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COMMENTARY

Detailed analysis at a single-cell level of cells undergoing pancreatic differentiation

Pluripotent stem cells (PSCs)-derived β-cells are expected to be a source for cell replacement therapy of type 1 diabetes mellitus patients. Several protocols have recently described the generation of pancreatic β-cells. The latest report from the Douglas Melton laboratory, fully utilizing single-cell ribonucleic acid (RNA) sequencing (scRNA-seq), provides in-depth knowledge of stem cell pancreatic differentiation[1].

Type 1 diabetes mellitus is a form of diabetes in which very little or no insulin is produced by the pancreatic β-cells. Pancreas or islet transplantation provides effective cures for maintaining normoglycemia for several years, and reduces the progression of complications. The limited number of donors, therefore, calls for alternative cells, and PSCs-derived β-cells are expected to be an attractive source for cell replacement therapy of type 1 diabetes mellitus patients. Several protocols have recently been reported for the generation of pancreatic β-cells from PSCs[2,3], but to further increase differentiation efficiency and maturity, it is necessary to characterize the individual cells that appear during differentiation. To date, several groups have reported on transcriptome analysis of differentiating bulk cell populations, but no research has comprehensively determined the identity and states of all the cell types at each stage of pancreatic differentiation at a single-cell level.

In 2014, Dr Douglas Melton's group at Harvard University reported a procedure for producing mature pancreatic β-cells, the so-called stem cell-derived-β (SC-β) protocol[2]. Veres *et al.*[1] characterized the cell types produced in the SC-β protocol using scRNA-seq and created a map of the developmental history of human pancreatic cells, namely, the progenitors, SC-β-cells and non-endocrine cells. Veres *et al.* sequenced >100,000 PSCs-derived pancreatic cells at different time points during the induction of the SC-β-cells, and then defined cell populations using computing analysis. The authors finally succeeded in creating a high-resolution map of the process by which endocrine cells differentiate from pancreatic progenitor cells. They applied pseudotime analysis to order the cells according to their gene expression profile and to track their lineage. As a result, they found that pancreatic cells prepared by the SC-β protocol contained four main cell types, namely β-like cells (SC-β-cells), α-like cells (SC-α-cells), endocrine cells that resemble enterochromaffin cells in the intestine (SC-EC cells) and non-endocrine cells (Figure 1).

An extended culture time at the final differentiation step is necessary to acquire the ability to secrete insulin in response to glucose. The authors examined the expression profile and *in vitro* glucose-stimulated insulin secretion tests during this culture period. Their results showed that the cells retained glucose-stimulated insulin secretion ability from the first week of culture to the fifth week using serum-free medium without exogenous signaling factors. Although the global transcriptional profile did not change a great deal, they observed increases in the expression of key genes of β-cell maturation, and glucose responsiveness was stably maintained and the insulin secretion level increased with the number of culture days. These results suggest that the long-term culture, in the final differentiation stage, can increase the maturity of SC-β-cells.

In contrast, the authors compared the amount of insulin secreted by SC-β-cells with that of cadaveric human islets from seven donors. After 5 weeks of culture in the final stage (after a total of 8 weeks of differentiation), the insulin secretion levels of SC-β was at approximately the same range as that of cadaveric human islets, but the magnitude of secretion was higher in the islets. Besides this, insulin secretion in human islets varied considerably from lot to lot, whereas SC-β showed little difference among batches. The fact that there are fewer variations in insulin secretion compared with human islets is a feature of SC-β-cells. This feature makes PSC-derived β-cells an attractive source for transplantation.

Veres *et al.*[1] found a population of cells with a gene expression pattern that expressed both glucagon and insulin, and markedly reduced insulin expression in further differentiation, then referred to these cells as SC-α-cells. The properties of these glucagon and insulin double-positive poly-hormonal cells that appeared in the *in vitro* pancreatic differentiation have been unknown for many years. From the comparative analysis of cadaveric human α-cells and β-cells, they concluded that SC-α-cells are closer to the precursor cells of α-cells, which appear in human fetal development, than to the de-differentiated β-cells described in type 2 diabetes mellitus. α-Cells are essential for regulating pancreatic islet hormone secretion and controlling glucose levels, and are ideal for creating aggregates containing β-cells and α-cells that are differentiated efficiently and separately. The observation by Veres *et al.*[1] that poly-hormonal cells are precursor cells of α-cells is an important finding for the establishment of a α-cell-specific differentiation procedure. In the future, research on how to efficiently generate

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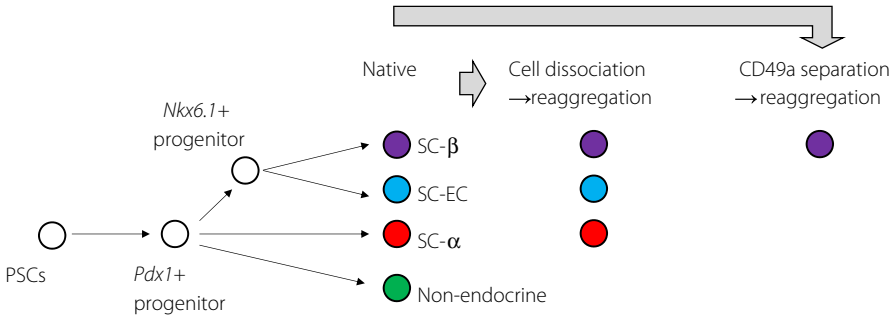

Figure 1 | Proposed model of differentiation of human pancreatic cells from pluripotent stem cells (PSCs) and purification of stem cell-derived β-cells (SC-β-cells) using reaggregation. Pancreatic cells derived from PSCs using the SC-β differentiation protocol were composed of four main cell types, namely β-like cells (SC-β cells), α-like cells (SC-α cells), enterochromaffin-like cells (SC-EC) and non-endocrine cells. Single-cell dissociation and reaggregation depleted non-endocrine cells, and improved β-cell function. Veres *et al.* succeeded in efficiently purifying SC-β by magnetic cell sorting using CD49a antibody and reaggregation, so that the final SC-β-cell ratio increased to 80%.

and purify SC-α-cells is expected to progress rapidly.

Another interesting finding is that a population of endocrine cells that resemble enterochromaffin cells exist in the small intestine is included in the four main differentiated cell types. This cell population was named SC-EC cells because its gene expression pattern closely resembles that of enterochromaffin cells that secrete serotonin in the intestine. Research using mice showed that enterochromaffin cell marker genes were elevated by β-cell-specific polycomb (Eed/PRC2) loss of function[4], suggesting that the cell lineages of β-cells and enterochromaffin cells are closely related. Furthermore, in the current SC-β protocol, non-endocrine cells are also differentiated. These cells then split into populations that express exocrine, mesenchymal and duct cell markers at the final differentiation periods.

Cell clustering is a critical process for functional maturation during islet formation, and Veres *et al.*[1] tried reaggregation of induced PSC-derived β-cells into pseudoislets, a procedure reported to recapitulate formation[5]. The authors showed that single-cell dissociation and reaggregation resulted in the removal of non-endocrine cells and an increase in the proportion of SC-β-cells (C-peptide$^+$ Nkx6.1$^+$ cells). Also, the authors identified CD49a as an SC-β-cell surface marker. Using CD49a antibodies, they succeeded in efficiently purifying SC-β using magnetic cell sorting, which resulted in obtaining clusters that contain up to 80% SC-β-cells. The highly purified SC islets are responsive to glucose *in vitro*, and have higher glucose-stimulated insulin secretion compared with unsorted and reaggregated SC-β-cells, but still a lower secretion magnitude compared with cadaveric islets.

Veres *et al.*[1] identified all cell types appearing during pancreatic differentiation from PSCs using scRNA-seq, reported that final maturation did not require additional growth factors, and showed that purification and reaggregation yielded SC-islets containing a high proportion of SC-β-cells. The scRNA-seq data allows researchers to check the expression of transcription factors and receptors of target cells in each differentiation process, and to study the further maturation of SC-β-cells. Furthermore, similar strategies using single-cell analysis will be informative for other organ differentiation studies, and promote the progress of regenerative medicine research.

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DISCLOSURE

The authors declare no conflict of interest.

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