Duct cells in development, regeneration, and transplantation: charting a path to new islets
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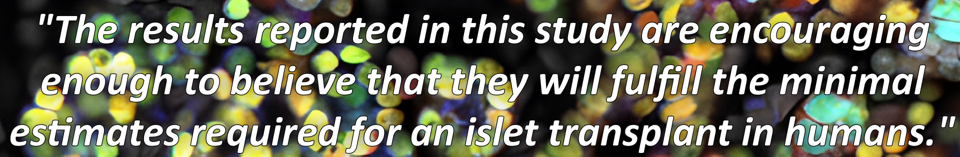

"The results reported in this study are encouraging enough to believe that they will fulfill the minimal estimates required for an islet transplant in humans."

C. Ricordi
Diabetes
1988

Human pancreas 17 wpc, PDX1 NKX6.1 SOX9 DAPI

CHAPTER| 8

General Discussion and Future Directions

General Discussion

Diabetes mellitus poses a significant threat to global health, impacting millions of people worldwide. The hallmark of type 1 diabetes mellitus (T1DM) is the autoimmune-mediated destruction of the insulin-producing beta cells residing in the pancreatic islets of Langerhans[1]. Patients with T1DM require lifelong administration of exogenous insulin for their survival. However, achieving optimal glycemic control remains challenging despite advances in insulin therapy and glucose monitoring technologies, leading to complications from hyperglycemia or hypoglycemia, in addition to a substantial self-care burden[2].

Currently, the most promising approach to cure diabetes is to restore insulin secretion by replenishment of beta cell mass, which can be performed with a whole-organ pancreas transplant (PT) or an islet transplant (IT)[3]. IT is associated with a lower complication rate and better overall survival while producing similarly effective outcomes compared to PT, making it suitable for a wider range of patients[4,5]. However, its widespread adoption is limited by several factors, including shortage of donor tissue[6]. Therefore, finding alternative sources for insulin-producing beta cells is an important goal in IT.

The existence of a beta cell progenitor in the pancreas has been a topic of debate for over a century[7,8]. Early studies suggested that the pancreatic ductal epithelium may harbor progenitor cells, supported by histological observations in human samples and lineage tracing studies in animal models. However, conflicting findings from various animal models and challenges in directly studying human pancreatic tissue have made it difficult to formulate definite answers[7,9]. The central question this thesis aimed to address was:

Can human pancreatic duct cells be used as a source for beta cell regeneration, thereby offering a novel approach to the treatment of diabetes?

The research described in this thesis involved multiple specific objectives: i) evaluation of the effect of islet purity in allo-islet transplantation, ii) the identification and characterization of potential progenitor cells within the human pancreas, iii) the development of novel methods to expand and differentiate these cells *ex vivo*, and iv) clarifying the role of key transcription factors in the differentiation of progenitor cells into beta cells. The findings from this thesis provides important insights into these areas. However, several challenges and limitations remain, highlighting the need for further research.

Evidence for beta cell regeneration in allo-islet transplantation

Our study explored the association of islet purity and metabolic outcome in patients undergoing islet transplantation. Demonstrating improved metabolic outcomes using lower-purity islets could provide evidence for beta cell regeneration from non-islet cells, such as duct cells, and potentially lead to compelling alternative strategies in clinical islet transplantation. These strategies could reduce

the reliance on high-purity islets and optimise the utilisation of donor material, including the non-islet portion. This area of research is important due to the limited availability of donor islets and the hypothesis that duct cells may form beta cells.

Islet purity is a key consideration in islet transplantation for two reasons, namely i) the delivery of a sufficient number of islets is considered essential for favorable metabolic outcomes post-transplantation, and ii) the procedure's safety depends on the ability to deliver these islets within a constrained volume to prevent complications such as portal vein thrombosis[10,11]. Therefore, islet isolation procedures include a purification step to address these concerns. However, purifying islets is challenging and often results in preparations that, despite containing a significant number of islets, are not transplanted due to insufficient purity[12,13]. Moreover, it is unclear how beneficial this purification is for graft function, as the influence of non-islet cells within the graft on metabolic outcomes remains uncertain. Interestingly, a few studies have reported that lower-purity islets are associated with improved long-term metabolic outcomes compared to high-purity islets[14,15]. The authors of these studies hypothesize that this effect might be attributable to beta cell neogenesis originating from ductal cells. However, these studies have small sample sizes and use inconsistent methods to measure islet purity, which complicates the interpretation of the impact of non-islet cells in IT.

In **Chapter 2** we examined the impact of islet purity on graft function in our patient cohort, using robust and reproducible methods to quantify islet purity and graft function. We found that high-purity islets resulted in better graft function three months post-transplant, but long-term graft function did not significantly differ across purity levels. This indicates that islet purity is not a critical determinant of long-term graft outcome. These findings have important implications. First, it is essential to use reproducible and unbiased methods to measure islet purity to accurately evaluate its impact on graft function, and to enable comparisons between different studies. In addition, our findings suggest that islet purity may be less critical for long-term outcomes than previously thought, potentially shifting the focus away from purity as an important factor when considering metabolic outcome in islet transplantation. This finding is also relevant in the ongoing discussions about the cellular composition of stem cell-derived islets, in which the impact of non-islet cells on function and safety after transplantation is debated[16]. Although our analysis included a large number of patients, long-term follow-up was limited, particularly due to exclusion of patients after receiving a second islet transplantation. This highlights the need for additional studies to validate our findings and to better understand the role of non-islet cells in graft function over time. In conclusion, our findings could help to update transplantation protocols and guide future research aimed at further improvement of the beneficial effects of IT. Importantly, the absence of improved long-term metabolic outcomes in lower-purity islets prevented us from finding any evidence of beta cell regeneration from duct cells in IT.

Investigating beta cell progenitors in the human adult pancreas

Previous work exploring the role of pancreatic ductal cells in beta cell regeneration has largely relied on rodent models, resulting in a wide range of contradictory findings, and shifting focus away from the investigations into the possible progenitor capacity of ductal cells[17,18]. However, rodent models are suboptimal for translating beta cell turnover findings to humans due to significant differences in beta cell physiology, highlighting the need for experimental studies using human tissues[19,20].

Progress in finding direct evidence for beta cell regeneration from ductal progenitors in humans has been modest, largely due to the limited availability of normal primary human pancreatic tissue for research. Early studies conducted over 20 years ago provided some of the first insights, showing that duct cells could be expanded from islet-depleted human pancreatic tissue[21-23]. These studies also demonstrated that, under specific culture conditions or genetic manipulations, expanded duct cells could acquire a beta cell phenotype, providing insights into the microenvironment and specific signaling pathways required for the differentiation of these cells into endocrine cells. However, the differentiation efficiency was low, and the highly heterogeneous starting population made it challenging to identify the origin of the generated insulin-positive cells. Subsequent studies used cell surface markers that could be used to enrich for duct cell subpopulations, to create a more homogeneous starting population. For example, duct cells expressing the surface marker carbohydrate antigen 19-9 (CA19-9) could be isolated and expanded on a plastic surface[24,25]. After expansion, the cells were cultured in suspension and proliferation-stimulating agents were withdrawn. This process resulted in the aggregation of ductal cells and their spontaneous differentiation into insulin-producing cells, though this occurred with low efficiency. Another cell surface marker, prominin-1 (CD133), was identified in fetal mouse pancreas labelling endocrine progenitor cells[26]. This marker was also used to isolate human adult pancreatic duct cells, which could be clonally expanded in 3D culture using Matrigel[27]. However, for differentiation towards beta cells these cells required genetic manipulation.

In **Chapter 4**, we optimised organoid culture techniques to expand human islet-depleted pancreatic tissue. After expansion, the duct cells exhibit characteristics such as self-renewal in clonal expansion experiments, and demonstrate similarities to fetal cells. We focused on cells expressing the stem cell marker ALDH, and found that $ALDH^{hi}$-positive ductal cells isolated after expansion were capable of clonal expansion and showed more similarities to $ALDH^{hi}$-positive cells derived from human fetal pancreatic organoids compared to human islet-depleted tissue, suggesting a more primitive state. In addition, we confirmed the potential for endocrine differentiation of expanded duct cells without genetic manipulation. The organoids derived from human islet-depleted tissue were capable of generating insulin-expressing cells *in vivo*, although the differentiation efficiency was still low, with only about 1.5% of the cells becoming insulin-positive. The low differentiation efficiency highlights the need to further refine the isolation and expansion protocols for ductal progenitor cells, as well as optimisation of the conditions that that promote their differentiation into functional beta cells. Despite the low number of insulin-positive cells generated in our experiments, our findings are significant as they confirm the progenitor potential of adult human duct cells.

One of the primary limitations of our study remains the heterogeneity of the islet-depleted tissue that was used to expand, which complicates the identification of the origin of the endocrine cells that are formed. However, creating a single-cell suspension from human islet-depleted tissue and plating it in Matrigel under the same organoid culture conditions used for human islet-depleted tissue chunks, did not result in the formation of similar complex organoids as can be generated with human fetal pancreatic progenitor cells. This highlights the importance of the progenitor cell niche required for the development of ductal cells into complex organoids. Similar challenges have been observed in Lgr5-positive intestinal stem cell cultures, in which self-renewal and proliferation is dependent on direct cell-cell contact of Lgr5-positive cells and supporting cells in their niche, such as Paneth cells[28]. Methods for culturing Lgr5-positive intestinal stem cells in complex organoids without the typically required niche have been developed, by using synthetic matrices and specific growth factors. These cultures can be maintained for a long time and directed to differentiate into various intestinal cell types[29]. Future research will have to determine whether the use of cell surface markers to isolate duct cells in combination with the culture additives can result in improved (clonal) expansion of duct cells directly after isolation, which could provide strong evidence for the progenitor capacity of duct cells.

Improved differentiation of primary human ductal cells

In the context of beta cell replacement therapy, the discovery of beta cell progenitor cells alone is insufficient without the parallel development of efficient endocrine differentiation protocols. While significant progress has been made in the differentiation of pluripotent stem cells (PSCs) into beta cells[30], similar protocols tailored for human ductal cells remain underdeveloped. One interesting finding from PSC differentiation studies is that cells commonly undergo a duct-like phenotype before further differentiation into insulin-producing cells, recapitulating islet formation as observed in human embryonic development[31]. Therefore, we hypothesized that the protocols used in these PSC procedures would serve as a valuable starting point for the development of new beta cell differentiation protocols for adult ductal cells. Besides the agents derived from PSC differentiation protocols, a range of other compounds that were able to induce beta cell neogenesis in animal or *in vitro* studies have been identified. These compounds, including factors like glucagon-like peptide-1 receptor agonists (GLP-1RA) or other small peptides such as islet neogenesis associated protein (INGAP), hold additional potential for inducing beta cell neogenesis, although their effect has yet to be verified in human ductal cells[32-35].

In **Chapter 5**, we aimed to address this gap by adapting an established differentiation protocol originally designed for PSC[36]. This protocol was modified to include several agents identified from PSC differentiation protocols and animal studies or *in vitro* experiments studying beta cell regeneration. We hypothesized that by combining insights from both fields, we could develop a more effective protocol for the generation of insulin-positive cells from primary human ductal cells. As proof-of-concept, we compared our adapted protocol with a traditional basic medium

used for spontaneous differentiation of ductal cells[24]. Our modified protocol, which included fibroblast growth factor 7 (FGF7), GLP-1RA and INGAP, increased the number of insulin-positive cells, showing that human duct cell differentiation can be improved using adapted differentiation protocols. However, even with our adapted protocol, differentiation efficiency remained low, forming only 5% of insulin-positive cells after *in vivo* maturation. Further understanding of the molecular mechanisms and pathways required for the differentiation process of adult ductal cells is essential for further development and optimisation of these protocols.

Gathering evidence for duct cell plasticity using lineage tracing

In the regenerative medicine field, clonal expansion and lineage tracing studies are key to identifying cells with progenitor characteristics. Lineage tracing refers to a collection of techniques used to track the fate of individual cells and their progeny with minimal disruption to their normal function[37,38]. This approach is useful for investigating the pluripotency potential of cells both *in vivo* and *in vitro*. It has been successfully applied in various contexts, including studies with human pancreatic cells[39,40]. Therefore, implementing effective and specific lineage tracing methods could provide an alternative strategy to show conclusive evidence regarding the plasticity and/or progenitor capacity of pancreatic ductal cells.

In **Chapter 6**, we optimised lentiviral transduction of primary human exocrine cells, achieving up to 90% efficiency using a VSV-G-pseudotyped vector with eGFP under a CMV promoter, with addition of protamine sulfate in a serum-free transduction environment. Despite this success, our attempts to create a duct-cell specific lineage tracing system using the duct cell specific promoters keratin 19 (KRT19) and carbonic anhydrase II (CAII) were unsuccessful due to a lack of specificity. Ongoing efforts testing other promoters are expected to yield duct-specific lentiviral constructs, which will enable precise and efficient labelling of these cells with our optimised protocol. This technique could be employed to trace the fate of adult duct cells in culture, or to facilitate genetic manipulations aimed at elucidating the signaling pathways required for duct cell proliferation and differentiation.

Further investigations into the transcriptional mechanism underlying beta cell differentiation

Understanding the transcriptional mechanisms that drive endocrine cell development is essential for the improvement of differentiation protocols. In the context of pancreas formation, transcription factors such as PDX1, NEUROG3, and SOX9, have been widely studied for their roles in directing progenitor cells toward specific endocrine lineages[41]. Recent investigations aimed at identifying proliferative islet cells in the adult human pancreas have uncovered a highly proliferative subset of alpha cells that also exhibited cytoplasmic expression of SOX9[42]. SOX9 is traditionally known as a nuclear transcription factor in the pancreas, where it plays an important role during the organ formation and the differentiation of progenitor cells into endocrine lineages[43,44]. Interestingly, the

ability of SOX9 to shuttle between the nucleus and cytoplasm (nucleocytoplasmic shuttling) is identified as a crucial process in regulating gene expression required for proper organ formation and function, such as in sex differentiation[45]. The discovery of cytoplasmic SOX9 in adult endocrine cells suggests that nucleocytoplasmic shuttling might be involved in the regulation of endocrine cell specification or function. However, this process has not yet been investigated in the context of human pancreatic development.

In **Chapter 7** we found that $SOX9^{cyto}$ is present during human pancreas development, and is initially expressed in both insulin-positive and polyhormonal cells during early gestation, but later becomes restricted to monohormonal insulin-positive cells. It did not mark a highly proliferative cell population. This pattern contrasts with cytoplasmic expression observed in adult proliferative alpha cells. These findings indicate that pancreatic biology and endocrine cell differentiation are more complex than previously understood, possibly involving spatiotemporal nuclear-cytoplasmic shuttling of transcription factors to regulate cell specification. This suggests the potential for enhancing beta cell differentiation protocols by manipulating the subcellular localization of transcription factors like SOX9. Future research should focus on understanding the dynamic regulation and localization of SOX9, as well as its function in endocrine cell specification. This deeper understanding of the transcriptional mechanisms that drive endocrine cell differentiation might eventually lead to new strategies or protocols for the development of new beta cells for cell-based diabetes therapies.

Future Directions

The challenge of addressing the massive global burden of diabetes underscores the need for novel therapies that are both cost-effective and scalable. Over the past two decades, significant advances have been made in differentiating human PSCs into functional pancreatic beta cells[46,47]. These methods have shown great promise, with ongoing clinical trials demonstrating their potential to replace lost beta cells in patients with type 1 diabetes[48,49]. However, challenges such as immune rejection, the risk of tumourigenesis, and difficulties in ensuring the long-term survival and functionality of transplanted cells persist in stem cell-based therapies[50-52].

While these technologies are an important step toward a potential cure, they have also shifted some attention away from the search for beta cell progenitors in the pancreas. However, identifying these putative progenitor cells is still vital, not only for our further understanding of beta cell turnover, but also for the development of new therapeutic strategies for diabetes. For instance, enhancing the possible endogenous regenerative capacity of the native pancreas through beta cell differentiation or replication mechanisms could overcome some of the challenges associated with cell therapies, such as time-consuming and expensive culture methods[19,53]. Therefore, it remains essential to obtain direct evidence confirming the existence of a progenitor cell in the human adult pancreas.

Characterization of the heterogeneous duct compartment

The pancreatic duct tree is an extensive system composed of a heterogeneous cell population, comprising various-sized ducts with distinct phenotypic and functional characteristics[54]. It has been hypothesized that only a subpopulation of these cells has progenitor capacity, but the specific locations of these cells—whether in large ducts, small ducts, or even in centroacinar cells that connect ducts to acini—remains unclear.

New high-resolution analytical tools offer enhanced opportunities to better understand pancreatic duct heterogeneity. Single-cell transcriptomics has become the leading method for studying heterogeneity in cell populations, reconstructing developmental pathways, and modelling transcriptional dynamics[55]. This approach is applied to a variety of cells, including those derived from PSC cultures, organoids, and other tissues[56,57]. Application of single-cell transcriptomics to the pancreatic duct tissue has revealed a previously unknown level of heterogeneity within the ductal tree[58-62]. This new information can be used to distinguish between duct subpopulations and identify the populations with progenitor capacity. Some approaches have already enriched duct subpopulations with progenitor potential[63-65]. Further investigations using high-resolution tools, particularly studies involving human fetal tissue where the pancreas is still rich in progenitor cells developing into endocrine cells, are crucial for deepening our understanding of normal pancreatic development and the characteristics of progenitor cells[66]. Ultimately, this research could provide spatial insights into the progenitor cell distribution along the pancreatic ductal tree in the human adult pancreas, in addition to the identification of cell surface markers that could be used to isolate these progenitor cells. This would facilitate additional studies on the plasticity of these cells and their differentiation potential into beta cells, paving the way for new therapeutic strategies.

Further optimisation of expansion and differentiation

Expanding and differentiating primary human pancreatic progenitor cells into beta cells *ex vivo* remains a complex process with significant challenges. One of the main challenges is the low efficiency of differentiation, with only a small fraction of progenitor cells successfully converting into hormone-positive cells. Future research should focus on optimizing the differentiation process by refining the combination of growth factors and peptides used to guide progenitor cells towards beta cells. Advances in bioengineering, such as the development of biomimetic scaffolds that mimic the extracellular matrix in the natural niche of progenitor cells, or dynamic culture systems like microfluidic devices, could also improve the expansion and differentiation of these pancreatic cells by providing a more physiologically relevant environment[67-69].

For example, organ-on-chip systems are microfluidic devices designed to replicate the physical and biochemical conditions of organs offering a more physiologically accurate model than traditional culture methods[70]. These systems can simulate the dynamic environment of the pancreatic ductal system, recreating fluid flow, mechanical stress, and nutrient gradients that are required for cell differentiation and proper function. This approach could be particularly beneficial

for optimizing the differentiation of pancreatic progenitor cells, as it closely replicates the *in vivo* conditions they would encounter within the body.

Future research should further refine these systems, exploring the optimal combinations of growth factors, extracellular matrix components, and dynamic culture conditions, that best support the differentiation and maturation of progenitor cells into functional beta cells. Such advancements are crucial not only for validating the potential of duct cells as a reliable source for beta cell replacement therapies, but also for enhancing our understanding of the mechanisms that regulate their differentiation.

Concluding Remarks

In summary, this thesis has provided insights into the potential of human pancreatic duct cells to serve as a source for beta cell regeneration, laying the groundwork for future research in regenerative therapies for diabetes. While there are challenges and gaps that remain to be addressed, the work outlined in this thesis offers new methodologies and insights that advance our understanding of beta cell regeneration and the role of pancreatic duct cells in this process. By exploring the potential of these cells as a source for beta cell replacement therapy, this research contributes to the broader field of diabetes research and opens up new avenues for therapeutic development.

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