Modelling the endocrine pancreas in health and disease

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Key points

The evolutionary differences in pancreas development, function and failure undermine the translation of successful pre-clinical studies from animal models into humans.

Establishment of novel therapeutic approaches for diabetes treatment requires comprehensive understanding of human endocrine pancreas formation and function.

The proper development of endocrine cells relies on the tight coupling of morphogenetic events with cell differentiation programs.

3D organoids and stem cell differentiation systems provide unique platforms for modelling human endocrine cell morphogenesis and differentiation.

Large animals such as minipigs offer novel systems for modelling diabetes closely to the disease development and progression in humans.

Establishment of organizations providing healthy and diabetic human primary pancreatic samples have increased our understanding of pathomechanism of diabetes.

Abstract [OK]

Diabetes mellitus is a multifactorial disease affecting increasing numbers of patients worldwide. Progression to insulin-dependent diabetes mellitus **[OK]** is characterized by the loss or dysfunction of pancreatic β-cells, but the pathomechanisms underlying β-cell failure in type 1 diabetes mellitus and 2 diabetes mellitus are still poorly defined. Regeneration of β-cell mass from residual islet cells or replacement by stem cell-derived β-like cells holds great promise to stop or reverse disease progression. However, the development of new treatment options is hampered by our limited understanding of human pancreas organogenesis due to the restricted access to primary tissues. Therefore, the challenge is to translate results obtained from pre-clinical model systems to humans, which requires comparative modelling of β-cell biology in health and disease. Here, we discuss diverse modelling systems across different species that provide spatial and temporal resolution of cellular and molecular mechanisms to understand the evolutionary conserved genotype–phenotype relationship and translate them to humans. In addition, we summarize the latest knowledge on organoids, stem cell differentiation platforms, primary micro-islets and pseudo-islets, bioengineering and microfluidic systems for studying human pancreas development and homeostasis ex vivo **[OK]**. These new modelling systems and platforms have opened new avenues for exploring the developmental trajectory, physiology, biology and pathology of the human pancreas.

[H1] Introduction [OK]

Diabetes mellitus is a major disorder arising from the malfunction of the endocrine pancreas. This disease develops mainly by autoimmune destruction (type 1 diabetes mellitus; T1DM)[1] or progressive loss or dysfunction of insulin-producing pancreatic β-cells due to insulin resistance and glucolipotoxicity (type 2 diabetes mellitus; T2DM)[2]. So far, no treatment can stop or reverse disease progression. Therefore, intensive efforts are underway **[OK]** to develop novel therapeutic approaches. Strategies that are currently being explored to replace lost and/or dysfunctional β-cells include **[OK]** in vitro differentiation of β-like cells from stem cells for replacement therapy and stimulating endogenous β-cell regeneration. Indeed, the findings of the Joslin Medalist study showing that a small amount of functional β-cells exists even after 50 years of autoimmune and insulin-dependent diabetes mellitus[3] strongly imply that β-cell regeneration might be a possible treatment for patients with diabetes mellitus. In addition, improvement of glucose homeostasis in T2DM patients upon bariatric surgery also suggests the reappearance of functional β-cells[4–6], though the exact mechanism is unknown. **[We have modified the previous sentence]**, **[OK]**. Human islet transplantation can also restore normoglycaemia in patients with T1DM[7], but its use is restricted due to the lack of transplantable islets. Thus, understanding the mechanisms underlying human islet development, homeostasis, function and failure is essential to trigger in vivo regeneration or allow in vitro differentiation of functional β-like cells from stem cells **[OK]**.

Over the past two decades **[OK]**, remarkable progress has been achieved in terms of understanding the **[OK]** mechanisms coordinating pancreas development. However, most studies have been conducted in animal models, such as rodents, *Xenopus* **[Yes]** and zebrafish[8–11]. In comparison, less work on developmental and regenerative biology has been conducted in large animals or in humans due to high costs and limited availability of biomaterial, respectively (Table 1 and 2). Although similarities between pancreas development, function and failure in animal models and humans exist, several reports have also highlighted key differences[12–15] **[OK]**. For instance, differences in early pancreatic development, organ morphology, endocrine cell ratio, islet composition, structure and physiology between rodents and human are present **[OK]**. Furthermore, the animal models, specifically rodent models of T1DM, do not perfectly mirror the cause and progression of T1DM in humans[16,17]. Additionally, studying human pancreas organogenesis have been limited due to the difficulty in accessing embryonic and fetal tissues and there are obstacles with performing longitudinal analyses **[OK]**. Yet, due to the recent increased availability and access to human tissues **[This sentence and the previous one have been changed slightly]**, several research groups have investigated key processes of pancreas organogenesis in humans[12,13,15]. This work together with intensive studies on in vitro differentiation of pancreatic cells partially deciphers the roadmap of human pancreatic lineage formation[18–21] **[OK]**. Nevertheless, these approaches are unable to

fully unravel the mechanisms that couple niche signals with cell-lineage allocation during human pancreatic and endocrine development to generate mature and functional β-cells in a dish.

In this Review, we first give an overview on the mechanisms linking pancreatic tissue morphogenesis and cell differentiation that have mainly been uncovered in animal model systems **[OK]**. Learning from these developmental programmes, we highlight the impact of 3D microenvironment, cell–cell and cell–matrix interactions (in other words the niche) on pancreatic differentiation and morphogenesis. Additionally, β-cells are not a homogenous cell population[22]. Thus, understanding such developmental processes and concepts of functional β-cell heterogeneity help researchers to **[OK]** design modelling systems, such as human organoids, human pluripotent stem cell **[OK]** differentiation, micro-islets and pseudo-islets as well as microfluidic systems to dissect endocrine lineage formation and function ex vivo. Furthermore, we review the modelling of β-cell maturation and failure in large animals with particular emphasis on porcine islet development and biology, which can act as a bridge between mouse and humans (as pigs and humans have similar physiology). Pigs can be genetically modified, and because of easier sampling and feasibility of performing longitudinal analyses, it is a valuable animal model to understand the development and pathomechanisms of diabetes mellitus. In addition, understanding islet biology in pigs is important as porcine islets are considered a tissue **[OK]** source for xenotransplantation for the treatment of diabetes mellitus in the future, which could compensate for the shortage of human islets **[OK]**.

[H1] Pancreas formation and homeostasis

[H2] Pancreas structure and function

The pancreas serves not only to adjust blood levels of glucose by secreting endocrine hormones, but also participates in digestion through the production and release of enzymes by its exocrine compartment. The exocrine pancreas consists of acinar and ductal epithelial cells. Acinar cells produce and release a variety of zymogens (proenzyme or inactive precursor of enzymes) **[Have been modified]**, which are transported through the pancreatic ductal system **[Au: is this what you mean? Pancreatic enzymes pass through pancreatic duct to reach duodenum]** to the duodenum to assist nutrient digestion. In comparison, endocrine cells cluster to form islets of Langerhans including α-cells, β-cells, δ-cells and pancreatic polypeptide (PP) cells, which produce glucagon, insulin, somatostatin and pancreatic polypeptide, respectively[23–26]. The precise balance of the function of these hormones regulates blood levels of glucose and contributes to energy metabolism[27]. In addition, the developing embryonic pancreas contains ghrelin cells (also known as ε-cells) and gastrin-expressing (G) cells, which disappear in the adult organ[28,29]. The

effect of these transiently generated cells on pancreas development is still elusive. The malfunction of the endocrine pancreas mainly leads to diabetes mellitus, while defects in the exocrine compartment result in pancreatitis and pancreatic cancer.

[H2] An overview of pancreas development

In mice, pancreas organogenesis comprises two distinct stages. During primary transition, pancreas specification and induction starts at embryonic (E) day 8.5 by epithelial evagination of the foregut endoderm (Fig. 1A). The ventral and dorsal buds fuse to form a multi-layered epithelium consisting of multipotent progenitor cells **[OK]** , which give rise to ductal, endocrine and exocrine cell **[OK]** lineages[26,30]. During this stage, the first wave (transition) of endocrine cell formation occurs de novo and mainly generates glucagon-expressing α-cells. During the secondary transition, morphogenetic events lead to the formation of microlumen structures in the pancreatic multi-layered epithelium, which subsequently fuse to form a continuous luminal network[31,32] (Fig. 1B). This process coincides with remodelling and stratification of the epithelium to form a single-layer branched structure in which endocrine progenitors exist[32,33]. By receiving appropriate niche signals from their microenvironment, the progenitors differentiate into different endocrine cell types and leave the ductal epithelium[34–36]. Finally, the clustering of endocrine cells in association with endothelial, immune, mesenchymal and neuronal cells generate islets of Langerhans[37,38].

In humans, dorsal and ventral pancreatic buds also emerge from the foregut endoderm[12]. Although these two domains show distinct gene expression patterns and their development depends on different signalling components, they eventually fuse together to form a single organ primordium[15,39,40]. In contrast to pancreas development in rodents, only a single wave of endocrine cell formation occurs in the developing human pancreas. Furthermore, unlike rodents, the pancreatic progenitors in humans do not express the transcription factor **[OK]** Homeobox protein NKX2.2 **[We added to this section]**, though this protein is later expressed in endocrine progenitors[12,41,42]. However, Neurogenin 3 (NGN3) is required for human endocrine cell differentiation and is transiently expressed in both human and rodents[43]. Notably, human pancreatic islets undergo endocrine cell rearrangement within the islets to acquire a final distinct morphology that is different from that of rodent islets[44] **[OK]**. Thus, there are similarities and differences during mouse and human pancreatic development. As the knowledge of the mechanisms involved in mouse development served as a blueprint **[Yes]** to differentiate human pluripotent stem cells into endocrine cells, it will eventually be important to understand human pancreas development in more detail to generate better functional human islets is dish **[Have been modified]** [45,46].

How different niche signals orchestrate the tight association between tissue patterning and cell differentiation during early pancreatic development remains largely unknown. Understanding these signals is important for endocrine induction and β-cell differentiation from stem cells in vitro and for identifying the factors that trigger in vivo regeneration. Furthermore, the mechanisms coordinating endocrine cell clustering and islet formation are poorly understood. Uncovering such mechanisms is not only crucial to understand the pathomechanisms of diabetes mellitus, but also to reconstruct islets from stem cell-derived endothelial, mesenchymal and endocrine cells to build islet biomimetics with improved maturation and function for long term culture.

[H2] Developmental defects impacting β-cell formation and function

Genome-wide association studies **[OK]** T1DM and T2DM have identified many candidate genes associated with β-cell loss and dysfunction[47,48], respectively, putting β-cells centre stage in diabetes mellitus aetiology. In T2DM, several parameters are known to trigger the onset of β-cell failure, such as genetic predisposition, diet and environmental factors. Defects in developmental programmes during early pancreatic organogenesis can also lead to β-cell dysfunction and T2DM. In this context, single nucleotide polymorphisms **[OK]** in developmental regulatory genes causing suboptimal generation of fetal β-cells increase susceptibility to T2DM[49–52] **[OK]**. The most extreme cases are monogenic forms of early onset diabetes mellitus[53]. More evidence of the impact of developmental programs on the onset of T2DM comes from the **[The sentence has been modified.]** the finding that islet mass at birth is vastly different between human individuals, possibly making people with less islet mass at birth more susceptible to develop diabetes mellitus[54]. Deletions or mutations in the coding sequence of several genes induce pancreatic agenesis (malformation of the pancreas where the entire or part of the pancreas fails to develop) **[OK and have been modified]**, permanent neonatal diabetes mellitus **[OK]** and maturity onset diabetes of the young (MODY). Most of these genes are key players in β-cell development and function, including *PDX1*, *PTF1A*, *HNF1B*, *NEUROD1*, *NKX2.2*, *HNF1A*, *GATA4, GATA6*, *KCNJ11* and *glucokinase* (*GCK*)[53,55–58]**.** Whether mutations in other genes regulating niche signals and tissue patterning also result in suboptimal β-cell formation and failure needs further investigation.

[H2] β-cell heterogeneity and endogenous β-cell regeneration

β-cells are a heterogeneous cell population composed of subpopulations that not only differ in their morphology (such as nuclear size **[Au: Do you mean 'nucleus size'? YES]** and insulin granularity **[Au: our journal style does not allow the use of 'etc'. I have removed it.]**), but also in their proliferative activity, glucose responsiveness, insulin **[Au: we do not allow the use of '/' so I have changed this to 'and' OK? Or do you mean and/or? Has been modified]** secretion, maturation state or in their

susceptibility to immune attack and metabolic stress[22,59–61]. Islet physiology is clearly affected by functional β-cell heterogeneity as proven by several studies. For instance, a β-cell pool (1–10% **[Au: Do you mean '1-10% of the entire b-cell population'? YES]**) has been identified, which exerts disproportionate control over islet responses to glucose[62]. These special pacemaker β-cells, termed hub cells, are highly metabolic and required for normal insulin release. Strikingly, these hub cells **[OK.]** are sensitive to pro-inflammatory and glucolipotoxic insults, which affect β-cell function and suggests that hub cell dysfunction might contribute to T2DM pathogenesis[62] **[OK]**

Differential activity of WNT– **[OK]** Planar cell polarity (PCP) signalling is another mechanism underlying β-cell heterogeneity. **[Au: Please explain briefly what the WNT-PCP signalling pathway does.]** PCP is defined as the polarity perpendicular to the apical-basal polarity and regulates the orientation of cells within the plane of an epithelium, the orientation of the mitotic spindle (asymmetric cell division) and intracellular organelle positioning by an evolutionarily conserved group of molecules called core PCP proteins[63]. In this context, Flattop (FLTP; also known as CFAP126 **[Au: I have included the UniProt symbol here.]**), a downstream effector and reporter of the WNT–PCP pathway is heterogeneously expressed among pancreatic endocrine cells and subdivides β-cells in mice into proliferative and metabolically active cells[22,64]. Importantly, stimulation of mouse and human β-cells with WNT–PCP ligands triggered expression of β-cell maturation markers and increased glucose-stimulated insulin secretion. This effect **[OK.]** suggests a link between β-cell polarization and functional maturation and implies that WNT–PCP signalling is a candidate pathway that reverts dedifferentiated cells to functional mature β-cells[64,65]. Additionally, in human islets the surface markers ST8SIA1 and CD9 can be used to discriminate between four functionally distinct β-cell subpopulations[66] **[OK]**. Importantly, this subtype distribution is altered in T2DM islets **[Au: Please reference this statement.]**. **[OK]** **[The sentence has been modified.]** This result together with the finding that distinct β-cell states increase or decrease in number in T2DM, age and depending on the BMI[67] highlights the importance of gaining a better understanding of β-cell heterogeneity and its implication in disease **[OK]**. Thus, β-cell heterogeneity should be an important consideration when modelling development, function or failure in vitro and for strategies of endogenous β-cell regeneration.

Stimulation of replication or redifferentiation and **[OK]** maturation of residual β-cells might be promising approaches to replenish the lost functional β-cell mass, as some β-cells remain in both T1DM[3,68] and T2DM[69,70]. Even though triggering human β-cell replication remains a challenge, several breakthrough studies give new hopes. Promising strategies include the inhibition of transforming growth factor β (TGFβ) signalling[71], treatment with the liver-derived Leukocyte elastase inhibitor (SERPINB1)[72] and the

use of Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) inhibitors[73] **[OK.]**. Yet, β-cell replication needs to be tightly controlled to not induce pathologic conditions, such as insulinoma or pancreatic carcinoma **[Au: Do you mean 'pancreatic carcinoma'? Yes]**. In addition, we must consider the apparent inverse relationship between β-cell maturity and proliferation[74], as there might be a risk of generating immature β-cells when forcing adult β-cells to replicate.

A safer approach might be to explore β-cell redifferentiation as a regenerative strategy. β-cells have been shown to adapt to immune and metabolic stressors in T1DM[75,76] and T2DM[70,77], respectively, by reverting to an immature state that at least in part accounts for the loss of functional β-cell mass in diabetes mellitus, but this phenomenon **[OK]** also has striking therapeutic potential. Adapting a dedifferentiated, immature cell state might be an active, protective process that allows β-cells to evade an immune attack (T1DM) or metabolic stress-induced cell death (T2DM), create a pool of precursor cells and become reactivated to redifferentiate into functional β-cells under specific conditions. Thus, the burning question now is which signals induce β-cell recovery. As bariatric surgery has been shown to resolve T2DM and prevent disease progression in patients who with morbid obesity, decreased metabolic pressure following body weight loss[6,78] seems to be such a trigger. Still, the molecular mechanisms underlying the effects of bariatric surgery-induced improved glucose handling are elusive and might be due to a sum of multiple changes including altered circulation of bile acids and gut hormones as well as changes in nutrient sensing and the composition of the gut microbiota[79] **[OK]**.

[H1] Pancreatic morphogenesis and differentiation [Au: The current heading is too long. The limit is 38 characters, including spaces. Please shorten this accordingly. Have been modified]

Currently, it is impossible to conduct longitudinal studies to understand human pancreas morphogenesis and endocrine cell differentiation at the cellular and molecular levels **[OK]**. Thus, establishing ex vivo modelling systems that can recapitulate developmental processes and allow prospective study at the tissue, single cell, subcellular organelle and molecular levels is important **[OK]**. In this section, we first discuss how epithelial polarity and morphogenesis effect endocrine cell formation and clustering during mouse pancreas development. To translate and/or compare these principles with humans, we review the state-of-the-art and potential of 3D organoids and in vitro β-cell differentiation platform to investigate human pancreatic morphogenesis and differentiation.

[H2] Morphogenetic events during endocrine cell formation and clustering

During embryonic development, the pancreatic epithelium segregates into tip and trunk domains. The tip structure differentiates into acinar cells, whereas the trunk domain contains bipotent progenitors, which depending on the signal received, generate ductal or endocrine progenitors[80–82]. The key transcription factor regulating commitment and differentiation of endocrine progenitors is NGN3[34,35]. This transcription factor is expressed at low levels in a subset of bipotent progenitors, which are long-lived mitotic cells **[OK]** and considered as an endocrine-biased progenitor pool. Upon transient increase in the expression levels of NGN3 (NGN3^{high}), these progenitors further differentiate into endocrine cells[83,84]. In mouse, there are two waves of NGN3^{+} **[they are not different. Ngn3+ cells include both low and high cells]** cell formation during primary and secondary transitions. By contrast, in humans[12] the primary transition and first wave of NGN3^{+} cells do not exist, highlighting another key difference between these two species **[OK]**. Several signalling pathways such as those involving Neurogenic locus notch homologue protein (Notch), WNT–PCP, epidermal growth factor receptor (EGFR) and sphingosine-1-phosphate[65,85–89] regulate endocrine differentiation, but much remains to be resolved, specifically the mechanisms underlying endocrine cell induction and allocation, that is how α-cells and β-cells are formed.

At NGN3^{high} stage, endocrine progenitors are unipotent and produce one endocrine subtype. Although the regulatory mechanisms of endocrine specification are unclear, it has been shown that during development NGN3^{+} cells consecutively generate α-cells then β-cells and PP-cells and finally δ-cells, suggesting that developmental timing is essential for cell-fate specification[90]. However, the underlying mechanisms of the sequential production of endocrine cells are unknown. One presumption is that the priming of the epithelial progenitors and the surrounding mesenchymal and extracellular matrix (ECM) niche might influence endocrine fate decisions. During mouse endocrine cell formation, the pancreatic epithelium consists of a branched peripheral region and a plexus core (Fig. 1B), which gradually remodels into a ramified epithelial layer[33]. As endocrine progenitors are located mainly within the plexus area[84], it is possible that remodelling of this domain and differential exposure to ECM components might define the specific fate of the new endocrine progenitors. In such a scenario, it would be exciting to identify the factors within the progenitors or their microenvironments that specifically promote α-cell and β-cell fate decision. Moreover, this process **[OK]** highlights the importance of proper epithelial morphogenesis for endocrine cell specification and allocation, which has only been investigated in a few studies. **[OK]**

Deletion of the small GTPase cell division control protein 42 homologue (CDC42), one of the major polarity regulators, impairs pancreatic tubulogenesis and reduces β-cell formation[31], underlining the association of cell polarity, morphogenesis and differentiation. Furthermore, in endocrine progenitors,

EGFR signalling through the phosphoinositide 3-kinase (PI3K) pathway reduces the apical domain size, which consequently inhibits Notch signalling and induces NGN3 expression **[Done]** for endocrine cell differentiation (Fig. 1C). Remarkably, using human embryonic stem cell (hESC)-derived endocrine progenitors, these pathways have been found to be evolutionary conserved[89]. Thus, these findings not only reveal the tight link between signal transduction, morphogenesis and endocrine differentiation in mouse and human, but also provide new molecular targets and small molecule inhibitors for triggering β-like cell generation from pluripotent stem cells.

Upon differentiation, endocrine cells delaminate from the epithelium into the surrounding mesenchyme. How changes in epithelial cell morphology regulate endocrine cell delamination and which signals coordinate this process are not fully understood. Epithelial–mesenchymal transition **[OK]** has been proposed to be involved, as delaminating endocrine cells express Zinc finger protein SNAI2 **[OK]** and downregulate levels of E-cadherin (also known as cadherin 1)[36,91]. Yet, the apparent switch from E-cadherin to N-cadherin (also known as cadherin 2), one of the hallmarks of classic epithelial–mesenchymal transition, does not occur during endocrine cell delamination, suggesting the involvement of other unidentified mechanisms. In line with this evidence **[Has been modified]**, delaminating endocrine cells narrow their apical domain, form a tether and finally detach from the epithelial lumen[84] (Fig 1C). This process is possibly regulated by actin cytoskeletal dynamics via small GTPases, such as transforming protein RhoA, Ras-related C3 botulinum toxin (Rac) and CDC42. Indeed, expressing a constitutive active form of CDC42 in mice stabilizes actin filaments and impairs endocrine cell delamination[92] **[OK]**. Despite these findings, much remains to be discovered on the cellular processes orchestrating endocrine cell delamination during development. Furthermore, whether and how morphological changes in endocrine cells impact cell-fate allocation and subsequent maturation needs to be explored.

After delamination, endocrine cells cluster to form proto-islets along the ductal region[23] (Fig. 1D). How endocrine cells find each other and whether they undergo long-distance migration is not known. One obstacle is the difficulty of monitoring endocrine cell movement in vivo. EGFR signalling is one of the players regulating endocrine cell migration. In mice, deletion of this receptor impairs endocrine cell motility and differentiation, supporting the interdependency of these two distinct processes during pancreatic development[93]. Additionally, live imaging of zebrafish larva has shown that G protein-coupled receptors **[OK]** and PI3K signalling regulate endocrine cell motility and islet formation **[OK]**. These pathways exert their effect through regulation of actin-based filopodia protrusions[94]. Nevertheless, the identity of possible external guidance cues orchestrating endocrine cell motility has been poorly

characterized. If such signals exist they might be derived from neurons, endothelial, mesenchymal cells or islet cells themselves. Towards this goal, the axonal pathfinding molecule Semaphorin 3a, derived from the peripheral mesenchyme, has been reported to induce mouse endocrine cell migration through activation of the Neuropilin 2 receptor[95]. These findings highlight the importance of cell dynamics and neighbouring tissue interactions for proper endocrine cell differentiation and islet formation. Therefore, the effect of cell polarity and tissue morphogenesis must be considered in any in vitro system attempting to generate functional β-like cells from stem cells.

[H2] 3D organoid systems modelling endocrine differentiation and morphogenesis

3D organoid systems were developed to overcome the limitations of in vivo studies in human. Organoids have great potential for developmental studies, disease modelling, drug testing and tissue transplantation. Cells derived from different tissues and organs can generate organoid structures, such as gastruloids, mini-brains and organoids of the gut, lung, kidney, liver and heart, to mention a few. These systems assemble as complex polarized epithelial-based structures with the ability of self-organization through sorting of the cells due to differential cell–cell adhesion. This sorting **[OK]** directs further fate decisions, making the organoids unique in vitro platforms to study developmental processes, such as tissue morphogenesis and patterning, cell plasticity and lineage decision[96–101]. Therefore, organoids are not only able to address specific mechanisms of human development but also reveal conserved features and key differences of animal models[102]. To form organoids, the cells of origin require self-renewal activity and multipotency. Thus, these structures are mainly derived from embryonic stem cells **[Has been changes]**, induced pluripotent stem cells, organ-specific adult progenitors or stem cells.

Organoids can be generated from mouse and human embryonic materials. Compared to the adult organ-derived organoids, the organoids **[OK]** from embryonic cells are easier to establish due to the higher **[Has been modified]** plasticity and regenerative activity of embryonic cells[102,103] (Fig. 2A-C). In a study led by Anne Grapin-Botton, mouse pancreatic progenitors were cultured to generate branched and differentiated pancreas epithelium with ductal, exocrine and endocrine lineages, but without mesenchymal, endothelial and neuronal cell types. Remarkably, the maintenance and expansion of these structures was dependent on fibroblast growth factor (FGF) and Notch signalling, revealing similar developmental dependencies and programs to those seen in vivo **[OK]**. This study also highlights the importance of epithelial heterogeneity and autocrine and paracrine epithelial signals for appropriate progenitor expansion and differentiation[104] (Fig. 2A). **[OK]**

Fetal pancreatic organoids can help identify novel players in endocrine cell development. For example, a functional genetic screen in organoids derived from isolated Transcription factor SOX9–enhanced green fluorescent protein **[OK]** positive pancreatic epithelial progenitors showed that the histone H3 lysine9 monomethylation (H3K9me1) methyltransferase PR domain zinc finger protein 16 (PRDM16) is a novel regulator of mouse islet development **[OK]**. Notably, analysis of *Prdm16*-deleted mice supported the data from the organoids, underlining the value and high similarities of these systems with in vivo pancreatic development[105]. Similar to mice, human embryonic pancreatic epithelial cells have the potential to form organoids. The expansion of these structures is promoted by epidermal growth factor (EGF), which inhibits their differentiation[106], suggesting the potential of modulating EGF signalling for triggering β-cell differentiation and/or regeneration. In another study, human pluripotent stem cells generated pancreatic organoids resembled the human fetal pancreatic epithelium after orthotopic transplantation. Notably, this platform has been used to mimic cystic fibrosis ex vivo, which reflected the disease phenotype and could be used for drug screening[107] (Fig. 2B).

Organoids have also been produced with cells derived from the adult pancreas, which are suitable models for regeneration studies as the cells are already committed to the pancreatic fate **[OK]**. In this context, isolated $CD133^+$ colony-forming units **[OK]** from the mouse adult pancreas form organoids, which upon treatment with R-spondin1 (WNT ligand) **[OK.]** expand and differentiate towards all pancreatic lineages, revealing the potential of WNT signalling in β-cell expansion and/or regeneration[108,109] (Fig. 2D). Consistently, the WNT pathway seems to be activated after pancreatic duct ligation **[OK]** to induce the regenerative response. When isolated ductal fragments from adult pancreatic duct ligation mice are treated with R-spondin1 they express Leucin-rich repeat-containing G-protein coupled receptor 5 (LGR5) and generate expandable organoids in 40 weeks **[OK]**. After in vivo engraftment, these organoids differentiate towards ductal and endocrine fate, indicating the presence of bipotent progenitors in the adult mouse pancreas[110]. In comparison, human $CD133^+$ cells from adult ductal epithelium have been clonally expanded to form organoids with self-renewing and ductal phenotypic characteristics. These structures can be differentiated towards endocrine cells when given essential transcription factors that are required for endocrine cell development ectopically[111], thus questioning their intrinsically multipotent characteristics **[OK Yes]**. By contrast, a subset of human pancreatic adult exocrine cells exhibiting high aldehyde dehydrogenase activity **[OK]** was shown to have multipotent progenitor-like features (Fig. 2D). In 3D organoids, these cells express crucial transcription factors of pancreatic progenitors and upon in vivo engraftment differentiate into endocrine cells[112], suggesting their potential for β-cell replacement. Generating organoids from adult stem cells has great potential and best recapitulates the in vivo

phenotype. Yet, not every organ, including the pancreas, has an adult stem cell population and the progenitors might not have self-renewing potential to generate an organoid model system.

3D organoids can assist in identifying the niche signals influencing proliferation, differentiation, and lineage allocation of human endocrine progenitors **[OK Is this what you mean? Yes]**. They also might help to model pancreatic tubulogenesis, cell–cell interactions, influence of the endogenous niche, and autocrine and paracrine interactions (for example, how β-cell loss might affect α-cell physiology). Furthermore, organoids can be used for modelling diabetes mellitus as well as for drug testing before clinical studies. How mutations in certain genes effect human pancreatic development and whether these developmental defects contribute to early β-cell dysfunction might be answered by using such model systems. The establishment of CRISPR–Cas9 gene editing technology[113] provides a powerful tool for genetically manipulating hESCs or induced pluripotent stem cells (iPSCs) by introducing or correcting mutations **[OK]**. Therefore, the combination of CRISPR–Cas9 and hESCs-derived or iPSCs-derived 3D organoids will assist in unravelling the development and function of disease-causing gene mutations and understanding diabetes mellitus pathomechanisms **[OK]**. Yet, organoids differ in shape and size and they lack their own blood supply and interactions with non-pancreatic tissues, thus, the cell-types are often immature and not fully functional. Henceforth, the challenge will be designing uniform structured organoids co-cultured with endothelial, neuronal and mesenchymal cells to improve cell maturation and the potential of these systems for modelling the human pancreas ex vivo.

[H2] Stem cell differentiation — a platform for β-cell development and disease modelling.

An approach, which has been successfully applied in the clinic and results in at least temporary normoglycaemia in patients with T1DM **[Au: patients with T1DM? Yes]**, is human islet allotransplantation. However, until now, this therapy option is restricted to a few selected patients with T1DM who have complicated glucose control due to the limited supply of pancreata from deceased donors and the need for life-long chronic immunosuppression[114,115]. In this respect, directed differentiation of hESCs and iPSCs into mature insulin-producing β-like cells is a promising alternative and might offer an unlimited source of transplantable material in the future. Apart from their great potential for cell-based therapy, stem cells also enable the study of mechanisms of human β-cell formation and maturation and can be used as a tool for drug discovery and disease modelling to identify new targets for diabetes mellitus therapy.

Since the first report of generating insulin-producing cells from hESCs by spontaneous differentiation[116], major progress has been made towards in vitro generation of β-like cells. Protocols in use are now

adapted to our knowledge of pancreas development in mice and to our still limited understanding of human pancreatic development. The underlying principle is a step-wise differentiation of hESCs or **[OK]** iPSCs through defined developmental stages ranging from definitive endoderm, primitive gut tube, posterior foregut, pancreatic progenitors, endocrine progenitors to β-like cells by exposing cells to various growth factors and small molecules in a specific dose and sequence to activate or inhibit embryonic signalling pathways, such as Nodal–Activin, WNT, retinoic acid, FGF, Bone morphogenetic protein (BMP) and Notch, which results in the expression of distinct transcription factors for each state [18–21]. Strikingly, through constant optimization of differentiation protocols the functionality of in vitro-derived β-like cells has greatly improved in the past 15 years **[Has been included]** from initially poly-hormonal, glucose-unresponsive β-like cells to mono-hormonal, glucose-responsive insulin-secreting β-like cells[18–21].

In contrast to the first established protocol, in which cells were grown as monolayers throughout the differentiation process, which produced immature β-like cells, more recent **[**can we say latest instead? last 3 years**]** protocols use 3D culture systems (for example shaking 3D cluster suspension or air-liquid interphase cultures[18–21]). Such 3D culture conditions mimic the islet architecture and morphology and strengthen cell–cell interactions, cell compaction and polarity, which are crucial for β-cell maturation and function[22,64]. However, despite extensive research and improvement of protocols, in vitro generated β-like cells resemble fetal β-cells at best and not fully mature β-cells[117] **[OK]**. Current efforts to differentiate endocrine progenitors into mature β-like cells are hindered by our poor knowledge of the pathways driving β-cell maturation in humans because of limited access to human fetal and neonatal pancreatic tissues. To this end, single-cell analysis using single-cell RNA sequencing or single-cell and **[OK]** imaging mass cytometry might be paradigm changing. These techniques are particularly useful when study material is scarce, for example studying human tissues, as the techniques can simultaneously measure the expression of thousands of genes or dozens of proteins in individual cells **[OK]**. The resulting high-dimensional profiles provide a comprehensive view of gene and protein expression and thus shed light on cell-to-cell heterogeneity. Furthermore, by using computational algorithms the temporal order and lineage choices can be reconstructed from single-cell snapshot data and thus enable the identification of the developmental progression of a given cell type and the driving pathways [118,119].

Modelling human pancreas organogenesis in culture through differentiation of hESCs into β-like cells might uncover molecular mechanisms driving β-cell formation and maturation. Interestingly, single-cell gene expression analysis of hESCs differentiation into β-like cells has revealed distinct subpopulations and progenitor states along the endocrine differentiation path and parallel paths to β-like cells [120]. The existence of multiple differentiation paths to β-like cells during development might underlie in part the presence of diverse β-cell subpopulations, which determines the functionality of the adult islet[22]. However, modelling β-cell formation and maturation in vitro is still hampered by the failure to generate

fully functional mature β-cells. Therefore, comparative expression analysis between human fetal or neonatal pancreatic tissue, in vitro generated β-like cells and human islet-derived β-like cells might identify missing factors and pathways that need to be targeted to improve differentiation protocols. Such analyses could also help identify surface markers that allow the specific enrichment of certain progenitor populations or proliferative and mature β-cell subpopulations, which might improve the generation of mature and glucose responsive in vitro derived β-like cells[66,121,122] **[OK]**. Apart from its possible impact on in vitro differentiation outcome **[Has been modified.]**, β-cell heterogeneity is also relevant for transplantation outcomes. **[Has been included]** In rodents, metabolically more active and mature β-cells are, for instance, sensitive to stress and die after transplantation into the anterior chamber of the eye, a transplantation site for non-invasive and longitudinal monitoring of pancreatic islet/ β-cell physiology and pathology[123]. In contrast, less mature β-cells survive, compensate and mature when islets get re-vascularized[64].

Differentiation protocols favour the generation of β-like cells. However, human islets are comprised of ~50% β-cells and 40% α-cells, while murine islets contain more β-cells (60–80%) and less α-cells (15–20%)[124,125]. Thus, human islets seem to possess more heterotypic cell-to-cell interactions that are most likely functionally relevant. Paracrine communication between β-cells and non-β-cells is known to regulate insulin secretion. For instance, besides intestinal L-cells, pancreatic α-cells are thought to secrete the incretin glucagon-like peptide 1 (GLP1), which stimulates insulin secretion and β-cell proliferation[126,127]. In addition, in mice **[OK]** δ-cells fine tune insulin secretion through urocortin 3-mediated somatostatin release and express receptors encoded by genes **[OK]** such as *Glp1r*, *Gcgr* and *Adra2a*, which are also expressed on the surface of β-cells, suggesting an important role of δ-cells for β-cell function[128,129]. So, it remains to be shown if optimal function is achieved using pure β-cells or whether the generation of islet-like structures is needed. Also, despite the fact that α-cells are abundant in T1DM their function and gene expression are severely compromised, which needs to be considered for cell-replacement therapy[130].

Another approach to improve differentiation protocols and functionality of β-like cells generated in vitro but also to allow the study of pancreas organogenesis and β-cell formation is to mimic certain aspects of organogenesis in vitro. It is known that the islet microenvironment (which consists of a network of ECM, mesenchymal cells, nerves and endothelial cells) has a critical role and signals to pancreatic cells and thereby regulates pancreas organogenesis and mature islet function in vivo and in vitro [131,132] **[Has been modified]**. A sophisticated 3D multi-lineage system has been described for liver bud formation from iPSCs by incorporating cell types from all three germ layers (that is, endoderm-derived hepatocyte-like

cells, mesoderm-derived endothelial and mesenchymal cells as well as ectoderm-derived neuronal cells[133]). Takebe, Treutlein and colleagues[133] compared the conventional 2D monoculture of stem cell-derived hepatocyte-like cell differentiation with a 3D multi-lineage organoid system by using single-cell RNA sequencing. Both systems recapitulated certain transcriptomic features of human hepatogenesis. However, heterotypic germ layer communication in the liver bud generated expression profiles that more closely resembled the characteristics of fetal liver development [133]. **[OK]**

A multi-lineage differentiation approach is particularly needed to model T1DM in vitro because besides β-like cells, thymic epithelia and hematopoietic stem cells need to be generated to mimic the disease. In this way, risk alleles associated with T1DM (such as *PTPN22* allelic variants) that are carried by isogenic human immune and endothelial cells can be engineered in stem cells, which enables controlled, mechanistic studies of disease mechanisms to be performed **[Has been modified]**. However, whether this kind of germ layer combination is applicable for β-cell differentiation from stem cells needs to be shown. A major challenge for such systems is to define culture conditions that provide optimal conditions for each lineage to form from the different germ layers **[OK]**. The best method is the generation of all cell types from pluripotent stem cells and the aggregation or condensation of the germ layer derivatives at the progenitor stage to recapitulate organogenesis **[OK]**. Multi-lineage differentiation approach **[Has been modified]** should provide the dynamic niche signals through neighbouring tissue interactions and therefore should most closely resemble in vivo development and generate more **[has been changes.]** functional cell types.

[has been deleted.] iPSCs have been generated from tissues taken from **[OK]** patients with diabetes mellitus in order to study the aetiology and pathology of the disease **[OK]** and for drug development (Fig. 2). These disease modelling systems are derived from reprogramming somatic cells taken from patients **[OK]** (such as skin fibroblasts[134]), which are readily editable by genetic engineering, and might even allow identifying personalized, patient-specific treatment options **[Has been modified]**. Diabetes mellitus is a polygenic, multifactorial disease in which gene–gene, gene–environment and gene–sex interactions as well as systemic effects contribute to disease pathogenesis. Therefore, iPSCs have been mainly generated from patients carrying mutations in a single gene **[Has been modified]** that cause MODY types 1, 2, 3, 5 and 8, or Wolfram syndrome[135,136]. **[The whole sentence has been deleted]**. iPSCs generated from individuals carrying insulin receptor mutations have been used to assess the consequences of genetic insulin resistance in a cell system without the complex regulatory mechanisms acting in vivo. Differences in gene regulation between mutant iPSCs and corresponding fibroblasts suggest that the outcome of insulin resistance is shaped by the cellular context and differentiation state[137]. **[OK]**

The Gadue[136]and Huangfu[56] groups addressed the function of GATA6 during human pancreas development using human pluripotent stem cells. Haploinsufficient *GATA6* mutations are associated with human pancreatic agenesis. By contrast, mice lacking *Gata6* expression exhibited no apparent pancreatic defects[138,139]. Surprisingly, unlike the pancreatic agenesis phenotype observed in patients both studies revealed that *GATA6* haploinsufficiency only has a mild effect on induction of pancreas development **[OK]** but impairs β-cell differentiation and function. Hence, these findings suggest that additional extra-pancreatic factors might contribute to GATA6-specific pancreatic defects in humans and/or the defects associated with *GATA6* haploinsufficiency might be overcome by certain factors present in the differentiation media.

A report by the Egli[140] group described the generation of iPSCs from individuals with Wolfram syndrome, which is a rare disease caused by mutations in *WFS1* and characterized by insulin-dependent diabetes mellitus, optic atrophy and deafness **[OK]** . The existing animal models for Wolfram syndrome do not recapitulate the symptoms observed in humans. Using the iPSC system, however, the investigators could show that increased levels of endoplasmic reticulum (ER) stress, decreased insulin content and a failure to react to glucose cause Wolfram syndrome. Strikingly, addition of 4-phenylbutyric acid, a chemical chaperone of protein folding and trafficking, to these iPSCs relieved ER stress and restored normal insulin synthesis and glucose-stimulated insulin secretion[140] **[OK]**

Studying disease mechanisms using iPSCs might identify novel targets for personalized medicine. One example of successfully applied personalized medicine is the treatment of neonatal diabetes mellitus, which is at least in part caused by activating mutations in the *KCNJ11* gene, which encodes the ATP **[Au: ATP did not need to be defined so I have removed the name.]**-sensitive inward rectifier potassium channel 11 Kir6.2 (also known as IKATP) **[OK].** The disease can be successfully treated with sulfonylureas, which inhibits the Kir6.2 [141,142] **[OK]**. In summary, stem cell differentiation platforms enable the study of human β-cell formation and disease processes, and might provide a valuable source of β-like cells for therapy in the future. iPSC technology is especially valuable when animal models generated by complete gene knockouts do not recapitulate the phenotype observed in patients as most genetic disorders are caused by exonic mutations or single nucleotide polymorphisms in non-coding regions that interfere with gene function, but do not result in a complete loss of gene function. However, as our knowledge of β-cell differentiation during development and postnatal β-cell maturation is incomplete, the major problem is that immature β-like cells are produced in vitro, which also hampers studying disease mechanisms using iPSC technology. Thus, identifying the pathways driving postnatal β-cell maturation is urgently needed to generate fully functional β-cells in vitro.

[H1] Modelling β-cell function and failure [Done]

Loss and dysfunction of β-cells are the main hallmarks of T1DM and T2DM, respectively. Therefore, understanding the cellular and molecular mechanisms underlying β-cell function and failure is of great importance to unravelling the pathomechanisms of diabetes mellitus. In this section, we discuss the advantages and obstacles of different modelling systems in studying β-cell physiology and pathology.

[H2] Primary islets

Mouse and human primary islets are extensively used for ex vivo endocrine cell studies[143]. Although compared with in vivo systems, isolated islets ultimately lose their microenvironment, innervation and vasculature, they still sustain their physiological function **[OK]**. Yet, studies based on human primary islets are restricted due to limited access to donor islets, especially from patients with early onset diabetes mellitus **[Au: Are you referring to T1DM or both T1DM and T2DM? both]**. In addition, the donor-to-donor variation due to genetic background, sex, ethnicity, diet, BMI, **[Has been removed]**, isolation procedure and the differences in size and composition of human islets are challenges for comparative studies of β-cell function. To overcome these issues, micro-islets with defined size, endocrine cell number and composition have been generated (by InSphero AG, Switzerland). This platform uses the ability of dispersed endocrine cells to spontaneously reaggregate[144] to form clusters with identical size and uniform cellular composition[145] (Fig. 3A). Thus, human micro-islets provide a model system for analyzing β-cell biology in respect to proliferation, heterogeneity and function in a standardized manner[64,145]. In addition, this system might be useful for screening small molecules in preventing β-cell apoptosis in ex vivo models of human T1DM.

Pancreas tissue slices is another platform in which islet function can be studied in their natural environment[146]. This system has been used for morphological and functional analyses of the endocrine and exocrine pancreas from mouse, porcine and human donors. Compared with isolated islets, this technology has advantages in preserving tissue morphology due to a less-damaging and faster preparation procedure[146]. Therefore, this platform not only offers the possibility to study islet morphology and function in their partially intact microenvironment, but also allows the investigation of the crosstalk between pancreatic and non-pancreatic tissues. Such interactions of islets with endothelial, mesenchymal and neuronal cells can also be monitored using noninvasive in vivo fluorescence imaging in living animals. Using this approach, isolated islets are transplanted into the anterior chamber of the eye, where the transplanted islet becomes vascularized and innervated[147]. After engraftment, islets preserve their

structure and function, providing a unique platform for longitudinal studies of islet proliferation, survival, stimulus-response and heterogeneity at single-cell resolution[64,123,147].

[H2] Pseudo-islets

Primary pancreatic tissues would be the closest to an in vivo physiological system to study β-cell function and failure. However, due to limited access to primary human endocrine cells **[has been specified]** and short-time period of culturing islets **[has been included.]** in vitro, several cell lines have been established to study β-cell physiology in vitro **[OK]**. Among these, Min6 and INS-1 insulinoma cell lines are the most commonly used for analysis of rodent β-cell function[148–150]. In comparison, several generations of immortalized human β-cell lines (EndoC-βH1-3) have been created as a suitable model for human β-cell studies[151–153] **[Has been modified]**. These cell lines not only fulfil β-cell characteristics and function in vitro, but also present a valid system for modelling T1DM and T2DM as well as for drug screening studies[154]. For instance, exogenous administration of mesencephalic astrocyte-derived neurotrophic factor (MANF) has been shown to prevent β-cell apoptosis induced by proinflammatory cytokines in human islets and EndoC-β-cell lines[155]. Furthermore, this cell line can model human β-cell dedifferentiation, which mainly occurs in T2DM[156]. Both Min6 and EndoC-β-cell lines are able to form 3D pseudo-islets either in free-floating or ECM-based culture conditions[157,158] (Fig. 3B). Compared with monolayer culture, these 3D pseudo-islets exhibit improved glucose sensing, highlighting the importance of cell–cell interaction, polarity and compaction for proper function of pancreatic β-cells. Additionally, the presence of islet derived-endothelial cells enhances the function of pseudo-islets, stressing the importance of crosstalk between endothelium and β-cells for appropriate glucose sensitivity and insulin secretion[158,159]. In the future, optimization of β-cell line-derived pseudo-islets co-cultured with endothelial and mesenchymal cells to generate islet biomimetics might offer improved physiology for in vitro β-cell function studies.

[H2] Islet-on-a-chip

One challenge of studying isolated islets is the lack of vasculature, intracellular flow and restricted media and oxygen diffusion in the static culture condition. This limited flow not only impacts the function and survival of β-cells located at the islet core but also results in ultimate loss of endothelial cells[160–162]. To overcome these complications, organ-on-a-chip **[OK]** (OOC) systems have been applied to analyze pancreatic islet function (Fig. 3C). These platforms use the microfluidic systems to generate micro-sized tissues in microchip chambers with dynamically perfused media in 2D or 3D culture[163–167]. The biggest advantage of OOCs is the direct control of liquid flow and rapid exchange of media, which removes the necrotic cells and increases the tissue survival. In addition, OOCs are perfect systems for parallel assays

for high-throughput analyses. It has been shown that continuous flow of media containing bovine serum albumin results in improved maintenance of islet endothelial cells in a microfluidic device[160,168]. Additionally, due to rapid assessment of islet quality and function, the microfluidic device offers a valuable system for fast quality control of human islets from donors before transplantation[169]. **[OK]**

OOCs can also be used to study the crosstalk between pancreatic islets and other organs. In this context, the interconnection between human micro-islets and liver spheroids has been assessed in a multiorgan-on-a-chip system. In this platform, insulin secreted by islets induces glucose uptake by liver spheroids supporting the presence of functional crosstalk between the two organs[170]. Despite these applications, OOCs still have some unsolved issues. For example, whether endocrine, endothelial and mesenchymal cells function optimally on OOCs is not clear as the required media is distinct for each cell type **[Has been modified]**. Moreover, the cell type source is a problem in respect to the differences in proliferation rates of distinct cell types. Furthermore, the time-period of keeping culture conditions on chips is shorter than for pseudo-islets and this shorter time **[OK]** together with insufficient evidence of functionality of the system, urge for further improvement and optimization of OCCs for future analysis of β-cell formation and function.

[H1] Modelling β-cell maturation and failure in large animal models [Au: The current heading is too long (maximum 38 characters, including spaces). I suggest shortening to 'β-cell studies in large animals. OK]

The systematic study of β-cell maturation in humans is not possible as material from embryonic, fetal and early postnatal stages, at which β-cell mass is established and β-cells functionally mature, is not available. In addition, longitudinal studies that enable assessment of disease onset and progression are required to understand the underlying pathomechanisms of diabetes mellitus, but these are not feasible in humans. Until now β-cell formation, maturation, heterogeneity and failure have been mostly studied in rodents. However, rodent models have limitations for translational research due to species-specific differences, for example, in gene expression but also in aspects of pancreas organogenesis and physiology (Table 1 and 2) [171]. Therefore, alternative model systems with physiological and pathophysiological similarity to humans are urgently needed.

Spontaneous development of T1DM in large animal models is rare but can be experimentally induced by pancreatectomy and chemical ablation of β-cells by streptozotocin **[Au: I have removed the abbreviation STZ as our journal style does not allow the use of abbreviations for single words.]** .

Pancreatectomy has been used to induce hyperglycaemia in pigs, dogs and non-human primates[172] **[OK]**. Streptozotocin has been used to induce diabetes mellitus in pigs and cynomolgus monkeys (*Macaca fascicularis*)[173].

Large animals are also used in T2DM research. Historically, dogs have been extensively used for metabolic studies. Obesity can develop spontaneously in dogs or can be induced by feeding a high-fat and high-fructose diet and is characterized by postprandial hyperglycaemia and hyperlipidaemia, but there is an absence of fasting hyperglycaemia[174]. In addition, the combination of a high-fat diet with streptozotocin in dogs serves as a model of obesity and mild T2DM[175]. In contrast to dogs, cats are more likely to spontaneously develop T2DM. Feline diabetes mellitus resembles human T2DM in several clinical, physiological and pathological aspects. Features common to humans that are also seen in cats include developing diabetes mellitus in middle age and the association with obesity and insulin resistance as well as severe loss of β-cell mass[176] **[Au: Edit OK?]**. In addition, besides *Macaca mulatta* monkeys, cats form amyloids (mainly aggregates of islet amyloid polypeptide) **[Has been modified]** in islets that are similar to those seen in humans with T2DM, making them a good model for human T2DM and the study of islet amyloidosis[176,177].Similar to humans, development of T2DM in old-world non-human primates **[Au: I have removed NHPs]** is most common in older, obese animals and reflects many characteristics of human disease such as compensatory insulin secretion and islet hyperplasia at onset of disease and replacement of islets with islet-associated amyloids at later stages[178]. Obesity in pigs is routinely induced by high-energy, high-fat and/or high-carbohydrate diets. However, diet-induced obesity in Göttingen minipigs does not elicit an overtly diabetic phenotype but rather serves as a model of the metabolic syndrome[174,179,180]. **[OK]**

In summary, large animal models have mainly been used for metabolic studies to assess insulin sensitivity, glucose tolerance or liver glucose uptake in animals that are obese or have diabetes mellitus **[OK]**. In addition the use of larger animal models enables invasive measurements and longitudinal sampling (for instance by chronic cannulation of vessels) that are not possible in rodent models or humans[174] **[OK]**. However, studies addressing the molecular mechanisms underlying β-cell maturation, function and failure are rare despite their great potential, which is briefly discussed in the following section **[OK]**.

[H2] Porcine NICCs — modelling β-cell maturation and failure

Pigs have a very similar embryonic development [181], anatomy (such as similar structure of the gastrointestinal tract) and physiology (for example blood levels of glucose) to humans (Table 1 and 2).

[Au: I have edited/rewritten the highlighted section for clarity. It is not my intension to change the original meaning of the text, if I have introduced errors then please correct me.] Genetic engineering in pigs is fairly easy. Techniques include genome editing techniques and somatic cell nuclear transfer, which generates reporter pigs [182] or porcine models of human diseases (including diabetes mellitus[183,184]) . The similarities between human and pigs **[Has been changed]** together with the ease in engineering transgenic pigs and the experimental evidence that transplantation of porcine neonatal islet-like cell clusters (NICCs) and adult islets into allogeneic pigs and non-human primates correct diabetes mellitus [185,186], make the porcine model an excellent large animal model for translational research and regenerative medicine[174] (Table 1). Alternatively, though ethically challenging, interspecies chimeras can be generated to produce pigs with human pancreases that serve as donors of human islets for transplantation but also allow the study of human pancreas organogenesis and disease [187–191].

Porcine NICCs are capable of proliferating and differentiating in vitro, making them an ideal source to study developmental islet cell plasticity and mechanisms of postnatal β-cell mass expansion, maturation and failure in a time-resolved manner (Fig. 4). NICCs can be easily obtained by enzymatic digestion and are composed mainly of exocrine tissue (duct and acinar cells) and 5% insulin-positive cells and 2% glucagon-positive cells. In vitro culture of NICCs, however, enriches the endocrine cell fraction up to 30%[192]. **[OK]**

Reconstructing the developmental trajectory of pancreatic β-cells to gain insight into the mechanisms of postnatal β-cell mass expansion and maturation requires time-resolved single-cell analysis. Single-cell transcriptomics of murine β-cells across different postnatal stages implicate reactive oxygen species **[Au: Is this what you mean by Ros? Yes]**, ER stress, serum response factor (SRF), mitogen-activated protein kinase (MAPK), TGFβ, WNT and platelet-derived growth factor (PDGF) signalling as factors in postnatal β-cells proliferation and maturation[193,194] **[OK]**. Modulating these pathways could be a strategy for reactivating and promoting the expansion and maturation of residual β-cells in patients with diabetes mellitus or to improve in vitro differentiation protocols. Yet, it remains to be shown if these pathways are evolutionary conserved and regulate human β-cell proliferation and maturation. As it is not possible to perform this type of analysis in humans, studying porcine NICCs across different fetal and postnatal stages might provide valuable molecular insights into β-cell biology **[OK]**.

[H2] Tailored diabetes mellitus models

In addition to experimental induction of diabetes mellitus (such as by streptozotocin ablation of β-cells), genetic engineering has been used to impair β-cell function and induce diabetes mellitus in pigs. Porcine

diabetes mellitus models mimic human disease mechanisms at the molecular level and enable the study of disease onset and resolving β-cell failure over time[195]. Models of diabetes mellitus have been generated for permanent neonatal diabetes mellitus (by the expression of mutant insulin Cys94Tyr in β-cells)[184], impaired incretin function (by expressing a dominant-negative form of glucose-dependent insulinotropic polypeptide receptor, GIPR), which is also observed in human T2DM[196] and MODY type 3 (by expressing a dominant-negative form of human hepatocyte nuclear factor 1α, HNF1α)[174,183]. Transgenic pigs expressing the mutant insulin Cys94Tyr are a model for ER-stress-induced permanent neonatal diabetes mellitus, also termed mutant *INS* gene-induced diabetes of youth **[Au: I have removed MIDY as this is not used again.]** [184,197]. Corresponding *INS* or *Ins2* mutations that disrupt the C(B7)–C(A7) interchain disulfide bond of the insulin molecule also exist in humans and in the Akita mouse model, respectively, and result in impaired trafficking and processing of proinsulin and accumulation of misfolded insulin in the ER[197].

Chronic ER stress inhibits β-cell proliferation[198] and is implicated in the pathogenesis of T1DM and T2DM[199]. *Ins*^{Cys94Tyr} transgenic pigs develop a stable diabetic phenotype at a very early stage and are **[OK]** characterized by elevated blood levels of glucose, impaired insulin secretion and reduced β-cell mass. **[OK]** This model can be used as a system to study the onset and progression of diabetes mellitus while monitoring the maturation and expansion of islets in vivo **[OK]**. Despite their functional heterogeneity, β-cells are also differentially susceptible to autoimmune attack[3,22,75]. As triggering endogenous repair by targeting specific β-cell subpopulations to stimulate their proliferation and/or maturation is a promising strategy to restore β-cell mass and normoglycaemia in patients with diabetes mellitus, the role of β-cell heterogeneity in the pathogenesis of the disease needs to be deciphered[171]. However, studying β-cell heterogeneity in health and during disease progression is difficult in humans due to the high donor-to-donor variability and limited access to material. Thus, the porcine model might offer an alternative as genetic variability is low and material is rich and easily accessible.

[H1] Conclusions

In term of clinical research **[Has been modified]**, findings from successful pre-clinical studies using mouse models frequently fails to translate to humans. Therefore, studying human primary tissues, establishing accurate and reliable in vitro models and using animal models that are similar to humans are crucial setups for translating our findings into clinics. Major progress has been made towards a better understanding of human β-cell biology over the last decade **[Done]** thanks to programs and networks such as the Juvenile Diabetes Research Foundation (JDRF) network program (www.JDRFnPOD.org), which

provides access to human pancreases, including tissues from very early life and from donors with T1DM. Furthermore, the integrated islet distribution program (IIDP, https://iidp.coh.org) and human islet research network (HIRN, https://hirnetwork.org) support research by providing isolated islets from organ donors with diabetes mellitus. These programs not only provide tissues for research but also enable extensive exchange between researches all over the world. This program together with the breakthrough in transcriptomic profiling at the single cell level makes it possible to obtain a comprehensive picture of human pancreatic lineage trajectory, differentiation and heterogeneity in health and disease. Additionally, the establishment of new technologies such as fluctuation localization imaging-based fluorescence in situ hybridization (fliFISH)[200], multiplex mass spectrometry imaging[201], single-cell Western blot[202] and single-cell resolution imaging might provide deeper insight into human β-cell development and function.

Future work should focus on the drawing of a conclusive picture on the functional state of β-cell **[Do you mean a 'b-cell'? Yes]**, which requires integrating single-cell-based genomic, transcriptomic and proteomics data sets as well as spatial information from imaging techniques. Importantly, pancreatic islets are micro-organs that interact and depend on their non-endocrine neighbouring cells, suggesting the need for establishing 3D multi-lineage systems to analyze their function and failure. This setup **[OK]** is important due to the possible contribution of non β-cells in triggering and assisting in the progression of diabetes mellitus (such as α-cell dysfunction or defects in the immune system that prevent the elimination of altered β-cells in T1DM) **[OK]**. The multi-lineage platforms are also important for the generation of stem cell-derived β-like cells in vitro, which is proven by the fact that, so far, these cells only become mature after transplantation **[Has been modified]**. In addition, we need to systematically analyse islets from patients with T1DM and T2DM from a large number of different donors to understand not only the genetic basis of the disease progression **[OK]**, **[The sentence has been modified.]** but also most importantly the onset of disease to identify biomarkers and mechanisms that can be targeted to prevent the disease. These analyses **[OK]** should address several main challenges, which we are still facing: to define pathomechanisms of T1DM and T2DM and identify novel targets for therapy; translating stem cell-derived islet differentiation into the clinic and identifying strategies to regenerate islet cell mass in patients with T2DM **[OK]**. In summary, the combination of the aforementioned tools and the availability of human tissues, optimized ex vivo modelling platforms and evolutionary comparative model systems **[Has been deleted]** aim to transform translational research and to generate in-depth understanding of pancreatic development and pathomechanisms, which will hopefully help to design improved therapeutic approaches for the treatment of diabetes mellitus.

Acknowledgements [OK]

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Author contributions [OK]

All authors contributed to researching data for the article, discussion of content, writing the article and reviewing and/or editing the manuscript before submission.

Competing interests

The authors declare no competing interests.

Related links

Juvenile Diabetes Research Foundation (JDRF) network program: www.JDRFnPOD.org

Integrated islet distribution program (IIDP): https://iidp.coh.org

Human islet research network (HIRN): https://hirnetwork.org

Table 1: Comparison of preclinical model systems with humans. **[Au: I have edited this table to conform with our journal style, please check that I have not changed anything incorrectly.]**

Parameters	Mouse	Domestic pig	Human
Ethical acceptance	Yes	Yes	Limited to research but organ donation for transplantation is well-regulated in many countries.
Sufficient sample material	Limited	Yes	Very limited.
Access to embryonic, fetal and postnatal material or **[OK]** sampling during disease progression.	Yes, but limited.	Yes, but limited.	Very limited or not at all.
Genetic engineering possible?	Yes, in vivo	Yes, in vivo	Limited (gene therapy). Ex vivo techniques involve manipulation of stem-cell derived β-cells and viral transduction of primary islets
Maintenance costs	Low	High	NA **[OK]**
Gestation period (days)	21	116	280
Life expectancy (years)	1–2	14–18	79
Diabetes mellitus models available	Yes[174]	Yes[174]	NA **[OK]**

NA, not applicable.

821 **Table 2:** Comparison of pancreas development in human, mouse and pig.

Features of pancreas development [Au: Edit OK? is this correct?]	**Mouse**	**Domestic pig**	**Human**
Appearance dorsal–ventral bud	E9.0–E9.5[203]	19 dpc (earliest time point analyzed)[204]	30–33 dpc[12,13]
Nkx2.2 gene expression in multipotent progenitor cells **[Has been changes]**	Expression of *Nkx2.2*[23]	Unknown	No expression of *NKX2.2*[12,13]
Primary transition	Yes (E9.5–E12.5)[23]	Unknown	No[12,13]
Appearance of endocrine cells	During primary transition, mainly generation of glucagon-positive and a few short-lived insulin-positive cells. The majority of β-cells and α-cells are formed during the secondary transition, which begins at ~E13.0[24]	Glucagon-positive and insulin-positive cells are present at 19 dpc (earliest time point analyzed), glucagon-positive cells are more abundant. From ~ 60dpc, insulin-positive cells are more abundant than glucagon-positive cells. SST-positive δ-cells appear at ~31 dpc[204].	β-cells appear first at ~8 wpc, α-cells emerge at ~9 wpc[12,13,205] . SST^+ cells emerge at ~9–10 wpc. PP-positive cells emerge at ~17 wpc[205].
Islet formation	Islets form close to birth (E19–21)[24].	Initially, endocrine cells are densely packed but from 82 dpc they are dispersed throughout the entire pancreas as single cells or small cell clusters[206]. Endocrine cells cluster in small islets ~10–13 days after birth. Many single insulin-positive cells remain scattered in the exocrine tissue. The adult distribution pattern is reached several months after birth[207].	Islets apparent at 12 wpc[205,208].
Polyhormonal cells	During primary transition, formation of glucagon-insulin-positive cells[24]	Glucagon-insulin-positive cells are present at 19, 25 and 28 dpc and not detected in embryos and **[OK]** fetuses at later developmental stages or in postnatal tissue [204].	Cells coexpressing insulin and glucagon are observed during 9 to 21 wpc[205].
β-cell ratio	0.85[125,209]	0.89[125,209]	0.64[125,209]
Regional differences in islet cell composition	PP-cells are enriched in head-derived islets, while α-cells are enriched in tail-derived islets[125]	PP-cells are enriched in head-derived islets, while α-cells are enriched in tail-derived islets[125,210]	PP-cells are enriched in head-derived islets, while α-cells are enriched in tail-derived islets[125,211]

Islet architecture	Adult islets: Core (β-cells)-mantel	• Adult islets
		  ○ Small islets: Core
			• Fetal islets (~14wpc)
			  ○ Core (β-cells)-

	(α-cells, δ-cells and PP-cells **[OK]**) segregation[125]	(β-cells)-mantel (α-cells, δ-cells and PP-cells **[OK]**) segregation[125] ○ **[Au: Please provide the first author initials for this observation. If this is someone that is not on this Review then we will need email permission from them to include is information here. Has been deleted]**	mantel (α-cells, δ-cells and PP-cells **[OK]**) segregation[205] • Adult islets ○ Small islets: Core (β-cells)-mantel (α-cells, δ-cells and PP-cells **[OK]**) segregation ○ Large islets: trilaminar plates – β-cells and α-cells are intermingled in the islet core[212]

822 Dpc, days post-conception; E, embryonic day; PP, pancreatic polypeptide; SST, somatostatin; wpc, weeks post-
823 conception **[Have been included]**

824

825 ## Figure legends

826 **Figure 1: Early pancreas development, endocrine cell formation and clustering.** (A) In rodents and
827 humans dorsal and ventral pancreatic buds are derived from the foregut endoderm. (B) During secondary
828 transition, the pancreatic epithelium consists of a branched peripheral region and a plexus core, which
829 gradually remodels into a ramified epithelial layer. (C) Endocrine progenitors mainly reside within the
830 plexus area. Upon differentiation, endocrine cells reduce their apical domain size, form a tether and
831 finally detach from the epithelial lumen. (D) Delaminated endocrine cells cluster together and form proto-
832 islets. E, embryonic day. **[Au: The panel labelling might change after our artist redraws it, I will**
833 **update the figure legend labelling here and in the main text when it comes through.]**

834 **Figure 2: 3D organoid systems for modelling human pancreatic morphogenesis and differentiation.**
835 3D spheroids (cell aggregates without central lumen), cysts or spheres (circular, polarized epithelial layer
836 with a central lumen) and organoids (complex, polarized epithelial structures with a central lumen) are
837 generated from embryonic or adult pancreatic progenitors in 3D culture condition. **[Done. Also can you**
838 **please tell me what the difference is between the red and green cells so I can get our artist to label**
839 **them. Has been included in the figure comments]** Different cell types from embryonic pancreatic
840 epithelium including multipotent progenitors, bipotent progenitors and ductal epithelial cells can generate
841 3D organoids. The reprograming of human fibroblasts into induced pluripotent stem cells (iPSCs) and
842 their subsequent differentiation towards pancreatic progenitors generates 3D organoids. Human
843 embryonic stem cells (hESCs) can be directly differentiated towards pancreatic progenitors to form
844 organoids. Adult isolated multipotent pancreas-derived progenitors (cells with high aldehyde
845 dehydrogenase activity ($ALDH^{high}$) or $CD133^{+}$ colony-forming units) **[OK These cells need to be**

defined in the diagram.] produce organoid structure in 3D environment. E, embryonic day. **[Au: The panel labelling might change after our artist redraws it, I will update the figure legend labelling here and in the main text when it comes through.]**

Figure 3: In vitro modelling systems to assess β-cell function. (A) Dissociation and reaggregation of human islets with different size and composition produce micro-islets with similar size and endocrine cell composition. **[Au: are green cells = α-cells, blue = β-cells and yellow = δ-cells? The color need to be consistent to the other figures]** (B) Mouse Min6 insulinoma and human EndoC-βH cell lines **[has been corrected.]** generate pseudo-islets in 3D culture condition. **[Has been added]** (C) Primary human islets co-cultured with non-pancreatic cells on microfluidics device. The advantages and difficulties of each modelling system are listed below each technique. (?, needs to be experimentally tested) …. **[Au: I have edited out the mention of red and green text as our journal style does not allow this. I will get our artist to have this clearly labelled in the diagram]**.

Figure 4: The pig as a translational animal model to systematically study β-cell formation, maturation, function and failure. (A) Neonatal islet-like cell clusters (NICCs) can be isolated from neonatal pigs and are composed of exocrine and endocrine cells (depicted are only α-cells and β-cells) with no defined structure. **[We would like to ask for change the word "Used in" to "Applications" in part a and b of this figure.]** (B) Adult porcine islets are organized into trilaminar epithelial plates similar to human islets (depicted are only α-cells and β-cells).

Reference [Au: I have edited a few of the references (highlighted in yellow), please make sure that my changes have not affected the coding of the referencing program you have used.]

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