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Importance of multiple endocrine cell types in islet organoids for type 1 diabetes treatment

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Abstract

Almost 50 years ago, scientists developed the bi-hormonal abnormality hypothesis, stating that diabetes is not caused merely by the impaired insulin signaling. Instead, the presence of inappropriate level of glucagon is a prerequisite for the development of type 1 diabetes (T1D). It is widely understood that the hormones insulin and glucagon, secreted by healthy β and α cells respectively, operate in a negative feedback loop to maintain the body's blood sugar levels. Despite this fact, traditional T1D treatments rely solely on exogenous insulin injections. Furthermore, research on cell-based therapies and stem-cell derived tissues tends to focus on the replacement of β cells alone. *In vivo*, the pancreas is made up of 4 major endocrine cell types, that is, insulin-producing β cells, glucagon-producing α cells, somatostatin-producing δ cells, and pancreatic polypeptide-producing γ cells. These distinct cell types are involved synergistically in regulating islet functions. Therefore, it is necessary to produce a pancreatic islet organoid *in vitro* consisting of all these cell types that adequately replaces the function of the native islets. In this review, we describe the unique function of each pancreatic endocrine cell type and their interactions contributing to the maintenance of normoglycemia. Furthermore, we detail current sources of whole islets and techniques for their long-term expansion and culture. In addition, we highlight a vast potential of the pancreatic islet organoids for transplantation and diabetes research along with updated new approaches for successful transplantation using stem cell-derived islet organoids.

INTRODUCTION

According to the Center for Disease Control and Prevention, 1.9 million Americans have type 1 diabetes (T1D).¹ There are several complications associated with diabetes including

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blindness, stroke, renal failure, heart attack, and lower limb amputation.² Despite the gravity of these complications, the most common T1D treatment, injection of exogenous insulin, does not address them.³ Exogenous insulin injections, when used properly, have the potential to mitigate the high blood glucose associated with this disease. However, insulin injections are not a cure, as they do not address the changes in β and α cell number and function underlying the development of diabetes.

The development of T1D is rooted in improper secretion of both insulin and glucagon.^{4,5} This is due to the fact that the heterotypic interactions between glucagon-secreting α and insulin-secreting β cells in human islets is paramount in regulating glucose homeostasis.^{6,7} Therefore, it is logical to conclude that treatments based on the injection of exogenous insulin alone are insufficient to treat T1D or adequately replicate the non-diabetic state. One major source of concern associated with exogenous insulin injections for the treatment of T1D is hypoglycemia.⁸ Should insulin injections lower blood glucose levels below a healthy level (90—110 mg/dL), transport of glucose to the brain is severely limited.^{8,9} This is hazardous and may lead to death. Despite the dangers of hypoglycemia, patients with T1D experience symptomatic episodes of hypoglycemia an average of 2 times each week.⁸

Even ignoring the failure to address the α cell dysfunction associated with T1D, exogenous insulin treatments are unable to replicate the function of the endogenous hormone.¹⁰ As a matter of fact, patients who receive several insulin injections each day experience glycemic volatility, indicating that their blood glucose levels vary between hyperglycemic and hypoglycemic.¹⁰ This is due in part to the fact that injected insulin is unable to reach the islets with sufficiently high concentration.¹⁰ Therefore, glucagon secretion is not suppressed as a result of the exogenous insulin.¹⁰ A higher dose of injected insulin is necessary to overcome the subsequent low ratio of insulin to glucagon.¹⁰ However, this high insulin dose may lead to hypoglycemia.¹⁰ Maintenance of blood glucose levels in the non-diabetic body is a delicate balance between insulin and glucagon. The complex interplay between these hormones is difficult to recreate using exogenous hormone injections, leading to glycemic instability.

In light of each of these limitations, islet transplantation is preferable to exogenous insulin injections. However, there too are severe limitations in the availability of pancreatic islets for transplantation as a means of curing T1D. This is due to the wide gap between the available cadaveric donors and the number of T1D patients who would benefit from a transplant.¹¹ Furthermore, there are many T1D patients who would benefit from a transplant that are not eligible for one. This is because insulin therapy is often used preferentially to transplantation. While pancreas and islet transplants are the only means for restoring endogenous insulin secretion and the fine-tuned glycemic control that results, these methods are limited to patients who experience additional renal failure or severe hypoglycemia and glycemic instability despite exogenous insulin treatment.¹²

An appealing solution to the organ donor shortage is the use of stem cell-derived pancreatic islet organoids as an alternative to cadaveric islets. Stem cells are capable of differentiating into multiple cell types. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are both commonly used for the development of pancreatic islet organoids.¹³

These cells may give rise to all of the major endocrine cell types within the islet of Langerhans: insulin-producing β cells, glucagon-producing α cells, somatostatin-producing δ cells, and pancreatic polypeptide-producing γ cells. Because each of these cell types plays an important role in metabolism and the establishment of normoglycemia in the non-diabetic state, it is critical that they are each present in organoids intended to treat T1D. In this review, we discuss the unique function of each pancreatic islet cell subset and their roles in maintaining normoglycemia in the body. We highlight the sources of whole islets, techniques for their long-term expansion and culture, and their utility for transplantation and research. In addition, we summarize updated new approaches for successful transplantation using stem cell-derived islet organoids. We will begin by highlighting the critical functions of each major endocrine cell type within the islet of Langerhans individually. Then, the effects that each cell type exhibits on its counterparts will be discussed.

ROLE OF HEALTHY BETA CELLS

β Cells are the most prevalent endocrine cell type located within the pancreas in the islet of Langerhans.¹⁴ In human islets, β cells, accounting for approximately 50%–75% of the volume of islets, are located in high amounts within the anterior head, body, and tail of the pancreas.^{14–16} Murine islets are generally arranged with a core of β cells surrounded by α , δ , and γ cells at the periphery.¹⁷ In contrast to this and in conflict with the islet architecture described in some textbooks, studies suggest that β cells within human islets are more randomly distributed among the other endocrine cell types.^{15,17,18}

β Cells produce, store, and secrete the hormone insulin in response to changes in blood glucose levels.¹⁹ They secrete insulin in response to high ATP/ADP ratios within the cells. It is in this way that the β cells exhibit glucose responsive insulin secretions.²⁰ When glycemic levels are high following a meal, there is a high concentration of glucose within the extracellular space of β cells.²⁰ The glucose is transported into the cell by glucose transporters like GLUT1 in human and GLUT2 in rodent and then metabolized.²¹ Furthermore, glucokinase within the β cells phosphorylates glucose, leading to increased levels of ATP and decreased levels of ADP.²⁰ Consequently, the ATP binds to potassium channels within the β cells to depolarize the cell which allows calcium to flow in through voltage-gated calcium channels, resulting in insulin exocytosis.²⁰ In addition, insulin is an anti-hyperglycemic peptide hormone, which functions to lower blood glucose levels.¹⁹ It binds to receptors located on the cell-membrane of target cells such as liver, fat, and skeletal muscle cells.^{22,23} Insulin binding on receptors of liver cells promotes the receptor-mediated endocytosis of glucose and its subsequent conversion into glycogen thereby decreasing circulating blood glucose levels.²³ In addition to triggering glucose uptake in target cells, insulin also antagonizes several hyperglycemic hormones such as glucagon, growth hormone, glucocorticosteroids, and epinephrine.¹⁹ In this way, insulin functions to maintain a healthy blood glucose level following postprandial spikes in blood sugar.

Several studies have investigated pancreatic development, with a focus on β cell differentiation.^{24–30} Through the use of animals, especially murine models, scientists have established the role that several transcription factors such as Pancreas/duodenum homeobox protein 1 (PDX1), Neurogenin-3 (NGN3), and V-maf musculoaponeurotic fibrosarcoma

oncogene homolog A (MAFA) play in β cell development, survival, and function *in vivo*.^{25,31–38} MAFA is a β cell maturation marker, as it regulates the transcription of insulin gene in response to glucose levels.³⁹ PDX1, expressed early on during development, is essential for the function and survival of β cells.^{25,32} Pdx1 null mice and humans with homozygous PDX1 mutations exhibit pancreatic agenesis.³² Furthermore, humans with heterozygous missense and frameshift PDX1 mutations are unable to secrete insulin and exhibit maturity onset diabetes of the young.³² In general, PDX1 is thought to regulate β cell development by activating genes associated with β cell identity and suppressing those critical to α cell identity.³³

ROLE OF HEALTHY ALPHA CELLS

After β cells, the second most prevalent endocrine cell type within the pancreatic islets is the α cell.¹⁷ The percentage of α cells comprising the human islet is generally 20%–40%.^{15,18,40} These cells produce and secrete the hormone glucagon.⁴¹ Glucagon is released into the bloodstream in response to low blood glucose levels. Within α cells themselves, there are voltagegated Ca^{2+} channels, such as the P/Q-type Ca^{2+} channel, that open in regard to low blood glucose levels.⁴¹ As a result, there is an influx of Ca^{2+} into the cell, leading to glucagon exocytosis.⁴¹ This hormone increases the amount of glucose present in the blood by interacting with glucagon receptors (GCGR) on the liver and triggering both gluconeogenesis, the synthesis of glucose, and glycogenolysis, the catabolism of glycogen into glucose.⁴¹ The regulation of glucagon secretion is less understood than that of insulin, but it is thought to involve a combination of intrinsic and paracrine signals.^{41,42} The dysfunction of α cells directly contributes to T1D development.^{4,5} Further involvement of α cells in islet functionality and diabetes are described in the following sections.

ROLE OF HEALTHY DELTA, GAMMA, AND EPSILON CELLS

Another subtype of endocrine cells within the islet of Langerhans is the δ cell. The function and therapeutic potential of these cells are less widely understood compared to the more prevalent β and α cells. δ cells make up about 5%–10% of human islet cells, and they secrete the hormone somatostatin.^{17,43} Somatostatin, also referred to as growth hormone inhibiting hormone, is released by the gastrointestinal tract, the hypothalamus, and the central nervous system in addition to pancreatic δ cells.⁴⁴ Somatostatin exists in 2 forms in the body: somatostatin-28 and somatostatin-14, the latter being secreted by pancreatic δ cells.⁴³ δ cells influence β and α cells through paracrine interactions. They do so using long neurite-like projections that extend between them.⁴³ Research also indicates that δ and β cells are electrically coupled, further suggesting their interactions.⁴³ Somatostatin secretion from δ cells is induced by glucose concentrations, beginning at a concentration of 3 millimolar and increasing proportionately as glucose concentration increases.⁴³ Somatostatin is an inhibitor of both insulin and glucagon.⁴⁵ One study investigated this function using somatostatin-null (*Sst*^{-/-}) mice.⁴⁵ They demonstrated that *Sst*^{-/-} mice had significantly higher plasma levels of both insulin and glucagon following intravenous administration of glucose or arginine compared to the wild type mice.⁴⁵ A similar difference was apparent during *ex vivo* experiments done on isolated mouse islets.⁴⁵ Islet isolated from *Sst*^{-/-} mice secreted approximately twice as much insulin and glucagon

in response to the glucose and arginine compared to the control.⁴⁵ This suggests that the source of insulin and glucagon suppression is δ cell-derived somatostatin rather than gastrointestinal, neural, or neuroendocrine sources of the hormone.⁴⁵ In β cell destruction models such as streptozotocin (STZ)-induced diabetic rats, there is an observed increase in the quantity of δ cells as well as an increase in pancreatic somatostatin.⁴⁶

γ Cells, also referred to as pancreatic polypeptide (PP) cells, make up approximately 1%—2% of the islet of Langerhans.⁴⁰ These cells secrete the hormone pancreatic polypeptide, a 36 amino acid peptide that is released after eating in a biphasic manner.⁴⁷ The first phase of pancreatic polypeptide secretion is stimulated by the vagus nerve.⁴⁷ This first phase is shorter than the second, which occurs in response to hormones including cholecystokinin.⁴⁷ Levels of pancreatic polypeptide in the plasma remain elevated for up to 6 hours following eating.⁴⁸ Its release is also stimulated by hypoglycemia and exercise via adrenergic stimulation.⁴⁸ Pancreatic polypeptide inhibits gallbladder motility, pancreatic exocrine, and gastric acid secretions.^{48,49} It does so by binding to dorsal vagal complex receptors and causing changes in the vagovagal reflex arc.⁴⁸ Additionally, the pancreatic polypeptide is thought to promote feelings of satiety following a meal via the vagus nerve.^{48,49} Studies show that transgenic mice that overexpress pancreatic polypeptide exhibit weight loss, decreased food intake, and decreased gastric emptying.⁴⁸ Furthermore, when the pancreatic islets from lean mice were transplanted into their genetically obese littermates, their obesity was reversed.⁵⁰ This suggests that decreased pancreatic polypeptide levels and sensitivity can contribute to obesity. Diabetes is sometimes associated with elevated pancreatic polypeptide levels, with about 10% of diabetes patients exhibiting high basal pancreatic polypeptide levels.⁴⁷ However, more research on the differences between healthy and diabetic γ cells would be helpful to fully understand the function of this cell type.⁵¹

In addition, there is a small proportion of epsilon (ϵ) cells present in the islet of Langerhans. ϵ cells make up less than 1% of islets, and they were not discovered until the early 2000s.⁵² They produce the hormone ghrelin which is 28 amino acids in length.⁵² Ghrelin, also referred to as the hunger hormone, stimulates appetite.⁵² It also induces the secretion of growth hormones from the pituitary.⁵² Even though ϵ cells make up a very small percentage of islets, this type of cells are detectable in stem cell-derived pancreatic islet tissue. For example, researchers performing single-cell RNA sequencing may evaluate the expression of GHRL which encodes a precursor to ghrelin.⁵³

DIABETES IS A BI-HORMONAL DISORDER

There are several changes in β cell survival and function as a result of the development of diabetes mellitus. T1D, also referred to as insulin-dependent diabetes, is associated with a decrease in the number of β cells.⁵⁴ In T1D, the immune system attacks β cells, leading to decreased insulin production and a failure to maintain blood glucose levels.⁵⁴ The abnormal β cell populations and their association with the development of diabetes mellitus often overshadow the role that α cells play in the disease. However, the importance of the hormone glucagon in diabetes cannot be ignored. In 1975, the bi-hormonal abnormality hypothesis replaced the previous unihormonal concept.⁴ This relatively newer hypothesis states that improper secretion or function of insulin alone is

not responsible for the hyperglycemia associated with diabetes.⁴ Insufficient insulin levels must be coupled with the presence and hypersecretion of glucagon for the development of diabetes mellitus.⁴ This is supported by a study comparing the results of β cell destruction on glucagon receptor null (GCGR^{-/-}) and wild type mice.⁵⁵ In this study, 100 milligrams followed by 80 milligrams per kilogram of body weight of the chemical STZ was injected intravenously in order to induce the destruction of β cells.⁵⁵ This process was performed in 2 groups of male mice, one with global glucagon receptor knockout and one wild type group.⁵⁵ Only the wild type mice showed traditional symptoms of T1D, including hyperglycemia, hyperketonemia, polyuria, and cachexia.⁵⁵ Through this study, researchers were able to recreate the conditions of T1D and demonstrate the critical role that glucagon plays in symptom development.⁵⁵ These results were further supported through experiments restoring hepatic glucagon receptors in GCGR^{-/-} mice.⁵⁶ Once again, this study demonstrated that STZ-mediated β cell destruction only led to clinical symptoms such as hyperglycemia in wild type mice expressing glucagon receptors.⁵⁶ After utilizing an adenovirus containing GCGR cDNA to restore the glucagon receptors, hyperglycemia developed in the mouse models, further suggesting that the action of glucagon is critical in T1D.⁵⁶ Hence, T1D treatment using glucagon receptor inhibition has been investigated extensively.⁵⁷⁻⁶⁰

The interplay between the synergetic regulation of insulin/glucagon and hyperglycemia has been demonstrated by a recent report showing that insulin receptor substrate 1 plays a crucial role in the regulation of glucagon secretion. It changes glucagon transcription in response to exogenous insulin,⁶¹ which provides a mechanism underlying how insulin signaling regulates glucagon secretion.⁶² As T1D is characterized by insulin dysfunction combined with high levels of glucagon, a diabetes treatment that can restore both β and α cell function is highly desired. Okamoto *et al.* demonstrated that an insulin receptor antagonist, S961, induced symptoms of diabetes in mice, including insulin resistance, hyperglycemia, and ketonemia.⁶⁰ When a monoclonal antibody was used to block the glucagon receptor, normoglycemia was restored. Interestingly, the mass of β cells doubled with glucagon receptor blocking compared to treatment with S961 alone, with a 5.8-fold increase in β cell mass compared to the control which received no monoclonal antibody or S961. Additionally, treatment with the monoclonal antibody increased α cell mass by 5.7-fold.⁶⁰ This study poses a potential therapy for diabetes while demonstrating the essential role of glucagon in symptom development. In a clinical trial, a glucagon receptor antibody, REMD-477, has been studied to examine its efficacy for T1D treatment. The results indicated that this glucagon receptor antibody improves glycaemic control in patients with T1D and reduces insulin usage.⁶³

ENDOCRINE CELL INTERACTIONS THAT INFLUENCE ISLET FUNCTION

In native pancreatic islets, there are complex interactions between each endocrine cell type to maintain healthy conditions within the body. In particular, β cells and α cells operate in a negative feedback loop to establish normoglycemia. In individuals without diabetes, the spike in blood sugar accompanying a meal leads to increased secretion of insulin from β cells and decreased glucagon secretion from α cells.⁶⁴ These conditions lead to a decrease

in plasma glucose levels. Should this level dip below the healthy level and hypoglycemia develop, α cells will begin secreting glucagon, thereby raising plasma glucose levels.⁶⁴

Since human islets of Langerhans are predominantly composed of a heterogeneous mixture of β , α , δ , and γ cells, there is an ample opportunity for paracrine signaling between each cell type.^{65,66} As opposed to the cytoarchitecture of other species such as mice, human β cells are not arranged in a cluster, rather they are intermingled with other islet cell types.^{18,67,68} Studies show that 71% of human β cells are in contact with other endocrine cell types, further supporting the importance of paracrine signaling, especially with human β cells.¹⁸ These paracrine signals include hormones, peptides, neurotransmitters, and metabolites.⁶⁵ The majority of β , α , and δ cells are located along blood vessels within the islet.¹⁸ However, the order of cells appears random, suggesting that the order of paracrine interactions between these cell types is not decided by the microcirculation.¹⁸ Paracrine interactions play an instrumental role in regulating the secretion of glucagon from α cells. Both insulin and somatostatin are released under hyperglycemic conditions, and these hormones are capable of inhibiting glucagon secretion.²⁸ It is hypothesized that insulin inhibits the secretion of glucagon indirectly, through the action of somatostatin.⁶⁹ Insulin secreted by β cells stimulates the secretion of somatostatin from δ cells in mouse islets.⁶⁹ Furthermore, the ability of insulin to regulate glucagon release was lost in somatostatin-secreting δ cell insulin receptor knockout mice, suggesting that the action of insulin on α cells is indirect and requires the involvement of δ cells.⁶⁹ In addition to insulin, several factors released by β cells are capable of inhibiting the secretion of glucagon by α cells including γ -aminobutyric acid (GABA), ATP, and zinc ions.⁷⁰ The influence of paracrine signaling on glucagon regulation is apparent when α cells are studied separately from whole islets. For instance, a study using isolated primary rat α cells showed that glucose level regulates K_{ATP} -channel activity in the cells, resulting in sugar level responsive glucagon secretion. Such glucose level responsiveness can be interrupted by means of ion channel modulators, such as diazoxide, a potassium channel activator. The activated signal of potassium channel would come from tissue surrounding the α cells.⁷¹

Although α cells do not typically release high levels of the hormone glucagon-like peptide-1 (GLP-1), in circumstances such as following IL-6 treatment, β cell regeneration after STZ treatment in neonatal rats α cells produce GLP-1.⁷⁰ This hormone binds with the G-protein coupled receptors present on β cell membranes, leading to increased insulin secretion.⁷² Furthermore, *in vitro* studies show that glucagon stimulates the secretion of insulin by β cells.⁷⁰ Meanwhile, the pancreatic polypeptide secreted by γ cells agonizes neuropeptide Y4 receptors which are expressed by several somatostatin-containing cells including those in islets.⁷³ The result of this agonism is decreased secretion of somatostatin by δ cells.⁷³ Additionally, pancreatic polypeptide interacts with the pancreatic polypeptide receptor present on α cells to regulate the secretion of glucagon.⁷⁴ At lower concentrations (1 nM), pancreatic polypeptide decreased glucagon secretion whereas it increased secretions at higher concentrations (100 nM) in rodent islets.⁷⁴ The various paracrine interactions between β , α , δ , and γ cells are visualized in Fig 1.

SOURCES OF WHOLE ISLETS FOR CLINICAL APPLICATIONS

Because of the crucial role each islet cell type within pancreatic islets plays, it is important to obtain whole islets for transplantation, as well as for use in a lab setting performing drug testing, disease modeling, and pathophysiological study. These islets can be obtained from cadavers or differentiated from stem cells (Fig 2). Key procedures to obtain donor islets and stem cell-derived islet organoids are visualized in Fig 2. The first attempt at treating T1D through pancreas transplantation was performed in the year 1966 by Richard Lillehei and William Kelly at the University of Minnesota.^{75,76} In this surgery, a 28-year-old female was simultaneously given a kidney and a pancreas graft.⁷⁵ Although exogenous insulin treatments were eventually necessary, this patient did not require insulin injections for an initial period of 6 days.⁷⁵ Despite this modest success, the surgery eventually failed due to the extensive use of steroids to prevent rejection.⁷⁵ Furthermore, the patient experienced pancreatitis due to the ligation of the duct, and the transplanted organs were subsequently removed.⁷⁵ Since then, several advances have been made in the area of allogeneic pancreas transplantation. The success of subsequent pancreas transplants owe in part to the development of effective immunosuppressive drugs such as cyclosporin A.⁷⁶

Whole-organ pancreas transplantation is usually performed in conjunction with kidney transplantation.¹² There exists a less invasive alternative for the treatment of T1D: islet transplantation.¹² The procedure for islet transplantation, known as the Edmonton protocol, was first outlined in the *New England Journal of Medicine* in the year 2000.⁷⁷ Before human islets can be transplanted, they must be digested using enzymes, purified, and cultured.⁷⁸ After this process is carried out, the islets are infused via the portal vein.⁷⁸ This is achieved by suspending the purified cells in transplant media containing heparin and infusing the solution using a catheter inserted into the skin.⁷⁸ Compared to the surgery necessary for the whole-organ pancreas transplantation, there are lower risks associated with islet transplantation.¹² However, islet transplants are limited in that they require more donors.¹² It is recommended that a patient receive at least 5000 islet equivalents for each kilogram of their body weight.⁷⁸ This further exacerbates the issue of donor limitations, as the number of cadaver pancreases available for transplantation is far less than the number of patients in need of a transplant.

Because of the severe limitations in donor availability, researchers have focused on the potential for stem cell-derived pancreatic islets for transplantation as well as diabetes research. While these tissues may originate from hESCs or iPSCs, pancreatic islet organoids derived from autologous iPSCs are advantageous in that they do not necessitate the lifelong use of immunosuppressants.⁷⁹ Furthermore, iPSCs do not face the same ethical constraints as ESCs which require the destruction of an embryo.^{79,80} Studies have demonstrated that iPSCs and ESCs exhibited similar differentiation capacities for the generation of functional tissues or organs.^{68,81–84} Hence, iPSCs became an unlimited source for the generation of human tissues.^{80,85} Although there is often a focus on the differentiation of β cells, there exist protocols for the derivation of pancreatic islet organoids containing all or most of the major endocrine cell types, outlined in Table I.^{67,68,81,82,86–88} As mentioned previously, the development of these multi-cell type organoids is preferred for cell-based therapies rather than generating solely β cells.

Typically, the generation of islet organoids from pluripotent stem cells *in vitro* is carried out in a stepwise manner that replicates the development of the native endocrine cells.⁹¹ The stages of this stepwise protocol generally include the differentiation of stem cells into the definitive endoderm (Stage 1), followed by the posterior foregut (Stage 2), pancreatic progenitor cells (Stage 3), endocrine cells (Stage 4), and ending with the development of mature islet cells (Stage 5).⁹¹ This protocol is recreated at varying levels of completion for the treatment of T1D. Some approaches utilizing stem cell-derived products to treat diabetes involve the procurement of β cells *in vitro* and subsequent use *in vivo*,^{92–96} the injection of pancreatic progenitor cells for *in vivo* maturation,^{97,98} and the development of heterogeneous pancreatic islet organoids *in vitro* comprising some or all of the major pancreatic endocrine cell types.^{67,68,81,82,87–90,99,100}

Although several studies focus on the development of insulin-producing β -like cells for diabetes cell therapies, there is an evidence that these cells are not fully mature as they often co-express both insulin and glucagon.⁹⁸ A proposed alternative is the injection of pancreatic progenitor cells *in vivo*.⁹⁸ Upon injection, these pancreatic progenitors continue to mature *in vivo*.⁹⁸ One study optimized the differentiation of hESC line H1 into pancreatic endoderm and endocrine precursor cells that highly expressed the marker genes PDX1, NGN3, and NKX6.1.⁹⁸ Populations of these cells were transplanted into mice that were previously given an intraperitoneal injection of STZ to model T1D.⁹⁸ Following implantation, the endocrine precursor cells continued maturing. Instead of co-expressing insulin and glucagon, as 40% of cells did before implantation, they began to form islet-like clusters.⁹⁸ Three months after implantation, 95% of these cells were monohormonal, including insulin-, glucagon-, and somatostatin-positive cells.⁹⁸ The mice were weaned off of exogenous insulin initially used to treat their STZ-induced diabetes, and the hESC-derived cells were eventually able to secrete insulin, as measured by C-peptide, in response to both intraperitoneal and oral glucose.⁹⁸

In addition to investigate the maturation of pancreatic and endocrine progenitor cells *in vivo*, researches aim to develop mature islet organoids *in vitro* that contain β , α , δ , and γ cells prior to implantation. Through temporal treatment with signaling molecules and 3D microenvironment, researchers have developed protocols for the differentiation of pluripotent stem cells into insulin-producing islet-like clusters (ILCs).^{67,101} For example, treatment of sodium butyrate and activin A leads to upregulation of definitive endoderm-associated genes such as SOX17, FOXA2 and HNF4 α .¹⁰¹ Eventually, these pancreatic endoderm cells begin expressing higher levels of PDX1, PTF1a, NGN3, and NKX6.1, each of which is associated with the endocrine cells.¹⁰¹ After maturation, characterization of ILCs through immunocytochemical staining reveals that they are made up of insulin, glucagon, and somatostatin-positive cells.¹⁰¹ Furthermore, these hESC-derived ILCs showed glucose-responsive C-peptide and insulin secretions.¹⁰¹ Wang *et al.* showed that collagen-based 3D scaffolds could significantly improve the generation of ILCs with high-level expression of C-peptide and higher sensitivity to glucose levels compared with that of 2D culture microenvironment (Table I).⁶⁷ Several research groups have developed protocols for the development of similar islet-like organoids, with variations in signaling molecules used or adding preselection through cell sorting technique (Table I).^{82,88–90,102}

The goal of many studies is to identify important microenvironmental signals during the pancreatic development *in vivo* and recapitulate them *in vitro* so as to effectively develop islets for treatments, disease modeling, drug screening, and other applications. Bi *et al.* demonstrated the efficacy of decellularized pancreatic extracellular matrix (dpECM) proteins and collagen type V as critical microenvironmental cues in islet development, showing that their presentation in the matrix-coating substrates during differentiation of human iPSCs and hESCs leads to the generation of pancreatic islet organoids containing insulin-, glucagon-, somatostatin, and pancreatic polypeptide-secreting islet organoids.^{68,81} This differentiation protocol leads to the formation of islet organoids with glucose-responsive glucagon as well as insulin secretions (Table I).^{68,81} Organ-on-a-chip techniques have also been utilized to recreate the native pancreatic microenvironment, including the key cell-cell interactions and mechanical stimuli.¹⁰³ Overall, stem cell-derived pancreatic cells and tissues offer a promising alternative to cadaveric islets for both research and clinical applications. However, a diverse array of chemical and molecular stimuli plays a critical role during *in vivo* development. More research is necessary to effectively recreate the native tissue microenvironment *in vitro* in order to improve differentiation protocols for the generation of mature islet organoids. Karanth *et al.* discovered that angiogenic factors, angiopoietin-1 or angiopoietin-2, facilitate the formation of islet organoids from induced iPSC differentiation with the elevated glucose responsiveness. The iPSC-derived islets generated under angiopoietin stimulation displayed glucose synchronized calcium ion influx in repetitive glucose challenges. Mechanistically, these islet organoids were able to regulate insulin exocytosis through actin-filament polymerization and depolymerization in response to glucose level change.⁸²

STRATEGIES FOR LONG-TERM CULTURE AND PRESERVATION OF ISLETS

Before transplantation, cadaveric islets must be isolated and purified. There is often a period of time during which the isolated islets must be maintained in a tissue culture before use.¹⁰⁴ In addition, due to the wide supply-demand gap for cadaveric islets, it requires the development of long-term culture and preservation techniques to maintain maximal function of pancreatic islets prior to clinical application. A plethora of investigations has been performed surrounding the best methods of culturing and preserving both cadaveric islets and pancreatic islet organoids for obtaining their flexible usage such as transplantation. Numerous studies have been done on pancreases obtained from animals such as mice, rats, and guinea pigs, as well as organoids derived from rodent stem cell lines.^{3,105–115} Additionally, several researchers have developed protocols for human islet preservation so as to best conserve β cell function. Importantly, some studies focus on the best means of culturing human stem cell-derived islet organoids.

As early as the 70s, research groups like Andersson *et al.* worked to develop protocols to maintain islets within a culture.¹⁰⁴ By adequately purifying and culturing these islets, there is a greater potential for utilizing multiple donors for source tissue and avoiding complications and rejection.¹⁰⁴ After pancreatic glands are isolated from cadavers and perfused with a chilled solution of Dextran 40 and electrolytes, the body and tail are injected with Hanks' Balanced Salt Solution (HBSS) and cut into small pieces.¹⁰⁴ The pieces are put into suspension and treated with collagenase.¹⁰⁴ After collagenase digestion

and fat removal through HBSS washing, the islets are added to petri dishes with culture medium changed every 2 days starting from day 3.¹⁰⁴ In this protocol, the culture medium is supplemented with 20% calf serum and antibiotics.¹⁰⁴ In more recent years, efforts have been made to identify molecules with which to supplement traditional culture media while still maintaining serum-free conditions.¹¹⁶ This helps avoid contamination, which is critical for transplanted tissues.¹¹⁷ Results of preliminary studies indicated that islets could be cultured *in vitro* for at least one week with no disturbances in β or α cell function.¹⁰⁴

In order to best preserve pancreatic islet tissue and function, differing conditions have been studied including various media formulations, additional molecules provided, and environments on which the tissues are cultured. Islets may be cultured in suspension, on surfaces coated with ECM, encapsulated or embedded in biomaterials, on scaffolds, in bioreactors, or even in microphysiological systems.^{118,119} The simplest and the best understood culturing environment of islets and islet organoids is the suspension culture.¹¹⁸ As compared to an adherent culture, the suspension culture allows the islets to maintain their three-dimensional architecture.¹¹⁸ Several experiments have shown that supplementation with insulin,¹²⁰ prolactin,¹²¹ sericin,¹²² human albumin,¹²³ ethanolamine, phosphoethanolamine, transferrin, triiodothyronine, and insulin-like growth factor 1¹²⁴ leads to better preservation of islet and β cell function. Alternative methods such as culture on ECM or in bioreactors restores the mechanical stimuli lost in suspension cultures such as the cell-cell and cell-ECM interactions and hydrodynamic shear stress.¹¹⁸ These methods prove to be more effective in maintaining islet cell phenotype.¹¹⁸

In cases where a longer period of time passes between obtaining and utilizing islets, cryopreservation is often used. This technique is useful in cases where islets are obtained from several donors. Cryopreservation of isolated and purified islets includes a supercooling process to around -40°C in ethanol after adding serum containing dimethyl sulfoxide (DMSO), a cryoprotectant that prevents cell injury caused by the development of intracellular and extracellular crystals.^{125,126} Then, the tissues are kept at low temperatures such as -196°C by submerging them in liquid nitrogen.¹²⁵ Studies show that after 46.5 days, cryopreserved islets had similar insulin extraction values on the day of thawing compared to before freezing.¹²⁵ When islet aliquots from cryopreserved islets and those that were cultured overnight were perfused with Krebs bicarbonate buffer containing basal (60 mg/dl) and high (300 mg/dl) concentrations of glucose, they showed similar primary insulin secretions.¹²⁵ However, the islets thawed after cryopreservation showed lower secondary insulin secretions.¹²⁵ Secondary insulin secretion follows the initial short spike in insulin concentrations.¹²⁷ This second phase of insulin secretion is characterized by a 2–3-hour slow increase in insulin concentrations that eventually plateaus.¹²⁷ While the difference in secondary insulin secretion between the 2 groups was not statistically significant, it reveals modest damage to pancreatic islets after cryopreservation.¹²⁵ This is further supported by the idea that secondary insulin secretions are typically made up of the newly synthesized hormone.¹²⁵

Variables commonly altered during the optimization of cryopreservation protocols include cryoprotectant chemicals and freezing rate.^{128,129} Although the most standard cryoprotectant used is DMSO, several research groups have investigated the effects of varying its

concentration and utilizing other cryoprotectants alone or in combination with DMSO.^{128–130} Combining DMSO with chemicals such as hydroxyethyl starch decreases its inherent toxicity to islets.¹³⁰ Another study found that using lower concentrations (1.5 M) of DMSO as a cryoprotectant shows better results than 2.0 M DMSO or various concentrations of ethylene glycol.¹²⁸ This conclusion was made on the basis of significantly improved percentage of recovered islets and stimulation indices.¹²⁸ Although the toxicity of DMSO makes its use as a cryoprotectant hotly debated, islets cryopreserved using DMSO have shown clinical success when transplanted after thawing.^{126,131} Warnock *et al.* demonstrated that a 36-year-old patient with T1D showed normoglycemia with the withdrawal of exogenous insulin injection following a simultaneous islet-kidney transplant using islets cryopreserved with DMSO.¹³¹

As previously mentioned, one approach for the treatment of diabetes is to use pancreatic or endocrine progenitor cells generated *in vitro* and allow their maturation *in vivo* or *in vitro*. Tanaka *et al.* developed a protocol for the expansion of iPSC-derived pancreatic islet progenitors in culture by introducing and subsequently removing the proliferation gene SV40LT.⁸⁶ Introducing this gene to a population of endocrine progenitor cells expressing NGN3 via lentiviral vector led to cell proliferation while inhibiting their differentiation into cells expressing NGN3, NEUROD1 and NKX2.2.⁸⁶ After the SV40LT gene was removed using Cre recombinase, the proliferation ceased, and the progenitor cells differentiated into β , α , and δ cells (Table I).⁸⁶ This protocol could be applied in the future to efficiently expand endocrine progenitors for the eventual generation of mature islet cells and pancreatic islet organoids both *in vitro* and *in vivo*.

UTILITY OF ISLET ORGANOIDS

The therapeutic potential of pancreatic islet organoids as a source for transplantable islets is clear, and it is highlighted in several papers.^{13,132–136} The utility of islet organoids in addressing T1D has been demonstrated in animal models.^{98,102,137} Protocols exist for the transplantation of islet organoids that hold the potential to remedy current challenges such as limited availability of cadaveric islets, need for immunosuppressive drugs, and even engraftment failure.¹³⁷ In addition to the potential benefits of implanting pancreatic islet organoids to treat diabetes and restore endogenous levels of insulin, glucagon, and other pancreatic hormones in patients, there are other applications for these organoids *in vitro*. Much of the research devoted to the potential of pancreatic organoids in diabetes disease modeling.^{138–144} By using islet organoids derived from human stem cells, researchers can investigate the mechanisms of disease progression and identify therapeutics without relying on animal models.¹³⁸ This is a valuable way to more accurately study human physiology instead of generalizing between different species.¹³⁸ Furthermore, by recapitulating the disease state in a laboratory setting, researchers can easily investigate the ability of drug candidates to ameliorate symptoms.¹³⁹ Moreover, this could be applied to patient-specific personalized medicine should autologous cells be used for organoids. By using pancreatic islet organoids on microphysiological systems, researchers can also investigate the interactions between the pancreas and other organs, like the liver, in a more precise, controlled manner.^{138,139} Multi-organ microphysiological systems create a unique opportunity to examine the interactions and crosstalk between organs at a level that is more

complex than a single cell culture or organoid yet simpler than *in vivo* studies. For example, Tao *et al.* utilized microfluidic organ-on-a-chip systems to model the liver-pancreatic islet axis.¹³⁸ The pancreatic islet organoids for this study expressed markers associated with β , α , δ , and γ cells, and the microfluidic device used allowed for the exchange of media and metabolites between the islet and liver organoids.¹³⁸ They found that this system was effective in modeling the ability of β cells to stimulate uptake of glucose by the liver in response to elevated glucose level.¹³⁸ This represents one of many studies that makes use of pancreatic organoids to model healthy and diseased islet function. Overall, pancreatic islet organoids are valuable beyond organ transplantation in their utility in disease progression studies and drug discovery.

CONCLUDING REMARKS

There is an urgent need to identify alternative sources for pancreatic islets as a means to treat diabetes. Islets derived from pluripotent stem cells, including iPSCs, are an appealing solution to the current organ donor shortage. However, β cell treatments, like exogenous insulin injections, fail to address the role of glucagon in the development of diabetes. Furthermore, each endocrine cell type in the islet of Langerhans plays its own critical role in normal physiology, both in the production and the secretion of their respective hormone and in their complex interactions regulating one another. For this reason, whole intact islets or islet organoids are preferred to reestablish healthy physiological conditions. However, there are many challenges currently facing the generation and transplantation of heterocellular pancreatic islet organoids.

One key challenge in the effective generation and isolation of pancreatic islet organoids *in vitro* is the absence of instrumentation and markers to reliably distinguish between islet organoids and other cell aggregates after differentiation.⁶⁸ Because the desired product is multicellular and heterogenous, flow cytometry cannot be used for purification. This leads to decreased efficiency in islet organoid generation. In contrast to the generation of uniform populations of β cells, preselection cannot be performed for the generation of pancreatic islet organoids composed of several endocrine cell types. During the development of monohormonal insulin-producing cells, preselection is often used to increase the final cell purity. For example, Reznia *et al.* demonstrated that selection of hESC-derived pancreatic progenitor cells that highly express both PDX1 and NKX6.1 leads to more efficient maturation into insulin-secreting cells *in vivo*.¹⁰² Pancreatic progenitor cells with high PDX1 and NKX6.1 expression were more effective in reversing diabetes in mice when transplanted subcutaneously in macroencapsulation devices compared to pancreatic progenitor cells with the low NKX6.1 expression.¹⁰² This preselection often requires using a reporter stem cell line allowing for single cell sorting through flow cytometry.^{145–147} For instance, reporter cell lines inserting green fluorescence protein into the INS locus for subsequent isolation¹⁴⁸ and transfecting cells with a plasmid containing both NKX6.1 promoter and neomycin-resistance genes for the subsequent selection of NKX6.1-positive cells¹⁴⁹ have been used as enrichment methods. However, increased heterogeneity and a lack of consistent cell surface markers for islet organoids makes these methods inapplicable to increase the purity and the efficiency for the generation of pancreatic islet organoids. Furthermore, there are resulting difficulties in quantifying the relative amounts

of each endocrine cell type within islet organoids.⁶⁸ Cryo-sectioning, immunostaining, and fluorescence microscopy may be utilized to measure the percentage of β , α , δ , and γ cells, but the presence of non-islet cell types leads to significant challenges.⁶⁸

Despite the current shortcomings in the efficient generation of pure islet organoids containing β , α , δ , and γ cells, there are many promising areas of further research. For example, gene editing may be used to address limitations in islet organoid purity.¹⁵⁰ One major risk of using pluripotent stem cell-derived tissues for transplantation is the potential for teratoma formation from undifferentiated cells.¹⁵⁰ This risk is high upon implantation of pancreatic islet organoids due to the aforementioned challenges in organoid purification and characterization. By selectively expressing a suicide gene in undifferentiated stem cells, this risk can be mitigated.¹⁵⁰ Wu *et al.* accomplished this by inserting the suicide gene inducible caspase-9, designated as iC9, into the endogenous SOX2 locus of the hESC line H1 using CRISPR-Cas9 technology (Fig 3).¹⁵⁰ In the resulting cell line, undifferentiated cells underwent apoptosis following treatment with an apoptotic drug AP1903 whereas differentiated cells were unaffected.¹⁵⁰ This approach poses a potential solution to eliminate undifferentiated stem cells. However, it is still challenging to apply this method to remove multiple progenitors or non-islet organoids due to the nature of heterogeneity during organoid differentiation.

Another significant challenge in the clinical use of pancreatic islet organoids is their limited survival following transplantation. Due to the shortage of oxygen at the transplantation site, organoids survival rate *in vivo* without the promotion of angiogenesis is low. In order to quickly establish a vascular network to provide nutrients and the exchange of oxygen and carbon dioxide to pancreatic islet organoids, efforts have been made focusing on using encapsulation devices that are able to generate oxygen in site or the formation of prevascularized islets before transplant.^{133,137,151–155} Wang *et al.* designed a scaffold which provides internal air supply continuously to deliver oxygen to the hydrogel encapsulated cells to support long-term function of transplanted cells.¹⁵⁵ This encapsulation device is promising for transplantation requiring high cell payloads. As an alternative approach, prevascularized islets, could be achieved by fusing islet cells with microvascular fragments¹³⁷ or with multiple cell types including human amniotic epithelial cells (hAECs), and human umbilical vein endothelial cells (HUVECs) (Fig 3).^{133,153,154} Nalbach *et al.* developed prevascularized pancreatic islet organoids by co-culture of pancreatic islet cells and microvascular fragments at an optimal ratio, allowing the formation of microvessels before transplantation.¹³⁷ These microvascular fragments were isolated by mechanic and enzymatic digestion of epididymal fat pads of mice, followed by filtration through a 500 μ m filter. These organoids demonstrate high angiogenic capabilities and restore normoglycemia in STZ-treated mice faster than freshly isolated islets.¹³⁷ Although the organoids developed in this study were derived from primary murine islet cells, a similar protocol could be applied in the future with autologous pluripotent stem cells and liposuction derived microvascular fragments.¹³⁷ The prevascularization leads to favorable engraftment, increased angiogenesis, and enhanced function of the islet organoids following implantation in murine models.^{133,137,154} Crosstalk between islet cells, hAECs, and HUVECs leads to the upregulation of angiogenesis genes such as vascular endothelial growth factor A as well as genes associated with proper β cell function like PDX1.¹⁵⁴

In addition, avoiding the immune system attacking transplanted organoids is critical for graft survival. Methods for a successful islet transplantation have been well discussed elsewhere.^{156–159} A commonly used approach is encapsulation of therapeutic cells prior to the transplant. Researchers have developed biocompatible microcapsules for cell encapsulation that can mitigate the buildup of fibrotic tissue to improve function of transplanted cells.^{160–163} Alagpulinsa *et al.* found that encapsulating hESC-derived β cells into microcapsules containing high concentration of CXCL12 supports β cell function and protects against the immune response and rejection.¹⁶² This study indicates that supplementation of immune-cell-repelling protein CXCL12 improves the likelihood of a successful transplantation. Another approach developed to protect stem cell-derived cells from immune recognition is to induce the expression of immune checkpoint protein programmed death-ligand 1 (PD-L1) (Fig 3).^{164,165} Treatment of human islets with interferon- γ (IFN- γ) led to a 20-fold increase in PD-L1 expression after 20 hours.¹⁶⁴ Furthermore, when IFN- γ was presented through multiple short exposures, the islets exhibited no changes in glucose-stimulated insulin secretions.¹⁶⁴ *In vivo* testing demonstrated that PD-L1⁺ islet organoids showed more prolonged effects in diabetic mice compared to organoids without PD-L1 expression.¹⁶⁴ Fig 3 outlines some critical methods of improving pancreatic islet organoids for use *in vivo*.

Another challenge using iPSC-derived organoids for cell-based therapy is immunological rejection if allogeneic stem cells were used, which requires the use of immunosuppressants to prevent rejection. While this can be avoided through the use of autologous stem cells as a source for pancreatic islet organoids, it too has its set of shortcomings. The process of reprogramming an individual's autologous cells into iPSCs is time-consuming and expensive. At the same time, the requirement for donor-recipient HLA compatibility is a major challenge for use of an off-the-shelf iPSC line.¹⁶⁶ The use of HLA-edited iPSCs may overcome this challenge (Fig 3).^{166,167} Using CRISPR-Cas9, Koga *et al.* demonstrated that HLA-A/B-knockout iPSCs are not rejected by donors with HLA-C-matched natural killer and T cells.¹⁶⁶ Furthermore, 12 HLA-C alleles were capable of 95% of the global population.¹⁶⁶ Therefore, these 12 HLA-edited iPSC lines could serve as a semi-universal source for stem cell-derived tissues.¹⁶⁶ These cells could be used as an unlimited source for pancreatic islet organoids while still avoiding immune rejection following implantation. Castro-Gutierrez *et al.* established a PD-L1 inducible expression and HLA class I knockout hPSC line by genome editing. Islet β -like cells generated from this genome edited, PD-L1 overexpressing cell line revoked CD8 T cell activation characterized by T cell stimulation assay.¹⁶⁵ It should be pointed out that disadvantages in the development of immune-evasive tissues are the loss of the capability of immune surveillance and the risk of tumorigenesis.¹⁶⁸ The use of HLA-editing inherently hinders the ability of the immune system to monitor tissues for the development of cancer, thereby impacting its ability to be applied clinically.¹⁶⁷ Wuputra *et al.* summarized approaches to manage and avoid tumorigenesis developed from iPSCs.¹⁶⁸ Taken together, this review emphasizes the importance of multiple pancreatic islet cell types in islets and the generation of the multicellular islet organoids from stem cells, particularly with updated new strategies to improve cell-based therapies using stem cell-derived islet organoids.

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Abbreviations:

DMSO	dimethyl sulfoxide
GABA	γ -aminobutyric acid
GCGR	glucagon receptor
GLP-1	glucagon-like peptide-1
hAECs	human amniotic epithelial cells
HBSS	Hanks' Balanced Salt Solution
hESCs	human embryonic stem cells
HLA	human leukocyte antigen
HUVECs	human umbilical vein endothelial cells
IFN-γ	interferon- γ
iPSCs	induced pluripotent stem cells
ILCs	islet-like clusters
LepR	leptin receptor
MAFA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
NGN3	Neurogenin-3
PDX1	Pancreas/duodenum homeobox protein 1
STZ	streptozotocin

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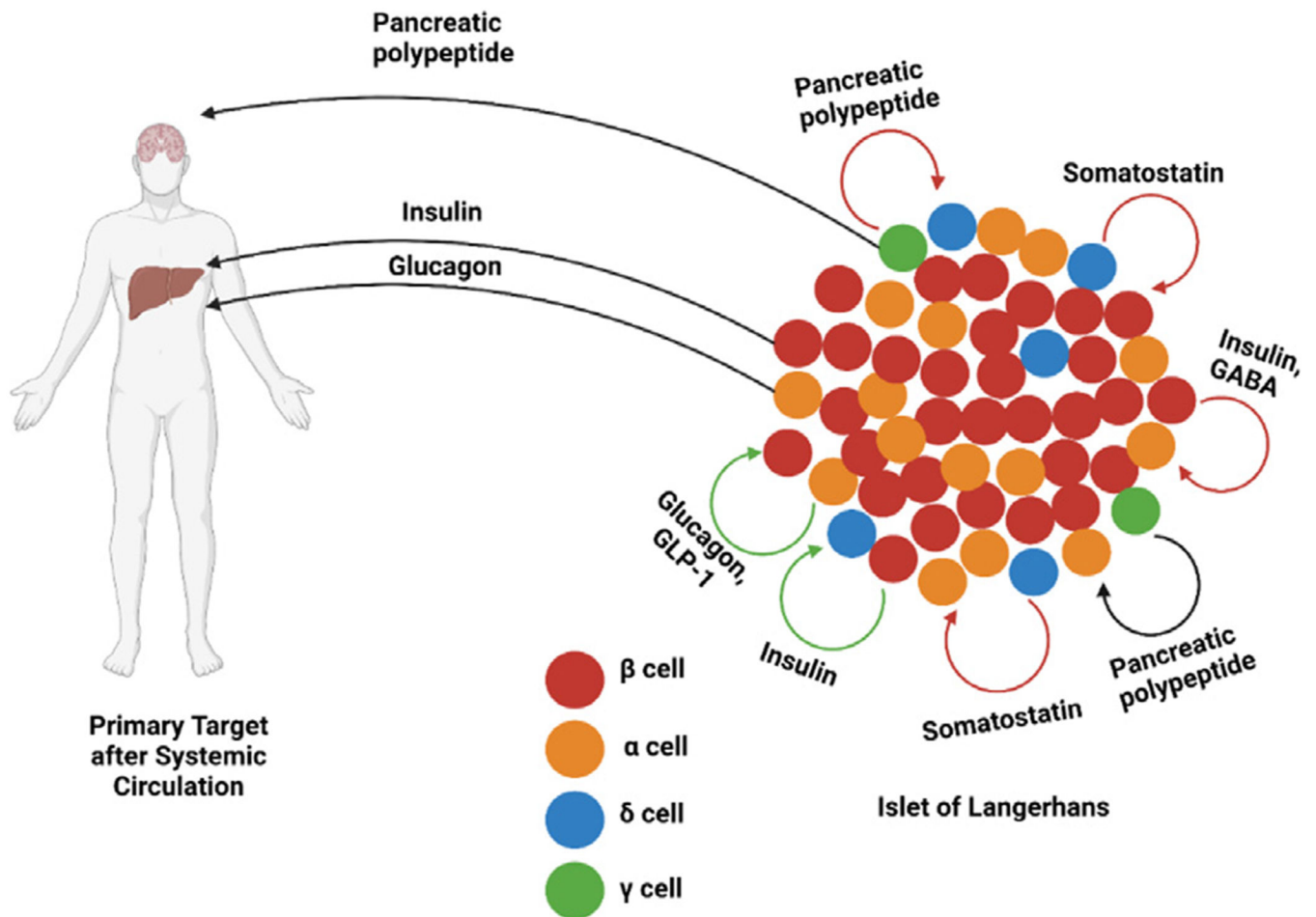


Fig 1. Paracrine and systemic targets of β -, α -, δ -, and γ -cell secretions, with red arrows indicating inhibitory paracrine interactions, green arrows indicating stimulatory paracrine interactions, and black arrows indicating concentration-dependent paracrine interactions. GABA: γ -aminobutyric acid. GLP-1: glucagon-like peptide 1. Created with [BioRender.com](https://www.biorender.com).

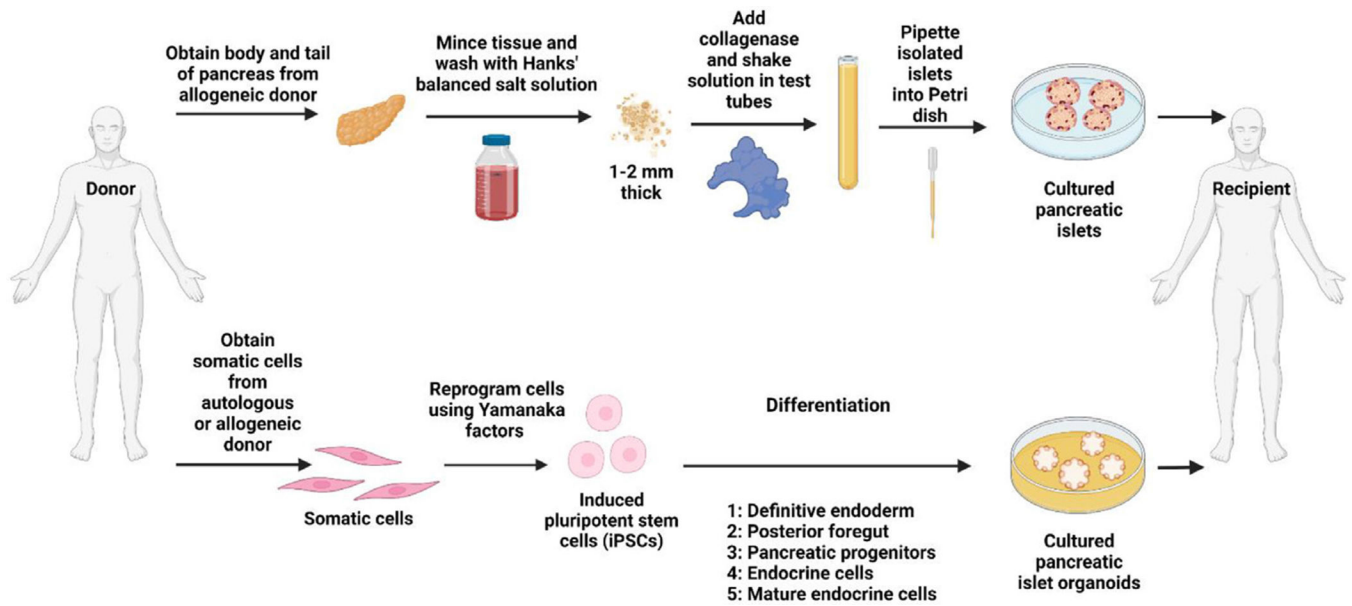


Fig 2.

Key procedures to obtain donor islets and stem cell-derived islet organoids for transplantation. Somatic cells obtained from autologous or allogeneic sources are reprogrammed into pluripotent stem cells using cell reprogramming technologies. Pancreatic islet organoids can be generated from induced iPSC differentiation. Created with [BioRender.com](https://www.biorender.com)

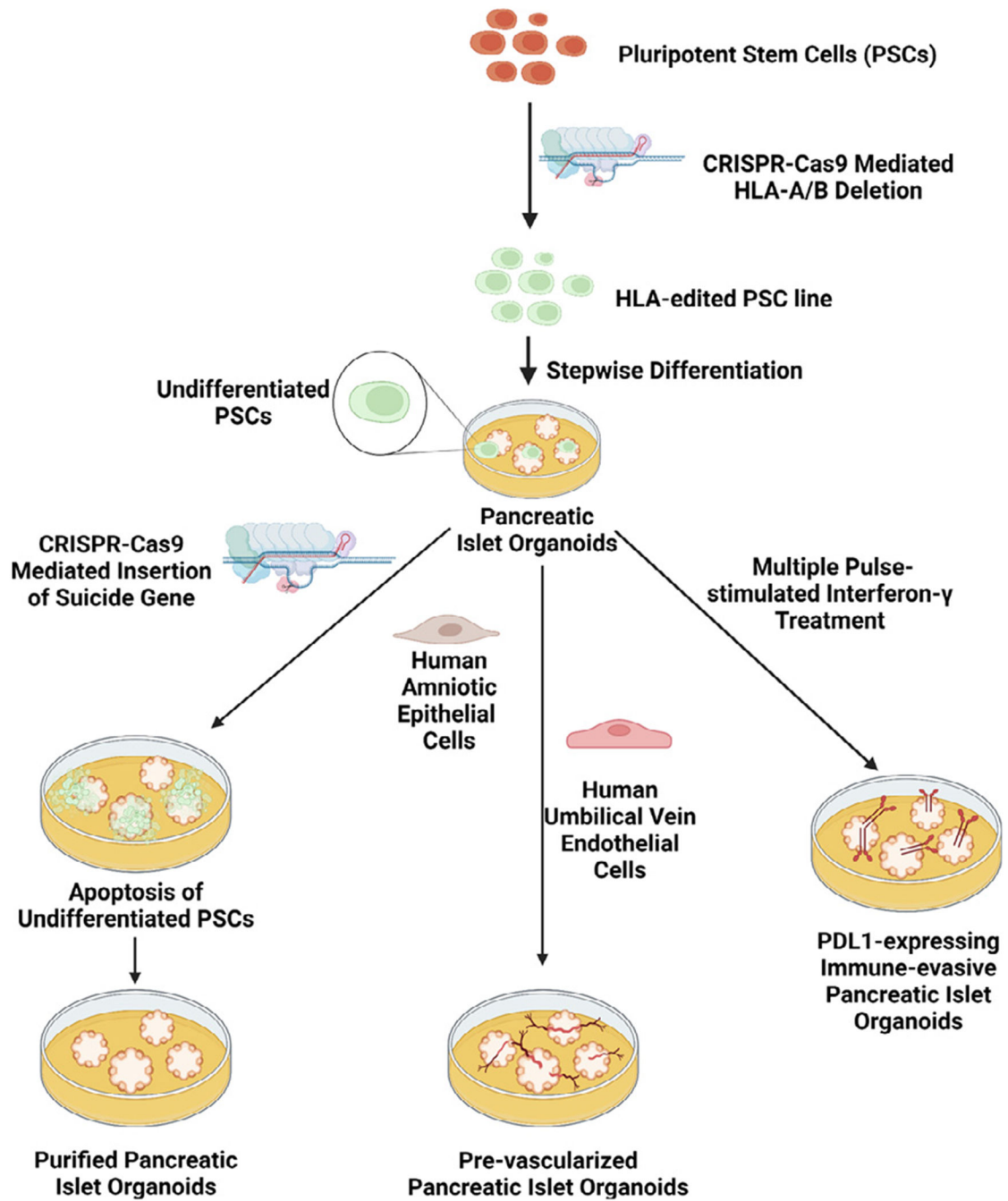


Fig 3.

Novel strategies to enhance the efficacy of using pancreatic islet organoids *in vivo*. Created with [BioRender.com](https://www.biorender.com)

Table 1.

Studies obtaining pancreatic islet organoids composed of multiple islet cell types

Cell Line	β cells	α cells	δ cells	γ cells	Novelty	Reference
iPSCs hIveNry	Detected	Detected	Detected	Not detected	SV40LT added to induce proliferation of endocrine progenitors and removed before differentiation	86
iPSCs TKDN4-M	Detected	Detected	Detected	Not detected	Suspension culture	87
hESCs HUES8, iPSC-1 and iPSC-2	Detected	Detected	Detected	Not detected	Modulation of signaling pathways in each stage of differentiation using small molecules and growth factors	88
hESCs H9	Detected	Detected	Detected	Detected	Collagen type I biomimetic scaffold	67
hESCs HUES8	Detected	Detected	Detected	Not detected	Dynamical regulation of TGF- β signaling	89
hESCs H9	Detected	Detected	Detected	Not detected	Isolation of CD177+ anterior definitive endoderm cells to inhibit Wnt signaling for further differentiation	90
iPSCs IMR90, hESCs H9	Detected	Detected	Detected	Detected	Decellularized pancreatic extracellular matrix in coating substrates	81
iPSCs IMR90, hESCs H9	Detected	Detected	Detected	Detected	Collagen type V presented in coating substrates	68
iPSCs IMR90 and DF4, hESCs H9	Detected	Detected	Detected	Detected	Angiopoietin stimulation in the middle of stepwise differentiation	82