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Using single-cell multi-omics screening of human fetal pancreas to identify novel players in human beta cell development

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Abstract

Islet transplantation from organ donors can considerably improve glucose homeostasis and well-being in individuals with type 1 diabetes, where the beta cells are destroyed by the autoimmune attack, but there are insufficient donor islets to make this a widespread therapy. Strategies are therefore being developed to generate unlimited amounts of insulin-producing beta cells from pluripotent stem cells, with the aim that they will be transplanted to treat diabetes. Whilst much progress has been made in recent years in the directed differentiation of pluripotent stem cells to beta-like cells, essential gaps still exist in generating stem cell-derived beta cells that are fully functional in vitro. This short review provides details of recent multi-'omics' studies of the human fetal pancreas, which are revealing granular information on the various cell types in the developing pancreas. It is anticipated that this fine mapping of the pancreatic cells at single-cell resolution will provide additional insights that can be utilised to reproducibly produce human beta cells in vitro that have the functional characteristics of beta cells within native human islets.

KEYWORDS

beta cells, development, gene analysis, human pancreas, stem cells, transcription factors

1 | INTRODUCTION

It is now over 100 years since the discovery of insulin, which has dramatically improved the outcomes for people with type 1 diabetes. Exogenous insulin therapy can, however, lead to an unstable glycaemic state and some patients can suffer from the untoward consequences of insulin-induced hypoglycaemia. Severe hypoglycaemia, which occurs in approximately 40% of insulin-treated type 1 diabetes patients, can be fatal, especially in those with impaired awareness of hypoglycaemia.[1,2] Islet transplantation has the potential to cure type 1 diabetes and it is effective at reducing the frequency of severe hypoglycaemia,[3,4] but it is not yet a widespread therapy because there are insufficient donor islets available for transplantation. Several research groups are therefore optimising protocols to generate beta-like cells from stem cells, with the aim of providing large quantities of these beta-cell replacements for transplantation therapy. Much success has been achieved so far, with a range of strategies being

developed to improve the generation and function of stem cell-derived beta cells.[5-8] However, the current protocols, which largely mimic mouse pancreas development, yield at best 30%–50% beta-like cells,[9] require an in vivo maturation stage and the beta-like cells generated do not yet have comparable metabolic attributes to human islets.[5,10] Recent studies using multi-omics technologies are beginning to shed light on the molecular landscapes of the human fetal pancreas at multiple developmental stages at a single-cell resolution.[11-14] The detailed information obtained from profiling pancreatic progenitor cells as they differentiate and mature into beta cells over different stages of development will be of critical assistance to the global effort of producing functional beta-like cells in vitro, which will not require additional maturation steps before clinical use. This review will provide an overview of recent developments in the multi-omics analysis of the developing human pancreas.

Novelty statement

- Some individuals with type 1 diabetes require islet transplantations to allow them to properly regulate their blood glucose levels.
- There are insufficient organ donors to provide human islets for all individuals who would benefit from a transplant.
- Protocols for generating beta cells from stem cells in vitro have not been fully optimised and improvements would benefit from a fuller understanding of the factors governing beta cell specification in the developing human pancreas.
- We describe here multi-omics methods that are being used to better understand beta cell developmental trajectories.

2 | HUMAN PANCREAS DEVELOPMENT

The pancreas is a dual-functional organ, with its exocrine and endocrine compartments regulating digestion and fuel homeostasis respectively. Derived from the foregut endoderm, human pancreas development begins with the formation of the dorsal and ventral buds approximately 26–30 days post-conception (dpc), and these fuse upon gut rotation at about 6 post-conception weeks (PCW) to form a single organ. The highly proliferative multipotent progenitor cells are initially marked by the presence of pancreatic and duodenal homeobox factor 1 (PDX1), then by the transcription factors pancreas-associated transcription factor 1a (PTF1A), SRY-box transcription factor 9 (SOX9) and NK6 homeobox 1 (NKX6.1), and they can differentiate into all the pancreatic cell types.[15-17] The importance of regulated expression of transcription factors in pancreas development has been established through mouse genetics, and information on transcription factor involvement in human pancreas development has been provided through the study of monogenic forms of diabetes. Figure 1a provides an overview of genes that have been implicated in pancreas development and the phenotypes associated with their mutation or deletion. This information on transcription factors driving beta cell development has been used to develop stem cell protocols in vitro in which small molecule- and protein-induced stepwise activation and inhibition of the associated signalling pathways mimic the sequential steps of pancreas development to generate beta-like cells. Thus, a homozygous point mutation in PDX1 was identified as the cause of pancreatic agenesis in humans and the phenotype was mimicked in a PDX1 null mouse model.[18,19] Ectopic overexpression of PDX1 was therefore used to drive pancreatic commitment in an embryonic stem cell line, which generated insulin-positive beta cells in vitro upon differentiation.[20] These cells showed a marginal increase in glucose-stimulated insulin secretion in vitro and reduced hyperglycaemia in diabetic mice.[20] SOX9, another transcription factor, is present in pancreatic cells with PDX1 expression between 8 and 11 PCW, but SOX9 protein expression decreases over the following weeks.[21] We have carried out fluorescent immunohistochemical staining of the stomach region of a human embryo at Carnegie Stage 13 (30–33 dpc) and identified the dorsal pancreatic bud containing multipotent pancreatic progenitors, which shows a dual expression of PDX1 and SOX9 at this stage, and the duodenum ($PDX1^+/SOX9^-$) (Figure 1b). This indicates that PDX1 and SOX9 are colocalised earlier than previously reported, although the earlier study did not use the fetal pancreas prior to 8 PCW.[21] The pancreatic progenitors undergo extensive proliferation with SOX9 becoming progressively restricted to ductal cells and barely detectable in the beta cells.[21] However, the knockdown of SOX9 in human fetal epithelial cells led to a reduction in the number of Neurogenin 3- (NEUROG3) and insulin-positive cells, indicating that SOX9 may contribute to endocrine specification.[21] NEUROG3 expression specifies endocrine lineage commitment and it is reported to be first detected at 8 PCW, the same stage of development at which the first insulin-expressing beta cells become detectable in humans.[22] NEUROG3 is central to the development of

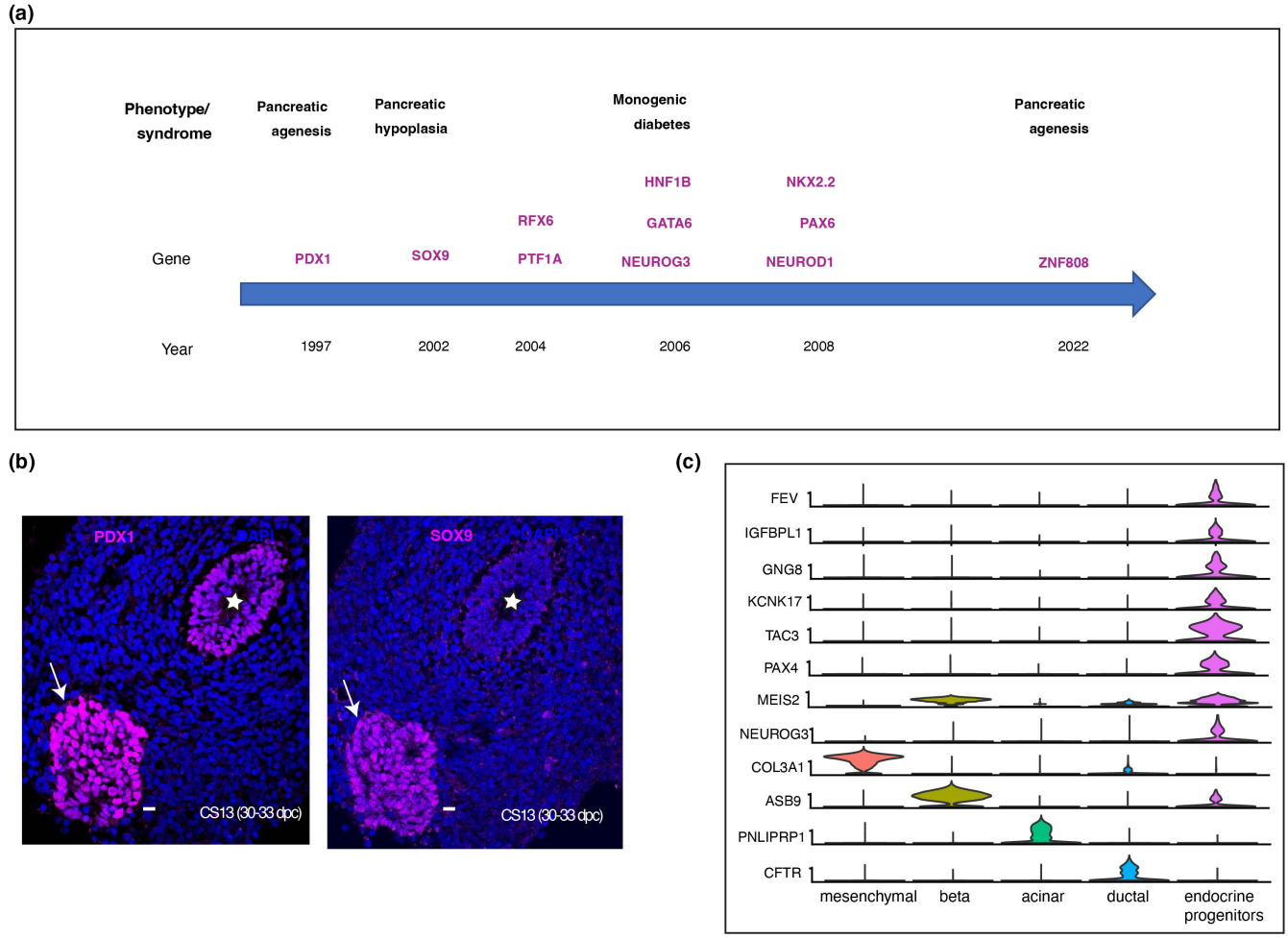

FIGURE 1 Genes driving human beta cell development. (a) Chronological line indicating some transcription factors that are important in pancreas and islet development.[15,21,23,24] Most of these genes were identified by studying the genetics of pancreatic agenesis or neonatal diabetes. Loss of ZNF808 was recently identified as a cause of pancreatic agenesis.[25] (b) Representative images showing immunostaining for PDX1 (left) and SOX9 (right) in a Carnegie Stage 13 (30–33 dpc) human embryo. Sections were de-waxed and antigen retrieval was carried out in citric acid followed by incubation overnight with primary antibodies (goat anti-PDX1, 1:100 and sheep anti-SOX9, 1:100). Signals were developed with fluorescently tagged secondary antibodies and images were obtained with a Nikon A1 inverted confocal microscope. Co-localisation of PDX1 and SOX9 indicates the dorsal pancreatic bud at this stage of development. Arrow = dorsal bud, star = duodenum. Scale bar: 20 μm. (c) Violin plot depicting the expression of endocrine progenitor-enriched genes (pink) from scRNAseq data of human fetal pancreas.[11]

all endocrine cell types as NEUROG3 knockout mice fail to develop endocrine cells.[23] NEUROG3 is therefore used as a marker for endocrine progenitors. Other transcription factors regulating human pancreas development have been well reviewed,[15] and mutations in genes known to cause neonatal diabetes add another layer of information that can be utilised in protocols to direct the generation of functional beta cells from stem cells in vitro. However, there are still many gaps in our knowledge of the critical genes involved in human beta cell specification and the high throughput from single-cell genomics technologies can offer additional insights into novel candidates essential for optimising beta cell differentiation protocols.

3 | MULTI-OMICS AS A TOOL FOR STUDYING PANCREAS DEVELOPMENT

It has been suggested that deep phenotyping of human beta cells, which includes information about cellular, epigenetic and positional gene expression patterns, will be required to generate functional beta cells in vitro.[26] A systematic analysis of human pancreas development is therefore required to generate the 'road-map' of maturing human beta cells from their progenitors. This will provide a thorough understanding of the transcriptional profiles of genes required for human beta cell maturation in situ, and such information can be utilised in improving protocols

for beta cell differentiation in vitro. Multi-omics is the integration of data generated from multiple layers of the cells such as the genome, epigenome, transcriptome and proteome and this approach can provide a comprehensive and integrative understanding of human pancreas development. These techniques, including single-cell RNA sequencing, spatial transcriptomics and epigenome sequencing, are already providing essential details about the individual cell types in human pancreas development, as outlined below.

4 | SINGLE-CELL RNA SEQUENCING

Single-cell RNA sequencing (scRNAseq) is a sensitive, high-throughput technique that identifies cell types and their transcriptional states. The ability to measure dynamic gene expression and transition states of single cells has made scRNAseq a more powerful technique for investigations of cell heterogeneity in embryonic development than standard bulk RNA sequencing. Innovations in scRNAseq are progressing rapidly and as such the power of this tool is increasing such that it is now possible to identify rare cell types, infer developmental trajectories, delineate gene-regulatory networks and determine differentially expressed genes between conditions.[27] However, compared to bulk RNA sequencing, a technique that reflects the average gene expression across thousands of cells, scRNAseq can have lower capture efficiency and higher dropout rates that contribute to the production of noisier data, making computational analysis more challenging. Data are therefore normalised to remove background noise and gene-specific biases so that gene expression in one sample can be compared to others.[27] scRNAseq usually involves a dissociation step, where tissues are mechanically or enzymatically dissociated into single cells, and live cells are then isolated using flow-activated cell sorting. The cells are lysed to generate as many mRNAs as possible, which are then captured by poly-T primers such that polyadenylated mRNAs can be converted to complementary cDNAs. Other nucleotide sequences such as barcodes and unique molecular identifiers (UMIs) are included to uniquely identify each cell. Single-cell transcriptomes are then obtained from the barcoded cDNA libraries by next-generation sequencing. When it is not possible to obtain fresh tissues, and thus single cells cannot be retrieved, nuclei may be isolated from archival or frozen samples for single nuclei RNA sequencing.[28] This approach has been used recently to create an atlas of human neonatal and adult pancreas cells, which has allowed the identification of tissue dynamics that take place during pancreas development.[29] To reduce cost and allow comparison between multiple batches when carrying out single-cell sequencing, cells from different donors or stages of development can be processed together in a single sequencing run.[30] In this approach the cells are conjugated with barcoded oligonucleotides which are then used to computationally resolve the cells into their various sources of origin. After quality control analysis of the sequenced data, cells are clustered based on transcriptional similarity and differentially expressed genes between distinct cell populations can then be identified. This allows for the identification of heterogeneity within a subpopulation of cells in a tissue and follow-up studies antibodies can be directed against surface markers to help distinguish cell subtypes, as has been carried out for human beta cells.[31] The use of scRNAseq to measure changes in the transcriptome of the human fetal pancreas over a range of gestational stages has recently allowed data mining of potential genes related to beta cell development.[11,13] In addition, these studies have provided information on pseudo-time ordering, which has led to improved understanding of the order in which genes are expressed during pancreatic cell lineage specification.[11,13] More detailed information on the use of scRNAseq to provide wider information on pancreas development may be found in a recent review.[32]

5 | SPATIAL TRANSCRIPTOMICS

In contrast to scRNAseq, spatial transcriptomics analysis does not involve tissue dissociation and this, therefore, provides a major advantage of allowing the determination of the transcriptome of cells in their native environment. This approach builds on scRNAseq to reveal information that is lost in the process of cell isolation that destroys the original tissue architecture. Spatial transcriptomics is a powerful technique that is used to resolve cellular trajectories occurring in situ and because of its applicability, it was named 'Method of the Year' in 2020.[33] Several versions of spatial transcriptomics have been developed and they vary in throughput and resolution, including Visium, Tomo-Seq and GeoMx Digital Spatial Profiling,[34] but only Visium has been used to study human fetal pancreas, to the best of our knowledge.[11]

Visium spatial transcriptomics by 10× Genomics combines histology with the quantitative spatial distribution of transcripts across tissue samples to reveal cellular heterogeneity and positional gene patterns.[35] Fresh frozen or formalin-fixed paraffin-embedded (FFPE) sections are mounted on spatial slides and for successful assessment of the spatial organisation, care must be taken during sectioning and mounting to preserve tissue morphology. RNA quality of the tissue should also be checked to ensure RNA integrity.

Visium spatial slides have 4 capture areas, each of 6500 × 6500 μm, which can hold four different samples. Each capture area contains 5000 spots, and each spot is about 55 μm, containing millions of positional barcoded oligonucleotides. Fresh frozen sections are methanol fixed, stained with haematoxylin and eosin and then imaged with a brightfield microscope, whereas FFPE samples are first de-waxed and de-crosslinked before downstream processing. The tissue sections are permeabilised to release mRNAs, which will bind to the spatially barcoded oligonucleotides present in the spots and reverse transcribed. By overlaying gene expression data from barcoded cDNA libraries on high-resolution images of tissue sections, distinct anatomical regions within the tissue and cell–cell spatial proximity can be identified. Visium spatial transcriptomics is a readily accessible approach, but its major limitation is that the 55 μm spot diameter does not allow single-cell resolution. However, this problem can be overcome by integrating Visium data with scRNAseq data and cell-type deconvolution of the spots can be achieved. We have recently used this integration approach to characterise gene expression profiles in the developing human pancreas and reveal cell–cell interactions and in situ spatial trajectories.[11] Cellular interactions are highly spatially-dependent and therefore the information gathered from spatial transcriptomics can identify the role of other cell types in directing the differentiation of a cell of interest. It is possible to predict ligand-receptor interaction from the scRNAseq data and use the spatial transcriptomic technique combined with immunostaining as orthogonal validation methods.

6 | ChIP AND ATAC-SEQUENCING

Understanding gene regulatory networks that govern human fetal beta cells by analysing chromatin or DNA accessibility can provide additional insights into optimising methods for generating functional beta cells in vitro. Gene regulation is controlled by transcription factors, which are genes that regulate the expression of other genes. Transcription factors have DNA binding domains that enable them to bind to corresponding regions on the DNA, making their actions specific, and they determine whether that part of the DNA is transcribed, allowing gene expression, or they may act as repressors to turn off gene expression. The short stretches of DNA regions, also known as enhancers, are important determinants of cellular diversity and lineage specification.[36] In the nucleus, DNA is wrapped around histones to form nucleosomes, the basic units of chromatin, and this controls whether a segment of the DNA is accessible to transcription factors. Post-translational modification of histones can offer another layer of gene expression regulation. The regions of the DNA where transcription factors are bound can be identified by transcription factor chromatin immunoprecipitation sequencing (TF ChIP-seq), in which chromatin is fragmented and antibodies are used to immunoprecipitate DNA-binding transcription factors of interest and thus 'pull down' these proteins and their bound DNA fragments. Since ChIP-seq identifies protein-DNA interactions, it can also map DNA-binding proteins and reveal histone modifications. The ChIP-seq protocol involves DNA and protein crosslinking and precipitation steps, so this method has several limitations such as the potential for antibodies to be non-specific, and as it requires at least one million cells it is not suitable for use with the limited numbers of cells that are available from the early-stage human fetal pancreas. A more sensitive method, with a higher signal-to-noise ratio and fewer input cells, known as CUT&RUN (Cleavage Under Targets and Release Using Nuclease) has, therefore, been developed.[37] In contrast to CHIP-seq, which requires DNA fragmentation, the CUT&RUN approach is applied to intact cells or nuclei that are incubated with a protein-targeted antibody. A nuclease is then used to selectively cut out the DNA to which the protein/antibody complex is bound. Chromatin is organised into a three-dimensional structure so enhancers can influence gene expression over a long range. Hi-C, a method in which cells are cross-linked with formaldehyde to maintain spatial links between chromatin segments, reveals chromatin interactions across an entire genome.[38] This method has been used to produce a three-dimensional map of long-range chromatic interactions occurring in human islets and this led to the identification of important enhancers driving adult human islet function.[39]

Gene transcription can only occur when the chromatin is opened, or loosely packed and another technique known as Assay for Transposase Accessible Chromatin with high throughput sequencing (ATAC-seq) is used to probe chromatin accessibility. This method uses a hyperactive Tn5 transposase to integrate sequencing adapters into open chromatin regions, which then reveals DNA accessibility upon sequencing. Unlike ChIP-seq, it does not require antibody binding and it can also reveal transcription factor footprints and the positions of nucleosomes. ATAC-seq requires about 500 to 50,000 cells but for all single-cell techniques, biological replicates are important to make a valid statistical inference. Regulatory programmes and type 2 diabetes risk variants occurring in adult islets and in development have been identified using this technique,[13,40] and novel endocrine-related transcription factors such as PLAGL1 have been identified in the human fetal pancreas.[13]

7 | NOVEL GENES THAT SHAPE HUMAN BETA CELL DEVELOPMENT

By integrating scRNAseq and spatial transcriptomics of human fetal pancreases at multiple stages of development, we have identified endocrine progenitor cell genes, including the transcription factors MEIS2 and FEV,[11] which had not previously been implicated in human islet cell specification. MEIS2 belongs to the three-amino-acid-loop-extension homeodomain superclass of transcription factors that are known to regulate growth and differentiation during embryogenesis in mammals. It is a direct regulator of PAX6 in lens development[41] and acts cooperatively with PDX1 to increase gene expression.[42] It is expressed in adult human islets by beta and epsilon cells.[43] Our analysis indicated that MEIS2 is expressed by endocrine progenitors and beta cells and, to a lesser extent, by ductal cells (Figure 1c). However, nothing is currently known about its role in human pancreas development, where it may be an important regulator of human endocrine progenitor differentiation. In addition, FEV whose expression is dependent on neurogenin3 in embryonic mouse pancreas was first detected in mouse islets in 2011[44] and has since been designated as a marker of a subset of endocrine progenitors in the mouse.[45,46] A recent study has identified its role in human pancreatic endocrine cell specification using stem cells that were differentiated towards the endocrine lineage.[13] Ideally, alterations in stem cell differentiation protocol outcomes following over- or under-expression of genes of interest should be validated in vivo, but this is obviously not possible in a human context.

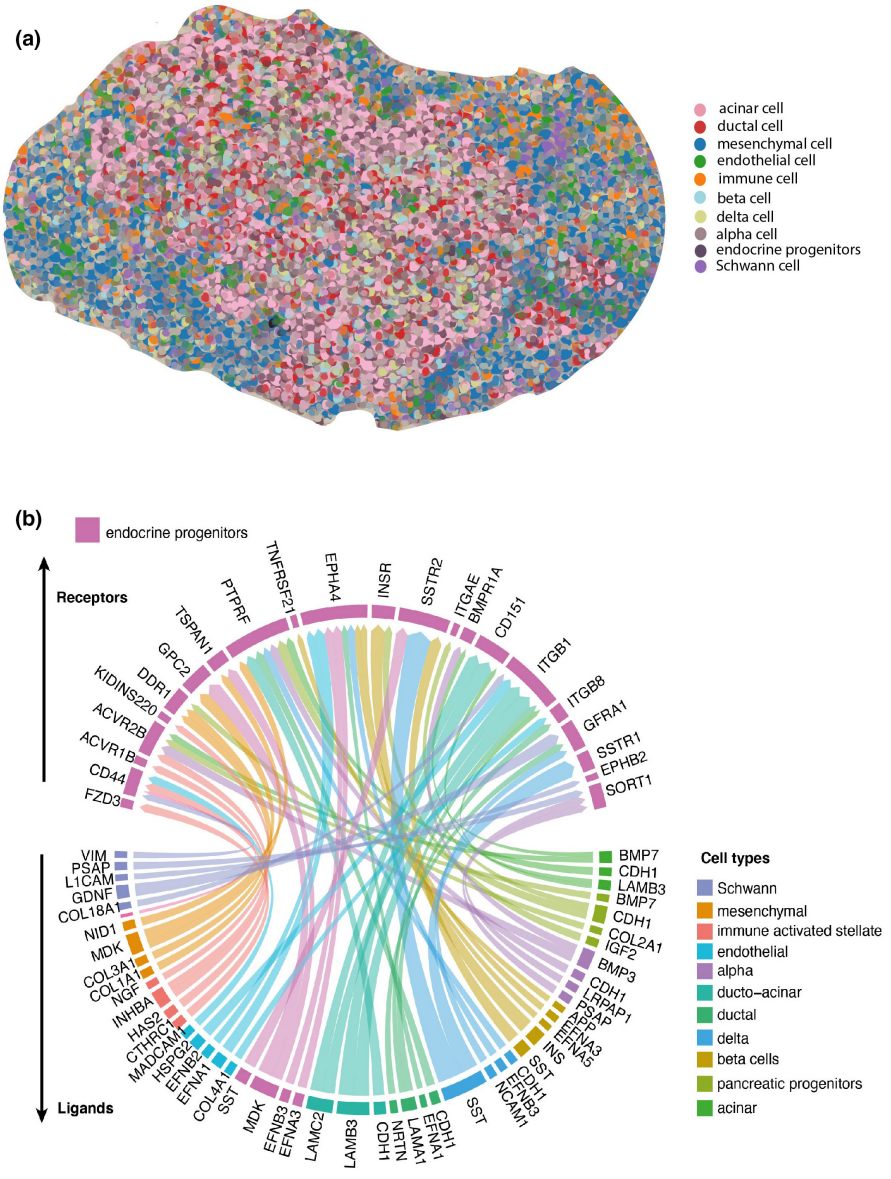

FIGURE 2 Cell–cell interactions within the developing human pancreas. (a) Schematic showing the spatial proximity of the different cell types identified by spatial transcriptomics of the developing human pancreas.[11] (b) Circos plot showing predicted ligand-receptor pairs from cell–cell connectivity analysis of human fetal pancreatic cells at 12–20 PCW.[11]

We have identified other genes that are highly expressed by human fetal endocrine pancreas progenitors, such as insulin growth factor binding protein like-1 (IGFBPL1), potassium two pore domain channel subfamily K member 17 (KCNK17), guanine nucleotide-binding gamma 8 (GNG8) and tachykinin precursor 3 (TAC3) (Figure 1c), which are also expressed by stem cell-derived endocrine progenitors.[5] IGFBPL1 was identified as important for increasing beta cell number by inducing alpha-to-beta cell transdifferentiation in a zebrafish model of beta cell regeneration, and this observation was replicated by treating isolated mouse and human islets with exogenous IGFBPL1.[47] IGFBPL1 has been implicated in the regulation of early brain development[48] and GNG is involved in ovarian development,[49] but nothing is known about their potential roles in beta cell differentiation. The contribution of these genes to endocrine progenitor specification and differentiation may be investigated by deleting or overexpressing them in stem cells and then investigating the effects that this has on the capacity of the stem cells to differentiate towards beta cells.

8 | PANCREATIC MICROENVIRONMENT

In an adult pancreas only approximately 1%–2% of the cells are endocrine, contained within the islets of Langerhans, and the remaining cells are mainly acinar, with lesser amounts of other cell types including ductal, mesenchymal and endothelial cells. It is known that co-ordinated interactions between multiple cell types such as mesenchyme, pericytes, endothelial cells and macrophages within the islet microenvironment regulate mouse beta cell development and maturity,[50-53] and the islet cellular environment must be replicated to produce functional beta cells in vitro.[54,55] During pancreas development, the endocrine progenitors are specified in the presence of other pancreatic cell types, and since the cellular microenvironment plays a crucial role in determining cell fate, an improved understanding of how other cell types influence endocrine progenitor specification and differentiation will be useful in improving protocols for generating beta cells in vitro. Cells have specific types of neighbouring cells within a tissue, between which there will be

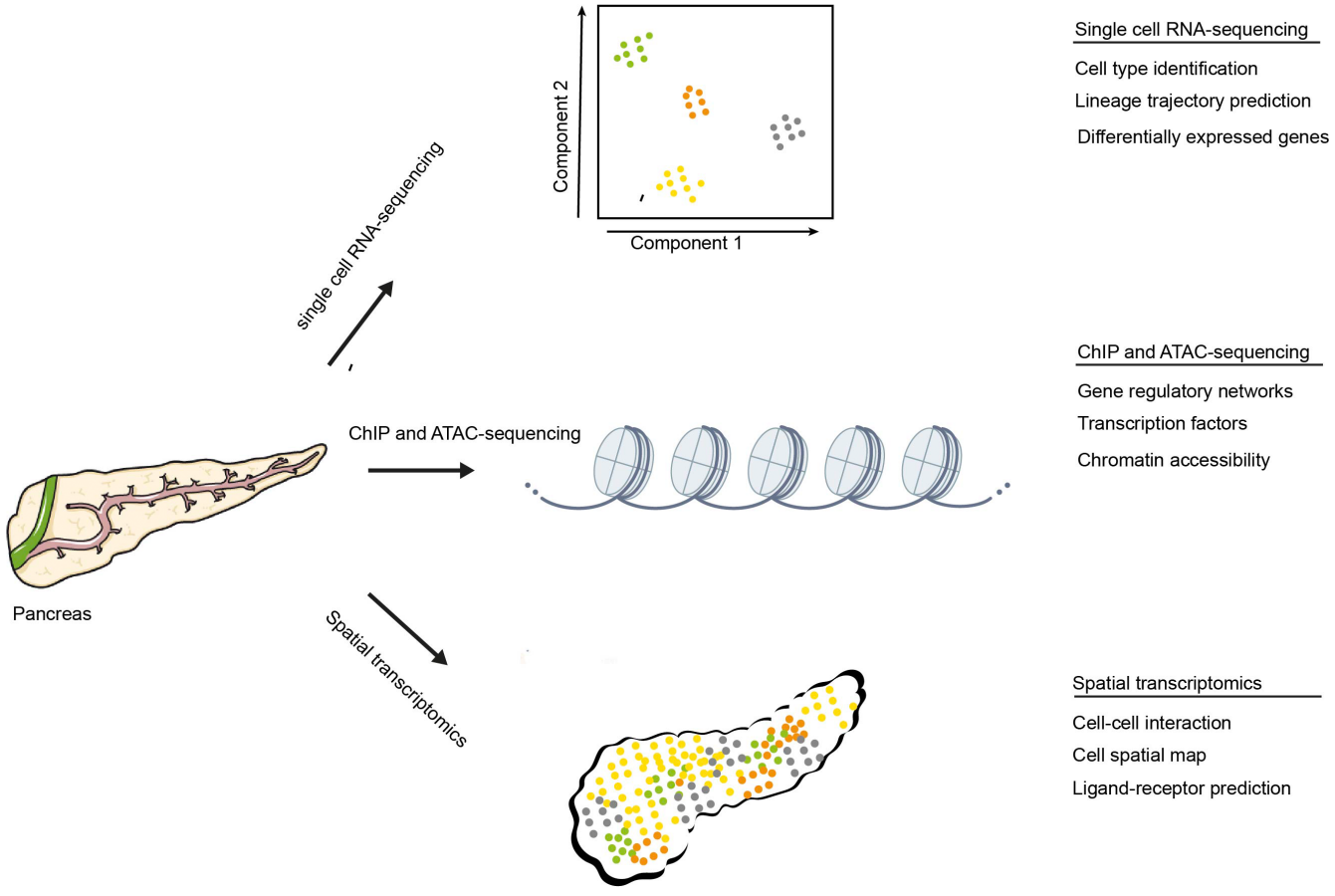

FIGURE 3 Single-cell multi-omic tools for studying human pancreas development. Schematic showing different single-cell genomic tools that have been used to study human pancreas development and the information derived from them.

cell-to-cell interactions. Using spatial transcriptomics, we have mapped the spatial proximity of the different cell types of the developing human pancreas, between 12 and 20 PCW[11] (Figure 2a). We identified that islet endocrine cells are in close proximity to ductal and acinar cells. Our data also revealed an unexpected interaction between immature Schwann cells and endocrine progenitor cells, indicated by their close spatial proximity and we have proposed that this interaction may be required for endocrine progenitor maturation.[11] Indeed, extensive development of the sympathetic nervous system and neuronal interactions with endocrine progenitors have been observed in the developing human pancreas by immunohistochemistry[56] and neural crest cells, which are precursors of Schwann cells, are important in regulating beta cell maturation and mass in mice.[57,58] We also identified potential ligand-receptor pairs by which the different cell types can potentially influence endocrine progenitors (Figure 2b). Thus, for example, it can be seen from Figure 2b that ligand-receptor pairs by which Schwann cells are predicted to interact with endocrine progenitors include VIM-CD44 and L1CAM-EPHB2, ductal cells by CDH1-PTPRF, acinar cells by BMP7-BMPR1A and beta cells by EFNA3-EPHB4 pairs. Close spatial proximity of the Schwann cells to the endocrine progenitors suggests that paracrine signalling will be important in influencing differentiation, and further work is required to identify which, if any, of the ligand-receptor pairs are critical for islet cell development. The lack of an in vitro niche containing the appropriate signalling factors required for full beta cell specification is thought to contribute to the immature cells produced by current stem cell differentiation protocols,[59,60] leading to the requirement for an additional in vivo maturation stage. A thorough understanding of the pancreas microenvironment in which the endocrine progenitors mature may, therefore, reveal signalling factors or ligands that can be included in current differentiation cocktails to increase the maturation of stem cell-derived beta cells in vitro.

9 | CONCLUSION

Cutting-edge tools in the rapidly evolving field of multiomics technologies are being applied to study human fetal pancreas development and regulation of beta cell gene expression, and these unbiased, discovery-driven approaches have produced major improvements in our understanding of the cellular heterogeneity, novel transcription and regulatory factors, lineage dynamics and spatial cell–cell interactions of the developing pancreas (Figure 3). The role of the microenvironment, including intercellular communication through direct physical contact and secreted molecules, in which the endocrine progenitors develop into beta cells is also being understood better. It is an exciting time for stem cell biologists to investigate the wealth of data that is being generated from these approaches to identify opportunities to increase the yield of functionally competent human beta cells in vitro. This, in turn, has the capacity to provide much-needed material for transplantation therapy for type 1 diabetes patients.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the preprint: Olaniru et al., single-cell transcriptomic and spatial landscapes of the developing human pancreas. BioRxiv. February 2022. 10.1101/2022.02.04.478971

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