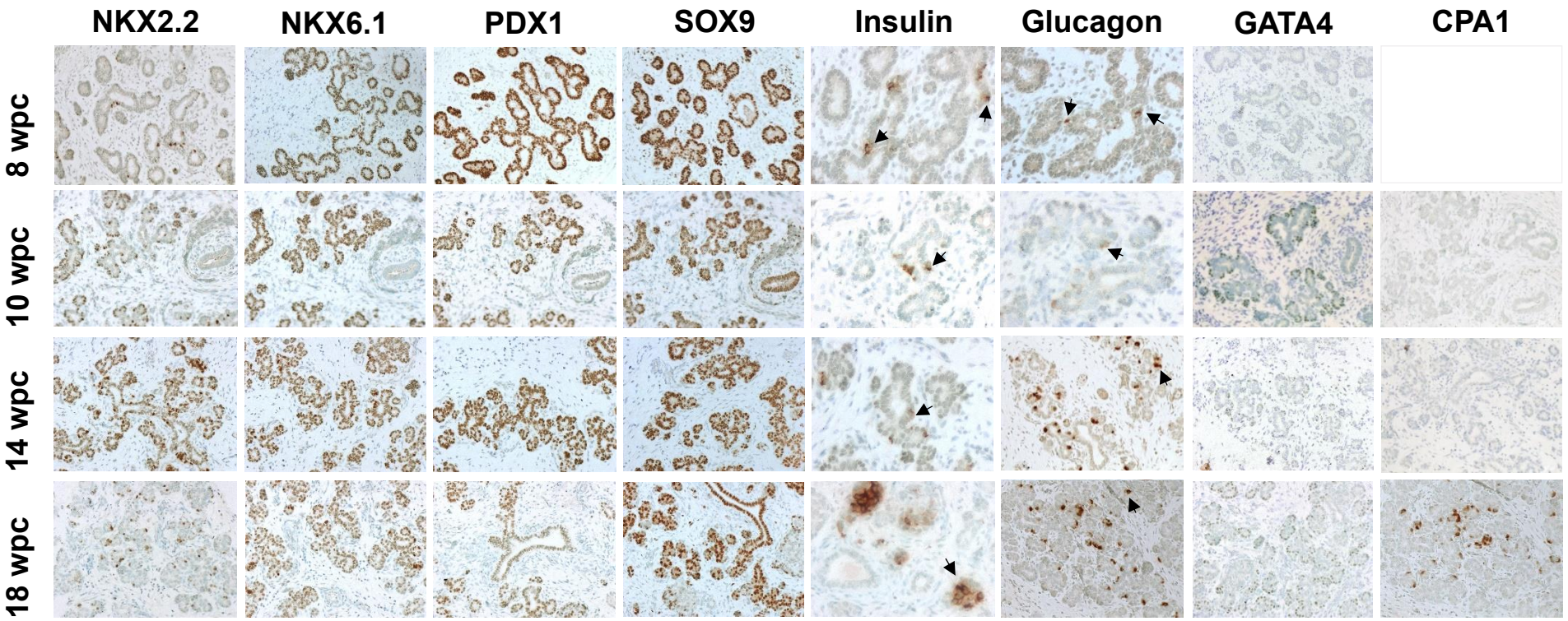


Figure 6. Immunohistochemistry staining for the key TFs active during embryonic and foetal pancreas development. Bright-field images counter-stained with toluidine blue at 20x or 40x (CS19 and INS and GLU) magnification. Pancreas Progenitor cell (PPC) markers NKX6.1 and PDX1 are active during early embryonic and persist throughout foetal pancreas growth. Endocrine cells producing insulin and glucagon are apparent from 8 wpc but increase considerably in number by 10 and 14 wpc. Arrows indicate genuine staining.

Organoid maintenance and differentiation:

Foetal pancreatic tissue was processed for organoid formation using the previously described method (Greggio *et al.*, 2013), as detailed in Figure 7. After culture of organoids for up to 30 days, organoid protein expression was characterised by immunofluorescence (Figure 8). Gene expression and flow cytometry were to be analysed too.

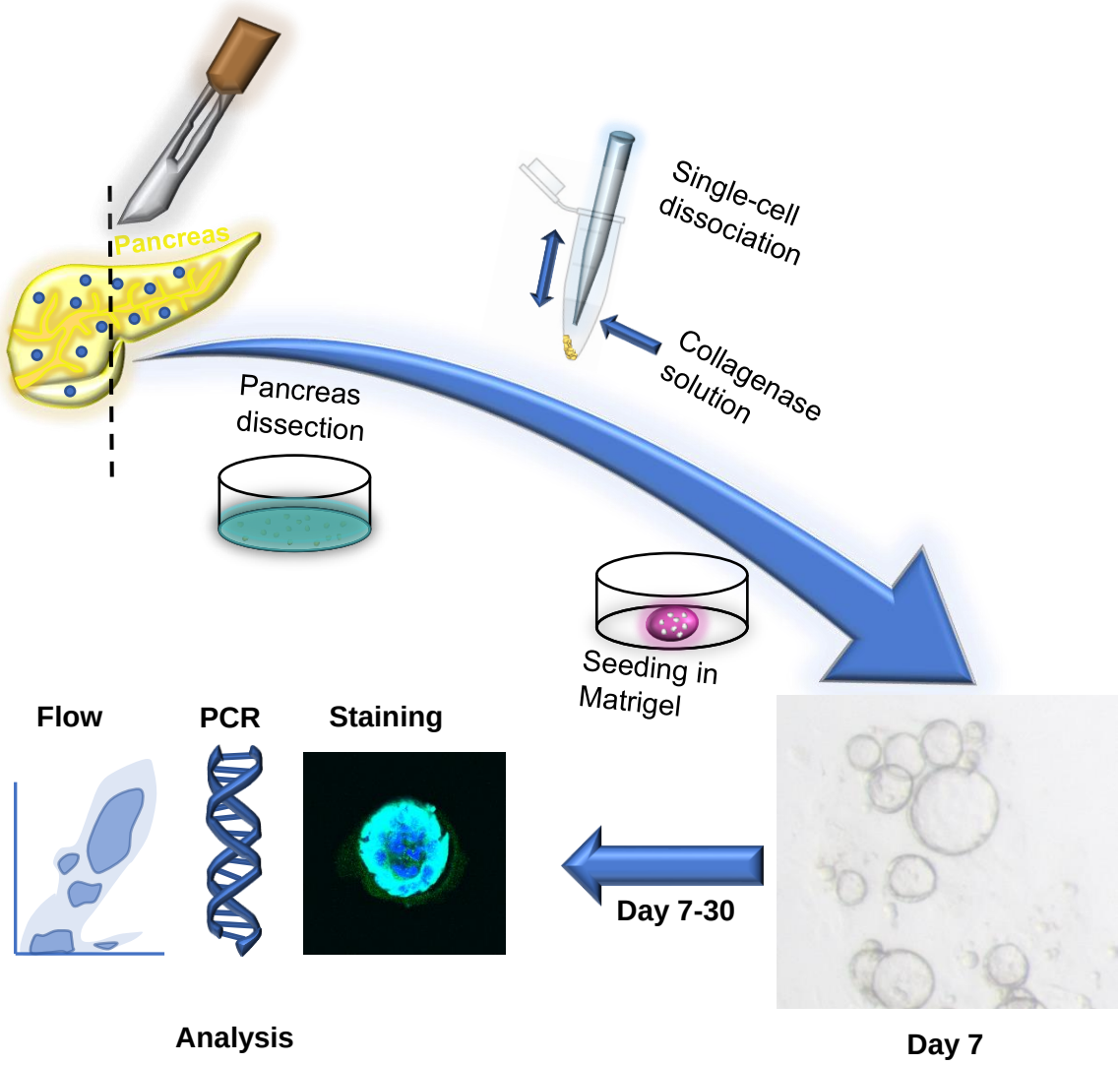


Figure 7. Schematic depicting the process of organoid formation from foetal pancreatic tissue. Samples are first dissected using sterile blades and cut into small 2-3mm pieces, whilst immersed in PBS. A single-cell-suspension is generated by multiple spin-PBS wash steps of pancreas fragments at 200g for 5 minutes at 4°C. Collagenase IV is then applied for 40 minutes at 37°C, with further mechanical dissociation of cells. The single-cell suspension is then seeded in Matrigel and incubated at 37°C before addition of pEM media for organoid maintenance. After 7 days, organoids form a round morphology and are ready to be split or be processed

for immunostaining, gene expression or flow cytometric analysis. Day 7 and staining images are for organoids generated from 10 wpc foetuses. Staining is for CK19 (cyan), DAPI (blue) and NEUROG3 (green), as taken with an inverted confocal microscope. Images are not to scale.

Organoids display ductal phenotypes:

Immunofluorescent staining of day 30 12 wpc organoids appears to show that the pEM maintenance media encourages formation of ductal populations, as evident by very strong cytokeratin 19 (CK19) protein expression (Figure 8A). CK19 is expressed in all foetal pancreas epithelial cells, becoming excluded when insulin starts to be expressed (Piper *et al.*, 2004). Further interpretation of organoid characterisation via immunofluorescence would require additional stains with the presence of negative and positive controls to determine whether the protein is genuinely absent or if the staining is a result of technical limitations. For example, unstained organoids and organoids stained for primary and secondary antibodies, in isolation or combination, would account for non-specific binding. Autofluorescence would be normalised by keeping exposure constant across samples and negative controls. A positive control of foetal pancreatic and liver tissue could be included too.

Although staining is detectable for NEUROG3 (Figure 8B), results are likely invalid. The signal is not nuclear, which is where NEUROG3 should mainly appear with some expression detected in cytoplasmic vesicles (Domínguez-Bendala *et al*., 2005). Cytoplasmic NEUROG3 has been reported *in vitro* and *in vivo* of the developing mouse hippocampus (Simon-Areces *et al*., 2013); repeat staining would determine whether the signal is nuclear or cytoplasmic. Therefore, NEUROG3 signal is likely non-specific antibody binding. Results for the PPC markers, NKX6.1 and PDX1, are inconclusive (Figure 8C). Figure 8B and 8C show the staining profile for two separate organoids collected from the same passage and stained under the same conditions. Expression for both markers appears highly non-specific and although SOX9, another PPC marker, is detectable in Figure 8B, SOX9 is not observed in Figure 8C. This indicates that the staining observed in Figure 8B may be non-specific staining, which is likely as the staining is mainly non-nuclear, or that the staining procedure was unsuccessful for the organoid imaged in Figure 8C. Overall, results indicate that the staining for these markers must be repeated to confidently characterise the organoids.

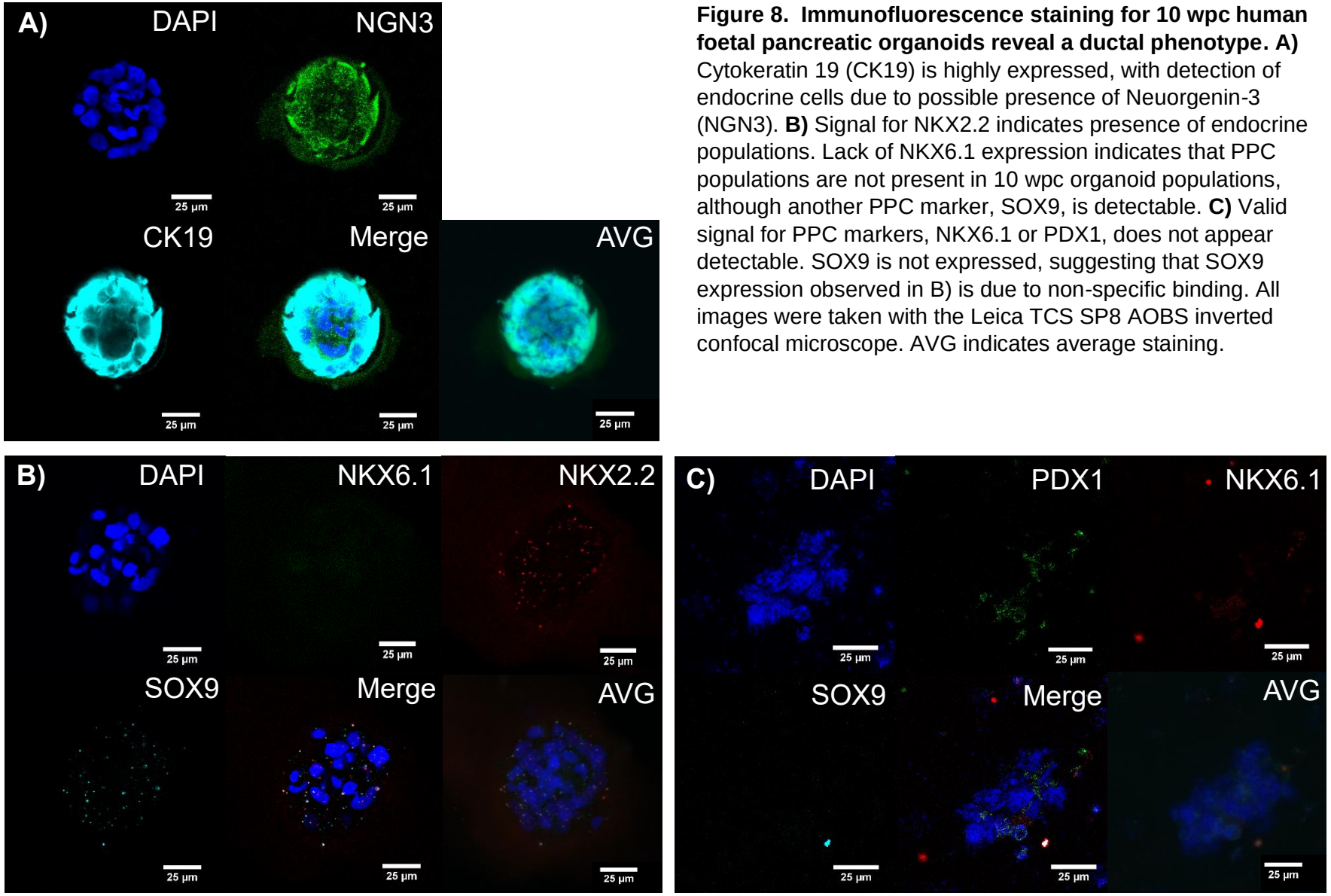


Figure 8. Immunofluorescence staining for 10 wpc human foetal pancreatic organoids reveal a ductal phenotype. A) Cytokeratin 19 (CK19) is highly expressed, with detection of endocrine cells due to possible presence of Neuorgenin-3 (NGN3). **B)** Signal for NKX2.2 indicates presence of endocrine populations. Lack of NKX6.1 expression indicates that PPC populations are not present in 10 wpc organoid populations, although another PPC marker, SOX9, is detectable. **C)** Valid signal for PPC markers, NKX6.1 or PDX1, does not appear detectable. SOX9 is not expressed, suggesting that SOX9 expression observed in B) is due to non-specific binding. All images were taken with the Leica TCS SP8 AOBS inverted confocal microscope. AVG indicates average staining.

Light sheet immunofluorescence microscopy as a tool for investigating pancreas development:

Light sheet immunofluorescence microscopy (LSFM) uses a thin sheet of light to image samples in the focal plane, allowing for thousand of individual “z-stacks” to be combined into a 3D image (Richardson and Lichtman, 2015). The use of a light sheet enables faster image scanning, a high signal-to-noise ratio, and reduced photobleaching and phototoxicity compared to confocal microscopy, which relies upon a pinhole of light (Chatterjee *et al.*, 2018; Jemielita *et al*., 2013; Reynaud *et al.*, 2008). The development of tissue clearing techniques has facilitated the rapid scanning of large samples, with a greater in-depth resolution compared to confocal microscopy (Richardson and Lichtman, 2015). Confocal microscopes will struggle with imaging depths beyond 1-2mm, without the use of expensive dipping objectives. Furthermore, LSFM can image large samples in real time, making it a vital tool for studying mammalian development; the high spatial and temporal resolution of LSFM enabled the study of the spindle assembly machinery in mouse zygotes with low phototoxicity (Reichmann *et al.*, 2018).

LSFM was utilised to image foetal pancreases in 3D (Figure 9). Sox9 expression is observed in the acinar and ductal cells of 9-20 wpc human foetal pancreases (Figures 9-13), as well as the duodenum of E13.5 mouse embryos (Figure 9E). 100% CUBIC2 provides poor focus with both the 5x and 20x objective, due to an unmatched refractive index (RI). Therefore, future experiments diluted CUBIC2 in PBS to final concentrations of 85% (Figures 11-12) or 80% (Figures 13-14). Clearing effectiveness discrepancies were not evident (data not shown) and 80% CUBIC2 provided the greatest focus (Figure 13). SOX9/PDX1 co-expression was analysed in 20 wpc human foetal pancreas (Figure 9). SOX9 is expressed strongly in both acinar and ductal cells, with the branched pancreas morphology apparent (Figure 10A). PDX1 also shares the expression pattern of SOX9; there is weak PDX1 expression in the ducts and stronger in the periphery of acinar cells (Figure 10B). Independent PDX1 localisation compared to SOX9 indicates the presence of β-cells, which are located away from acinar cells (Figure 10C). IMARIS was used for the 3D reconstruction of z-stacks, showing the spatial expression of SOX9 and PDX1 across all pancreas sections (Figure 10D-F). Co-expression of SOX9 and GATA4 were compared between 12 wpc and 20 wpc human foetal pancreases as well (Figure 11). The staining of 12 wpc foetal pancreas appeared unsuccessful (Figure 11A-B), although GATA4 autofluorescence could be used to trace blood vessel structures in 3D (Figure 11B). Analysis of individual z-stacks revealed

delicate expression of GATA4 at the periphery of acinar cells in 20 wpc foetal pancreas (Figure 11C-D). However, fluorescent aggregates were more apparent when 3D reconstruction was performed (data not shown).

Bright fluorescent patches are present in most LSFM images of CUBIC2-cleared pancreases. This indicates that the clearing or staining processes may result in artefacts. Although the fluorescent artefacts could be removed artificially using IMARIS thresholding techniques (Figure 12), there were concerns that genuine signal could be removed. This is apparent for the 9 wpc human foetal pancreas (Figure 12D). Dark patches appear when the bright fluorescent signal is removed from within the pancreas itself, as opposed to mainly from the surrounding tissue for the E13.5 mouse pancreas (Figure 12C). There is also the possibility that the high fluorescent patches were due to inadequate antibody penetration, resulting in an aggregation of antibodies. Therefore, the clearing and staining processes were investigated.

Unstained samples indicate no background autofluorescence (Figure 14A). Individual red and far-red secondary staining were performed to determine the extent of non-specific binding to endogenous cell proteins (Figure 14B-C). Minimal fluorescent signal was detected, showing high antibody specificity. More high-intensity fluorescence was detected for red compared to far-red secondary; far-red was used for all future experiments. $NaBH_4$, included to reduce potential amine-aldehyde residues after prolonged PFA fixation, provides clear fluorescence (Figure 14G+J)). Next, the effect of blocking on non-specific binding was investigated. The use of Superblock (Thermo Fisher Scientific) appeared to have no improvement on antibody specificity or staining (Figure 14H+K). Staining is potentially improved when $NaBH_4$ pre-treatment is included with the Superblock blocking step (Figure 14 I+L), indicating that further investigation of $NaBH_4$ is warranted.

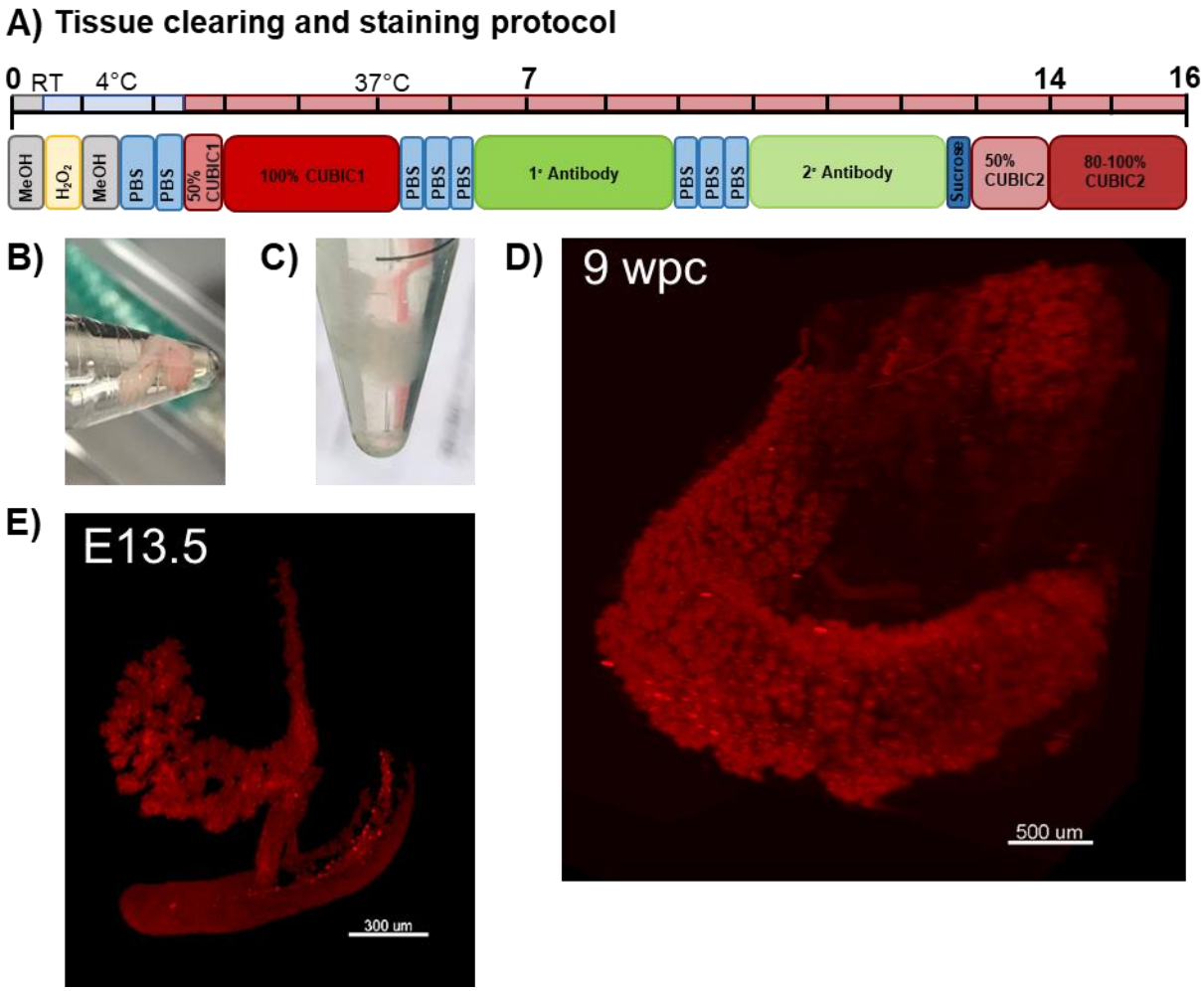


Figure 9. Imaging of mouse and human pancreas using light sheet immunofluorescence (LSFM). A) Protocol for preparing tissue for LSFM, after fixation in 4% PFA and storage in 70% ethanol. Day 1 involves dehydration steps in methanol (MeOH) at room temperature (RT). Samples are then bleached overnight in hydrogen peroxide (H_2O_2). On Day 2, samples are rehydrated in a series of methanol washes and washed overnight in phosphate buffered saline (PBS) at 4°C. From day 3, samples are cleared and stained at 37°C, with the previously described protocol (Nojima *et al*, 2017). **B)** Uncleared human foetal pancreas. **C)** CUBIC2-cleared human foetal pancreas. **D)** LSFM imaging of SOX9-stained 9 wpc human foetal pancreas using 100% CUBIC2. **E)** LSFM imaging of SOX9-stained E13.5 mouse pancreas and duodenum using 100% CUBIC2. SOX9 1:5000; images taken using the 5x objective and 3D reconstructions created using IMRAIS.

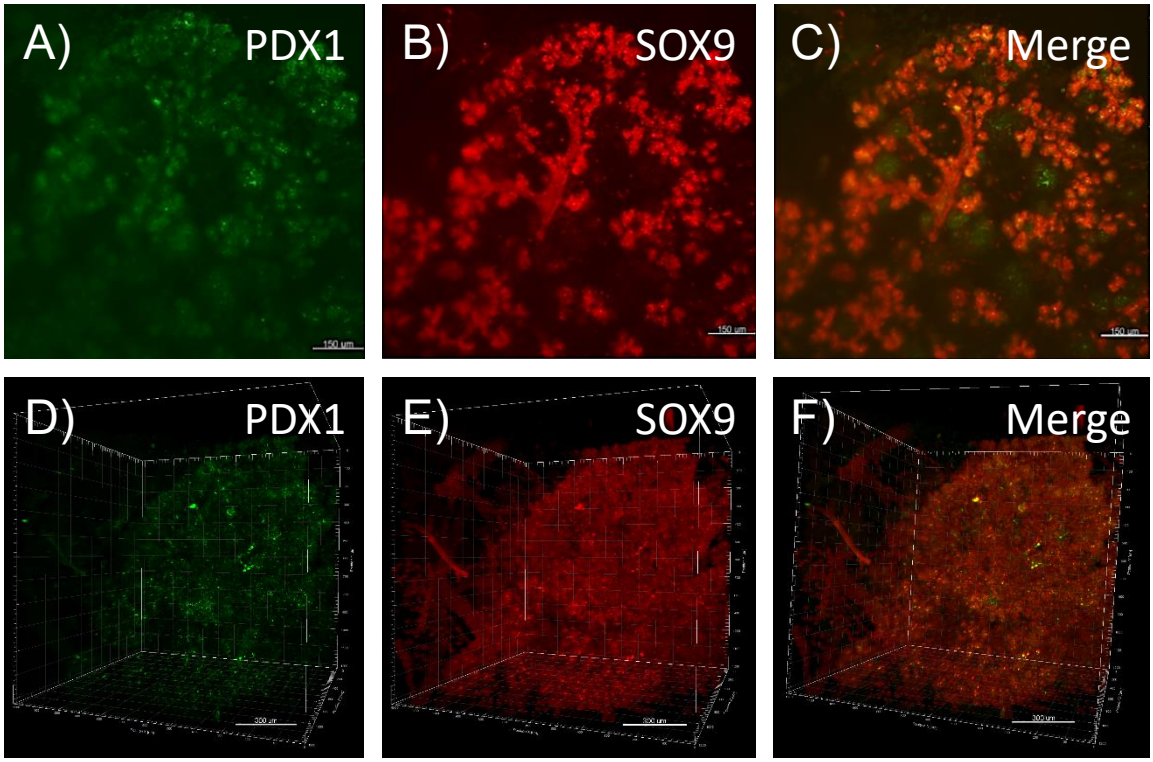


Figure 10. LSFM imaging of 85% CUBIC2-cleared 20 wpc human foetal pancreas stained for PDX1 and SOX9. A-C) Individual 2D z-stacks of PDX1, SOX9 and combined PDX1/SOX9 immunofluorescence, respectively. **D-F)** 3D reconstructions of PDX1, SOX9 and combined PDX1/SOX9 immunofluorescence, respectively. SOX9 1:5000; images taken using the 20x objective and 3D reconstructions created using IMRAIS.

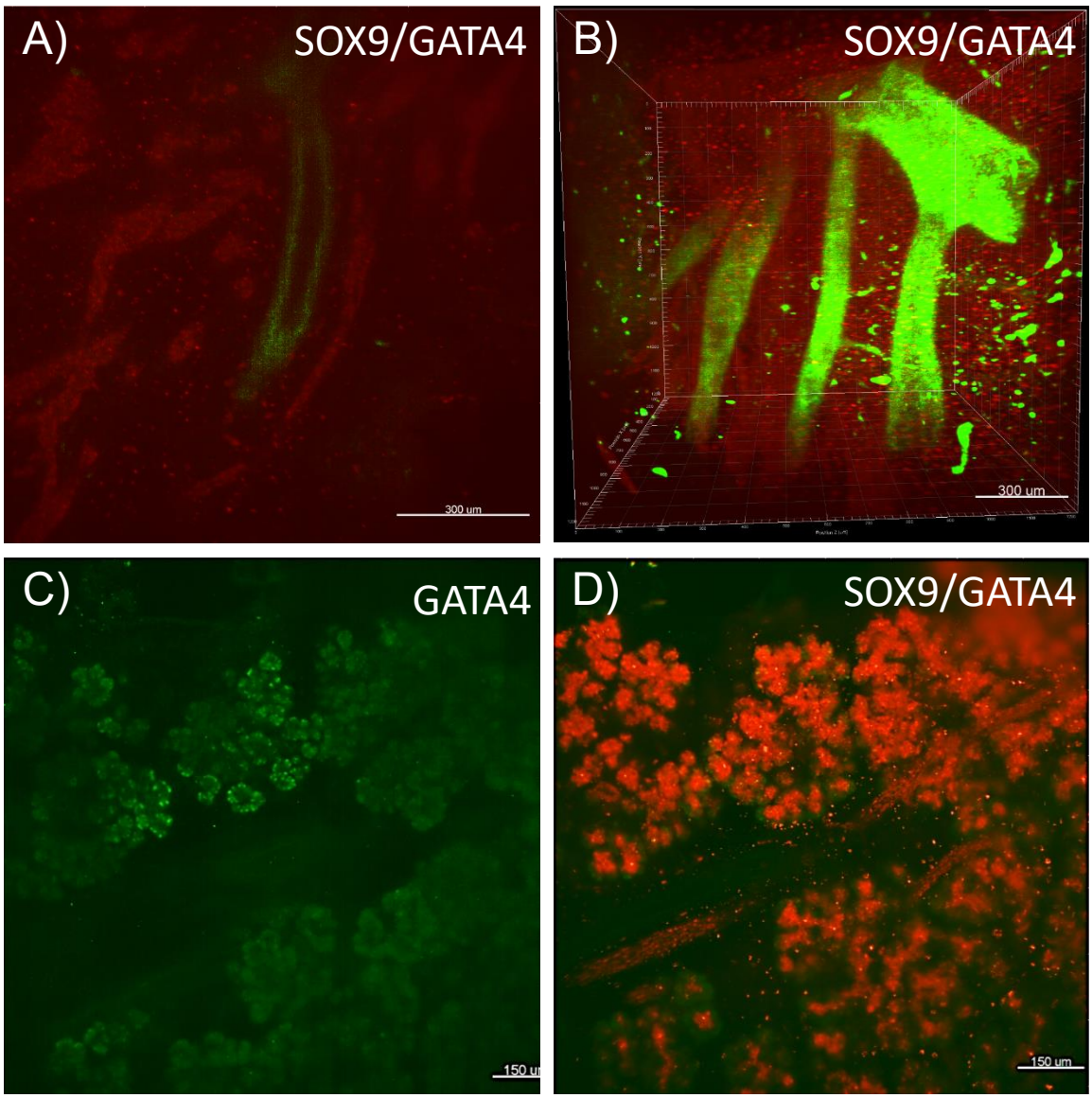


Figure 11. LSFM imaging of 85% CUBIC2-cleared human foetal pancreas stained for SOX9 and GATA4. A-B) 2D z-stack and 3D reconstruction, respectively, for SOX9/GATA4 immunofluorescence in 12 wpc human foetal pancreas. GATA4 autofluorescence outlines blood vessels. **C-D)** Individual 2D z-stacks for GATA4 and combined SOX9/GATA4 immunofluorescence, respectively. SOX9 1:5000; images taken using the 20x objective and 3D reconstructions created using IMRAIS.

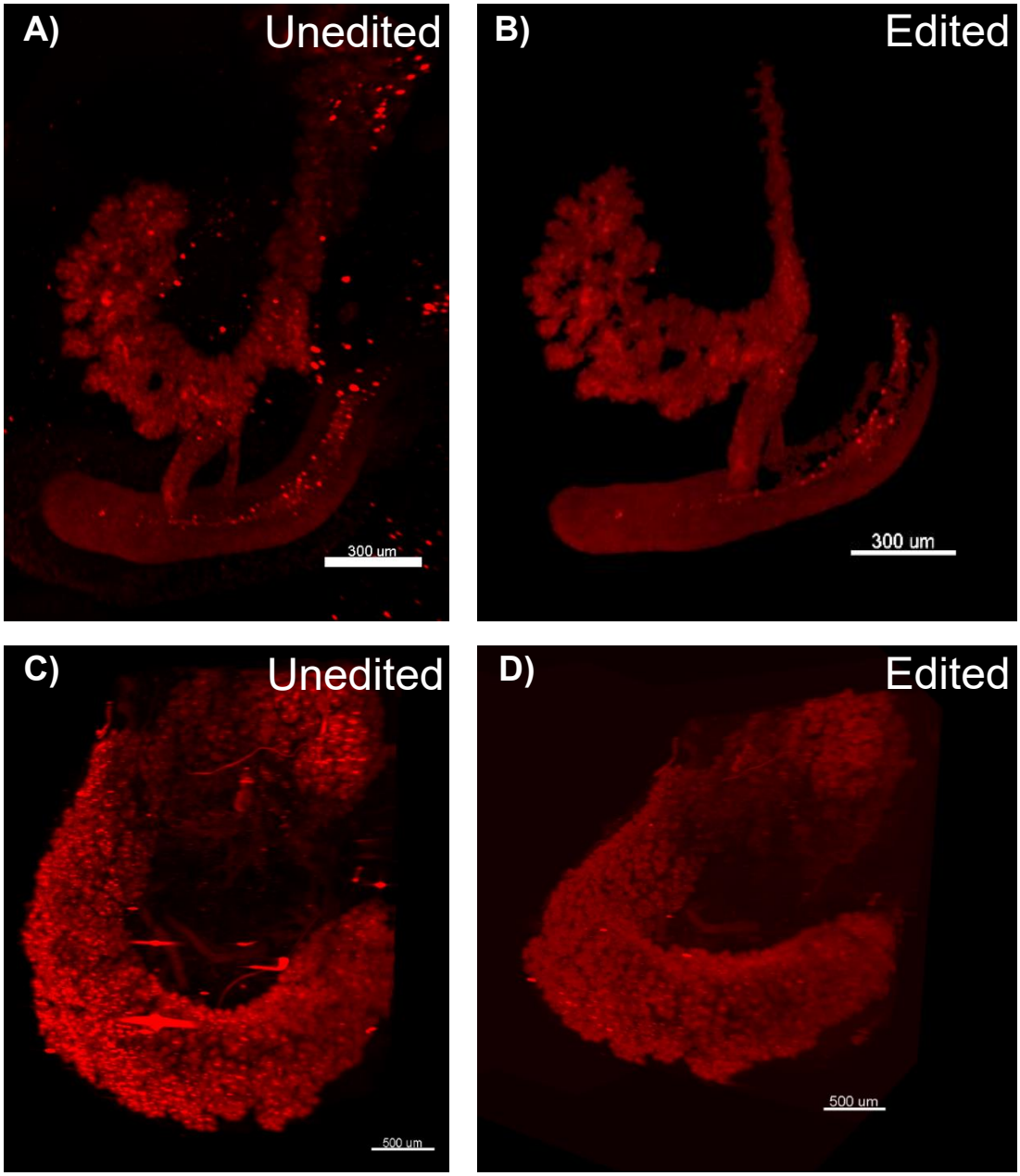


Figure 12. 3D reconstructions can be created and edited to remove fluorescent artefacts using IMARIS. A-B) Sox9-stained E13.5 mouse pancreas and duodenum. **C-D)** Sox9-stained 9 wpc human foetal pancreas. A and C are raw 3D reconstructions using IMARIS; B and D are edited using thresholding to remove noise.

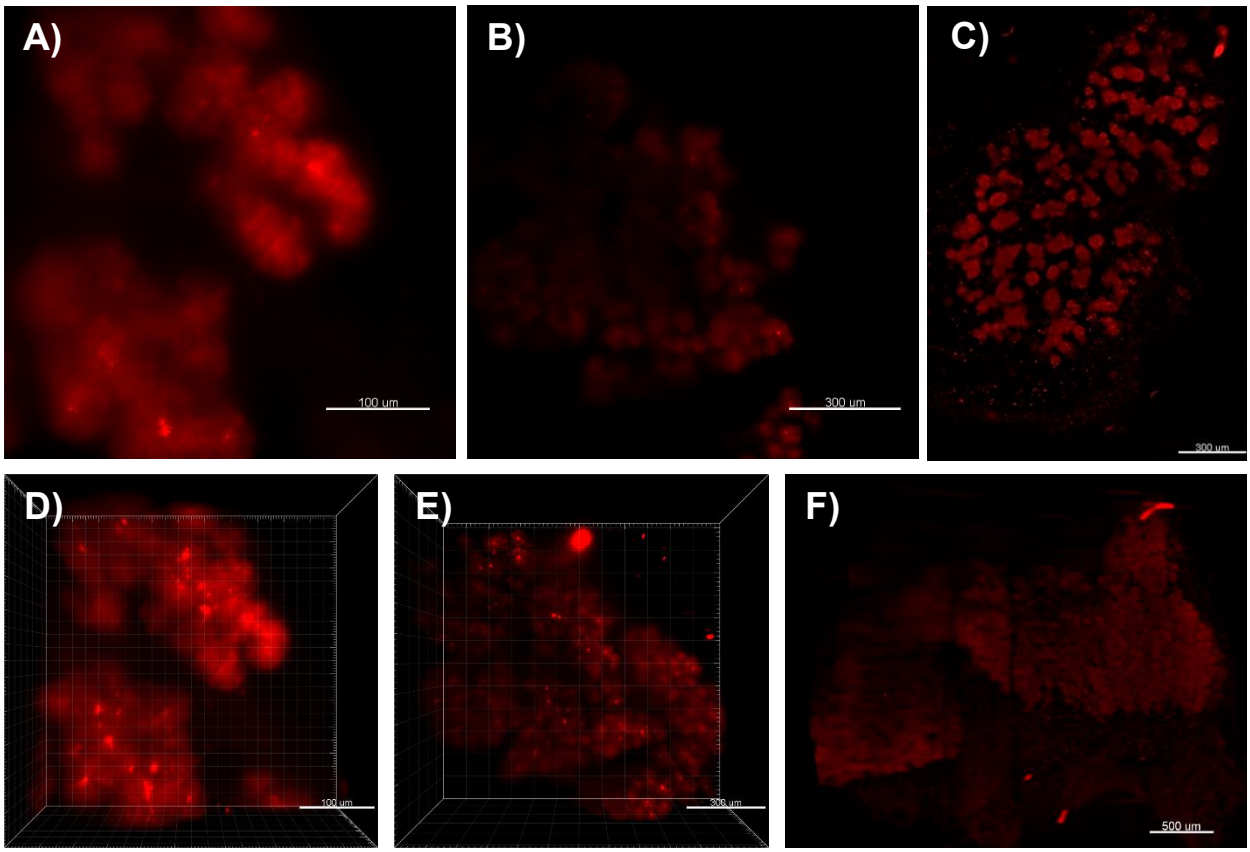


Figure 13. 80% CUBUIC2 provides adequate clearing and the optimal focus for LSFM imaging of pancreases. A +D) 100% CUBIC2 cleared 9 wpc foetal pancreas imaged with the 20x objective. **B+E)** 100% CUBIC2 cleared 9 wpc foetal pancreas imaged with the 5x objective. **C+F)** 80% CUBIC2 cleared 10 wpc foetal pancreas imaged with the 5x objective. 3D reconstructions (D-F) were generated using IMARIS.

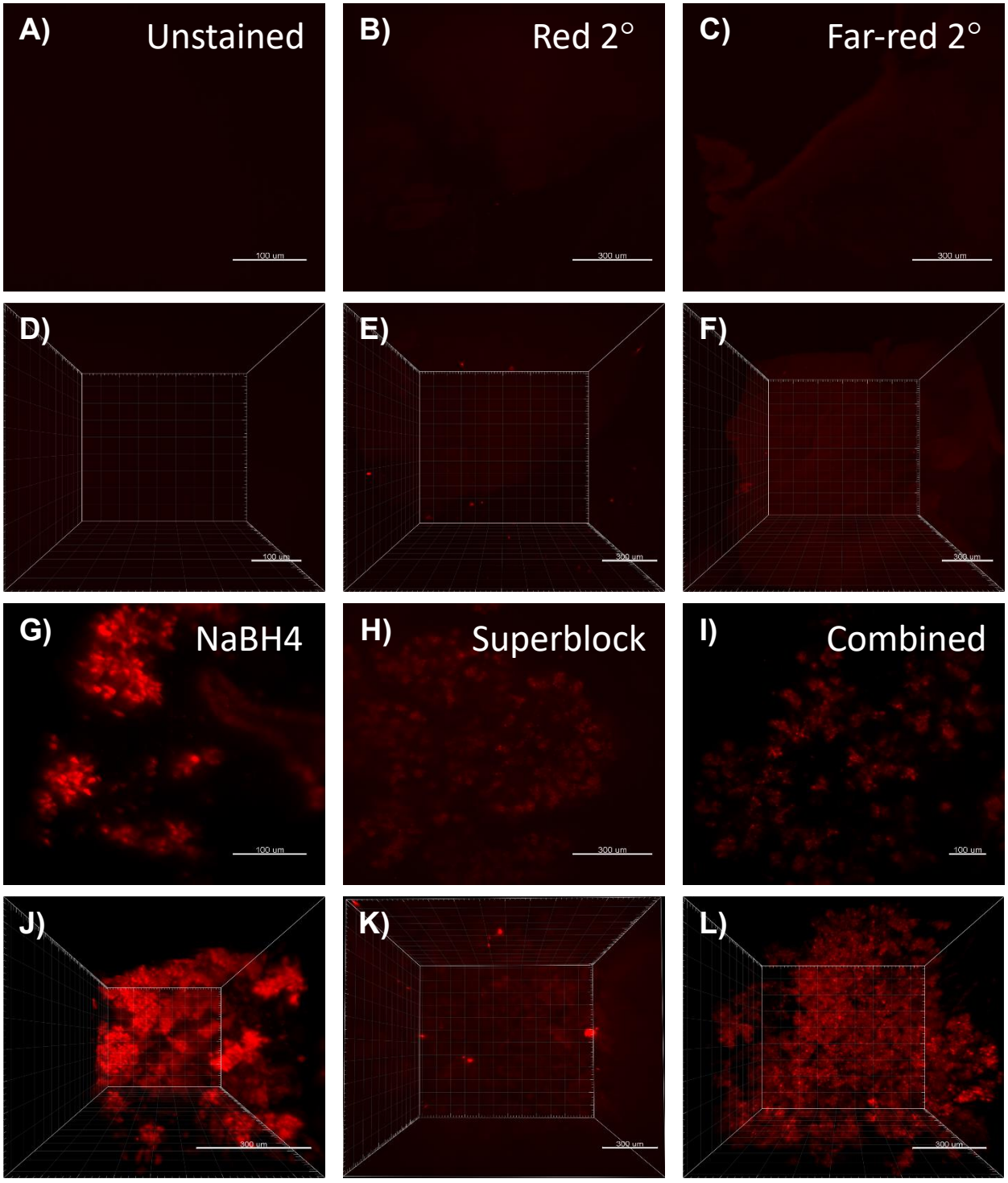


Figure 14. Optimisation of imaging conditions can improve specific fluorescence. A-C) Representative 2D z-stack images for unstained 80% CUBIC cleared pancreas sample, red secondary antibody (°) and far-red secondary antibody staining without SOX9 primary, respectively. **D-F)** 3D reconstructions of z-stacks shown from A-C, respectively. **G)** NaBH4 treated samples before clearing. **H)** Blocking using Superblock instead of Donkey serum. **I)** NaBH4 and Superblock treatments. **J-L)** 3D reconstructions of z-stacks shown from G-I, respectively. All

images were of a 17 wpc human foetal pancreas. SOX9 primary antibody was used at a dilution of 1:10,000. Secondary antibodies were used at 1:1500. IMARIS was used for 3D reconstruction. Fluorescence for all images was adjusted to: min:0, max: 3000, gamma:1.

Discussion:

Transcription factors active during pancreas development:

Identification of the main TF expression profiles during early human pancreas development has helped to shape PSC-derived β-cell differentiation programmes (Jennings *et al*., 2013), which previously relied upon mimicking the endocrine commitment events observed in other model systems, such as the mouse, chick and frog (Isaacs *et al.*, 1995; Pan and Wright, 2011). Immunohistochemistry staining for the main TFs active in embryonic and foetal samples revealed the expected expression of NKX6.1+/PDX1+/SOX9+ bi-potent progenitors within the central "trunk" regions, and GATA4+/CPA1+ cells located at the acinar "tips" (Figure 6). Low levels of insulin and very low levels of glucagon expression are observed from 8 wpc in endocrine islets adjacent to the central trunk regions. This supports the notion that CS21 embryos start producing insulin before glucagon has begun to be secreted from 8 wpc (Piper *et al*., 2004). The apparent lack of co-expression matches results for insulin-glucagon dual immunofluorescence, with only a very low proportion of cells co-expressing insulin and glucagon (De Krijger *et al.*, 1992; Piper *et al.*, 2004). There also appears to be an increase in β-cell areas between 10 and 14 wpc, which is consistent with Meier *et al*. (2010). Co-localisation of PDX1 with insulin expression is difficult to observe in foetal pancreas, due to the wide expression of PDX1 and slight discrepancies in section morphology. To see the expression of PDX1 and insulin in islet cells observed from 14 wpc in Piper *et al*. (2004), immunofluorescence would need to be performed on identical sections.

Although CPA1 is only detected from 18 wpc via immunohistochemistry (Figure 6), CPA1 is shown to be co-expressed with GATA4 from CS21 in the "tip" cells by immunofluorescence microscopy (Jennings *et al*., 2013). Therefore, lack of staining is likely due to inadequate antibody concentration and would be repeated. Only weak GATA4 expression is observed at the "tips" of acinar cells from CS19 (Figure 6), perhaps due to sub-optimal conditions and antibody concentration. LSFM also reveals the expression of GATA4 at the periphery of acinar cells in 20 wpc human foetal pancreas (Figure 11). This is consistent with the mutually exclusive expression domain for GATA4 and SOX9, as shown by immunofluorescence (Jennings *et al*., 2013). Further study of the spatial organisation of pancreas TFs could be performed in 3D using LSFM, providing a novel insight into human pancreas morphogenesis (Hong *et al*., 2019).

Despite considerable advancements in PSC-derived β-cell differentiation protocols recently (Nair *et al*., 2019; Russ *et al.,* 2015; Tremmel *et al.*, 2019; Velazco-cruz *et*

al., 2019), complete β-cell maturity and function are still to be attained. Generation of the large number of β-cells required for clinical transplantation has been a pertinent challenge, although improvements are being made (Dossena *et al.*, 2020). Therefore, investigation of new markers for PPCs could allow adaptation of PSC differentiation protocols to promote proliferation and dramatically expand PPC pools, prior to terminal differentiation towards β-cells. This would be enabled by further elucidating the regulatory networks of these markers.

Preliminary single nuclear RNA sequencing (sn-RNA seq) within our laboratory group has identified multiple genes encoding transcripts functioning as candidate PPC population regulators, including Cut-like homeobox 2 (CUX2) and single-minded homology 1 (SIM1) (unpublished data). Although the staining performed in Figure 6 for key pancreatic markers mainly complements previous research (Jennings *et al.*, 2013; Piper *et al*., 2004), the skills performed could be transferred to documenting the expression of these novel markers across a time-course of pancreas development. A deep understanding of fundamental immunohistochemistry techniques will be required for high-quality images with precious human tissue samples.

Identification of CUX2 or other TFs as key regulators in PPC maintenance could aid expansion of stem cells and improve our understanding of the steps occurring prior to terminal differentiation into β-cells, increasing the feasibility of β-cell transplants for treatment of type 1 diabetic patients. In addition, the development of gene editing techniques in pancreas organoid systems could be incredibly useful for studying genetic diseases that compromise pancreas development, such as maturity onset diabetes of the young (MODY), PNDM or pancreas agenesis. Genetic diagnoses are missing for many of these pancreatic developmental disorders. This suggests hitherto unidentified disease genes (Jennings *et al*., 2015; Petersen *et al.*, 2018).

Generation of a human foetal pancreatic organoid system:

Our work has shown that the pEM maintenance media used for the expansion of mouse embryonic pancreas organoids (Greggio *et al*., 2013) allows for the successful expansion of human foetal organoids. Human foetal organoids generated from a range of tissue ages (10 wpc-16 wpc) could be maintained for up to 30 days and appeared to display a mainly ductal phenotype (Figure 8). Alternatively, only ductal cells may have survived cell culture with our growth factor regime and other transcription factors remain to be characterised. Detection of non-nuclear NEUROG3 expression is likely due to non-specific antibody staining and background fluorescence commonly associated with the green AlexaFluor 448; high

non-specific signal is observed for PDX1 (Figure 8C). However, NKX2.2 expression is also detectable, in some cases in the nuclei. If genuine signal, this would indicate the presence of endocrine populations, supporting endocrine commitment driver, NEUROG3, activity in organoids (Jennings *et al.*, 2013; Jeon *et al*., 2009; Lyttle *et al.*, 2008; Piper *et al.*, 2004; Sussel *et al.*, 1998). In addition, there is no fluorescent signal for NKX6.1 in the green Alexa Fluor 488 channel, contradicting the NEUROG3 staining being due to autofluorescence (Figure 8B). Presence of islet cells is expected after the induction of NEUROG3 from 8 wpc in humans (Jennings *et al*., 2013). Thus, immunohistochemistry staining for insulin and glucagon supports low protein expression from 8 wpc, which increases considerably by 14 wpc (Figure 6).

Other papers report a shift of bi-potent progenitor organoid populations towards ductal cells when maintained in culture (Bonfanti *et al*., 2015; Greggio *et al.*, 2013; Huch *et al.*, 2013b). Although no branching is observed, the presence of other cell types (NKX2.2 expression for endocrine cells in Figure 8B) justifies the classification of the clusters of cells as organoids, as opposed to spheroids with different morphologies (Grapin-Botton, 2016). Lack of EGF, which is reported to inhibit endocrine differentiation (Bonfanti *et al*., 2015; Greggio *et al.,* 2013), could explain the presence of NKX2.2. Furthermore, this indicates that our culture system is not optimised for organoid expansion, with different conditions needing to be tested.

The impact of the transition from pEM maintenance media to a differentiation media on organoid morphology would be of interest. It would be expected that similar organisational changes would be observed in human foetal pancreatic organoids compared to mouse pancreatic organoids. For example, microlumens would likely initially form, followed by branched structures with acinar cells localised to the tips, as well as multipotent HNF1b+/SOX9+/PDX1+ cells, endocrine progenitor populations and terminally differentiated islet cells in the centre (Dahl-jensen *et al.*, 2016; Greggio *et al*., 2013). Furthermore, incomplete terminal β-cell differentiation *in vitro* has been suggested to be due to lack of maturity signals that usually occur postnatally (List and Habener, 2004; Ohmine *et al*., 2012).Therefore, inclusion of GLP1 in the differentiation media may promote endocrine differentiation (Davis and Sandoval, 2020). Notch signalling has a concentration dependent role in endocrine differentiation. For example, Greggio *et al.* (2013) report that notch is essential for mouse embryonic organoid maintenance and promoting SOX9 expression, which will then induce *NEUROG3.* However, small molecule-mediated inhibition of notch signalling in adult murine pancreas organoids promoted endocrine differentiation by

de-repressing NEUROG3 (Wedeken *et al.*, 2017). This indicates that a threshold of notch signalling is required for triggering and completing murine endocrine commitment, with a regulatory feedback loop (Shih *et al.*, 2012). High notch signalling inhibits endocrine differentiation, which remains to be addressed in human pancreatic organoid models.

Light sheet immunofluorescence microscopy (LSFM):

Up until recently most immunohistochemical and fluorescent staining has been performed on 2D slices created with microtomes. Serial sectioning can reconstruct 3D volumes, but the rendering is often poorly aligned with lack of focus (Oh *et al.*, 2014). Therefore, confocal microscopes have been designed to remove out-of-focus light, resulting in the development of laser-scanning and spinning disk confocal techniques, two-photon microscopy and LSFM (Mertz, 2011; Reynaud *et al*., 2008). Each optical section, referred to as a “z-stack”, can be combined to generate a 3D structure. 3D structures are essential when considering neuronal or branching pancreas networks (Hong *et al*., 2019). However, it is hard to image deep into samples with high resolution due to light scattering. Clearing the sample can alleviate this issue by removing lipids and membranes, resulting in homogenous scattering throughout the tissue when combined with pigmentation removal (Ariel, 2017; Tainaka *et al*., 2016). Reduced lateral scattering ensures that all wavelengths can penetrate the tissue.

Many methods for tissue clearing have been developed, as reviewed in depth by Richardson and Lichtman (2015). Care needs to be taken to match the refractive index (RI) of the clearing agent to the tissue and lens (Hell *et al.*, 1993), which is defined as “the ratio of the speed of light in a vacuum divided by the speed of light in the medium” (Richardson and Lichtman, 2015). CUBIC2 has an RI in the range of ~1.49 and functions by sucrose-urea-mediated hyperhydration (Table 5) (Richardson and Lichtman, 2015; Susaki *et al.,* 2014; Susaki *et al.*, 2015). This avoids the toxicity, tissue-shrinkage and fluorescence quenching concerns associated with solvent-based clearing (Becker *et al*., 2012). Our results demonstrate that a dilution of 80% CUBIC2 most greatly matches the LSFM 20x objective lens RI of 1.45 (Figure 9-13), although a refractometer needs to be used for an exact measurement. Cleared tissue can then be imaged using LSFM or other techniques. The slow laser scanning potential of confocal and two-photon microscopes limits them to very localised imaging. LSFM was chosen to image foetal pancreases, as it uses a single plane of light, resulting in far greater readout speed and allowing larger samples to be imaged (Richardson and Lichtman, 2015).

LSFM has been used to image adult mouse and human pancreases but there are currently no reports for the study of foetal pancreas development in the literature (Hong *et al.*, 2019); developing LSFM imaging methodology in foetal pancreas samples represents an incredibly exciting opportunity. B-cell distribution, islet-vasculature networks and Schwann cell plasticity (after β-cell ablation) have been investigated in adult mouse pancreases using confocal and LSFM microscopy (Fu *et al*., 2010; Kim *et al*., 2010; Lee *et al.*, 2014; Tainaka *et al.*, 2014; Tang *et al.*, 2013; Tang *et al.*, 2018a; Wong *et al*., 2017). Examples of cleared human pancreases are very limited (Butterworth *et al*., 2018; Fowler *et al.*, 2018; Noë *et al*., 2018; Tang *et a*l., 2018b). The neuro-insular network was mapped in detail in 3- and 8-week-old mouse and adult human pancreases using confocal microscopy, allowing comparison of ganglionic interactions during fatty infiltration (Tang *et al*., 2018a; Tang *et al.*, 2018b). Confocal tissue tomography of fructose-thioglycerol-cleared 1mm adult human pancreases revealed the relationship between islets and capillary density in 3D (Fowler *et al.*, 2018). Outside of diabetes research, development of LSFM methodology relevant to the pancreas is imperative for modelling the interaction of the vasculature, ducts and neuro-insular networks during cancer invasion in 3D (Hong *et al.*, 2019; Noë *et al*., 2018).

Previously, spatial visualisation and quantitative analysis of pancreas morphogenesis has been challenging, due to the limited imaging techniques not distinguishing fine structures or in enough detail (Hörnblad *et al.*, 2016). Optical projection tomography (OPT) has been used recently to outline the developmental time course of pancreas structures from early bud formation at E10.5, to fusion at E13.5 and full pancreas development by E17.5 (Hörnblad *et al*., 2011). Despite the effectiveness of OPT for revealing 3D structures, data acquisition is far slower and potential statistical analysis greatly reduced compared to LSFM (Isaacson *et al.*, 2020; Liu *et al*., 2019). We used LSFM to reveal the spatial expression of SOX9 within an E13.5 mouse embryo (Figure 9E), identifying SOX9 within the ducts, acinar cells, and duodenum.

Alternative clearing methods have been effective in pancreas and other tissues, as outlined (Hong *et al.*, 2019). For example, Passive CLARITY technique (PACT) uses a hydrogel embedding to allow slow penetration of SDS, reducing protein denaturation and improving clearing quality (Neckel e*t al*., 2016; Tainaka *et al*., 2016; Yang *et al.*, 2014). PACT has an RI of 1.45 and displays excellent clearing of foetal urogenital tissue for LSFM (Isaacson *et al.*, 2020). PACT was also utilised for the clearing of paraffin-embedded human adult pancreases to facilitate the 3D

reconstruction of neuro-insular networks (Butterworth *et al*., 2018). However, the increased image focus is not sufficient enough to justify the complex process of setting the sample in an acrylamide gel or purchase of expensive reagents for future investigation. Our CUBIC2 clearing method appears to more than adequately clear the tissue visually (Figure 9C), as well as providing well focussed images when cleared with RI matched 80% CUBIC 2 solution (Figure 13). The ECM is often not removed during the clearing process and blocks macromolecule access to target proteins (Calve *et al*., 2015; Lee *et al*., 2016). Therefore, antibody penetration remains the area to address for improved signal quality without fluorescent artefacts.

A distinct advantage of LSFM over confocal microscopy is the reduced photobleaching of samples (Reynaud *et al*., 2008). Therefore, LSFM is preferable for imaging weakly expressed proteins, which might have their expression masked when imaged via confocal microscopy. LSFM has also been used to image organoids of various tissue types with high resolution (Dekkers *et al.*, 2019). Resultantly, LSFM would be a valuable tool to characterise the expression profiles of PPC maintenance and endocrinogenesis TFs throughout murine and human pancreas development, in both embryonic and foetal tissue, as well as organoids. Overall, LSFM has great potential for studying human development and refinement of this technique could broaden our understanding of human embryology (Chatterjee *et al*., 2018).

Future work:

Further characterisation of organoids:

Organoid culture reproducibility can be challenging due to natural variation in samples, chemical compositions and individual cell interactions (Dahl-Jensen and Grapin-Botton, 2017). Moreover, the lack of samples and other unforeseen circumstances meant that further immunohistochemical characterisation of pancreatic marker expression could not be performed in the human foetal pancreatic organoid model. The expression patterns of existing stains were also unreliable. Therefore, immunofluorescence staining should be repeated, and further analysis of organoid protein expression performed. Mucin-1 should be analysed as a marker of duct polarisation (Greggio *et al*., 2013), as well as co-localisation of NKX6.1 and PDX1 to further investigate the presence of PPC populations within the foetal organoids. GATA4 and CPA1 co-localisation should additionally be investigated as acinar cell markers (Jennings *et al*., 2013). PSC- and iPSC-derived PPCs could be used as the *in vitro* comparison to the primary organoid system.

LSFM optimisation:

Multiple approaches can be considered for improving antibody penetration (Richardson and Lichtman, 2017). The initial PFA fixation step aids antibody penetration by dissolving cell membrane lipids, disrupting membrane integrity (Cheng *et al*., 2019; Fox *et al.*, 1985). A slight improvement in imaging quality may be observed when $NaBH_4$ is included to eliminate any Schiff's base reactions (Figure 14). A decrease of PFA concentration from 4% to 1% during the fixing stage aided antibody penetration in adult mouse brains (Gleave *et al*., 2013). Immunolabelling is always performed before the final clearing step to allow adequate antibody diffusion (Richardson and Lichtman, 2015). Our staining protocol already performs primary antibody incubation at 37°C with rotation, which is found to increase depth of antibody uptake in adult mouse brains and other systems (Richardson and Lichtman, 2017; Schwarz *et al*., 2015). Addition of an electric field or centrifugal force to a rotating system can result in improved antibody distribution, as well as reduced incubation times (Kim *et al*., 2015; Lee *et al*., 2016). Triton-X 100 is the most widely used non-ionic detergent for permeabilization, removing lipids from the cell membrane and allowing access to the nucleus (Jamur and Oliver, 2010). High concentrations are required to achieve optimal lipid removal. However, Triton-X 100 and urea both highly denature proteins (up to 41% protein loss) and decrease subsequent immunostaining strength considerably (Chung *et al*., 2013). Finally, some protocols clear and stain tissue for month-long periods (Richardson

and Lichtman, 2015); greater antibody incubation periods may be required to improve penetration and staining results.

Hydrogel effect on organoid and stem cell differentiation:

Matrigel is the staple substrate for 3D cell culture, especially for pancreas organoid formation (Bonfanti *et al.,* 2015; Greggio *et al*., 2013; Huch *et al*., 2013a; Loomans *et al.*, 2018). However, although the composition is similar to the BM surrounding pancreatic islets, there are also ~1500 other unique proteins which likely do not reflect the native pancreas ECM (Hughes *et al.*, 2010; Talbot and Caperna, 2015). Matrigel is also tumour-derived xenogeneic material, reducing clinical applications, and there is large batch-to-batch variability (Hughes *et al*., 2010; Vukicevic *et al.*, 1992).

Therefore, investigation of a range of designer matrices is essential for furthering organoid and PSC differentiation protocols (Aisenbrey and Murphy, 2020). Novel ECM components recently identified from the foetal human pancreas matrisome could be investigated (Ma *et al*., 2019; Sackett *et al*., 2018). Substrate stiffness also has a considerable impact on PPC maintenance or differentiation. The nuclear translocation of yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) is controlled by altering substrate stiffness (Elosegui-Artola *et al*., 2016; Shi *et al.*, 2020). Therefore, strain on organoids and stem cells in 3D cultures could be measured with atomic force microscopy (AFM) and the effect of altered hydrogel stiffness on subsequent proliferation or differentiation investigated (Candiello *et al*., 2018).

Conclusions and perspectives:

Diabetes has reached epidemic levels and current pharmacological treatments, especially for T1DM, are failing. T1DM patients can achieve euglycemia through insulin injections and pumps if glucose levels do not fluctuate drastically. If patients are predisposed to HAAF events, cadaver-derived islet are the best option, which will be overtaken by β-cells when more clinical trials are conducted, and differentiation protocols are improved. Safety concerns remain for both cadaver-derived islet cells or PSC and iPSC approaches, which are being addressed through encapsulation techniques and new immunosuppression regimes. Studying human development has revealed the fascinatingly complex interaction of TFs active during early foetal pancreas development, allowing growth factor and small molecule regimes to be modified to better simulate the signalling pathways active *in vivo*. The hurdle of consistently generating mono-hormonal β-cells appears to have been overcome; however, despite large improvements in β-cell function, they are still not fully mature.

The signals active during human foetal pancreas organoid differentiation still need to be optimised. Improved cell expansion and maturity could be achieved through the identification of new PPC markers, enabling directed endocrine commitment. Characterisation and gene editing of these novel markers could provide the missing link in pancreas PSC and primary organoid systems. Light sheet fluorescence microscopy has never been used to image human foetal or embryonic pancreases, with development of the technique allowing analysis of the spatial localisation of key TFs throughout development. LSFM is also compatible with imaging our organoid system; the 3D structural information provided will help to inform on how pancreas morphogenesis occurs. Moreover, 3D matrices are necessary for the correct cell-cell contacts and polarity during expansion and differentiation. Nevertheless, Matrigel is a limiting factor in correct β-cell function and translation to GMP-compliant regulations. As a result, novel hydrogels need to be designed that closely recapitulate the ECM components and dynamic signalling milieu of the native pancreas. Overall, the possibility of clinically available PSC-derived β-cell transplantation is becoming much more attainable, but there remain large areas of improvement before they are a reality.

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Appendix:

IMARIS animation:

Example animation created using LSFM highlights the applications of the IMARIS software. Scan QR code to provide the link to SOX9 staining of the E13.5 mouse embryo (Figure 9E).

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