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A Stem Cell Approach to Cure Type 1 Diabetes

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Treatment of type 1 diabetes with insulin injection is expensive, complicated, and insufficient. While cadaveric islet transplantations coupled with immunosuppressants can cure diabetes, the scarcity of acceptable islets is problematic. Developmental research on pancreas formation has informed in vitro differentiation of human pluripotent stem cells into functional islets. Although generating β cells from stem cells offers a potential cure for type 1 diabetes, several challenges remain, including protecting the cells from the immune system.

THE PROBLEM OF DIABETES

Diabetes is a metabolic disease that results from damage to β cells, which are endocrine cells in the pancreas that produce and secrete insulin (Ashcroft and Rorsman 2012). In type 1 diabetes (T1D), an autoimmune destruction of β cells results in a lifelong dependence on exogenous insulin (Katsarou et al. 2017). In type 2 diabetes, which is linked with obesity, β cells become dysfunctional and fail to supply sufficient insulin (DeFronzo et al. 2015). In T1D, invasion and autoimmune attack by both $CD4^+$ and $CD8^+$ T cells specifically destroys β cells and results in almost complete elimination of β-cell mass. The cause for this autoimmune attack in T1D may involve genetic and environmental factors, but it is not clear whether it is the immune system that loses the ability to distinguish between self and nonself, or whether β cells are defective in a manner that makes them a target for clearance.

The destruction or dysfunction of β cells results in high blood sugar levels because β cells secrete insulin and possibly other gene products that are essential for proper glucose homeostasis. β cells are highly sensitive to glucose, constantly monitoring blood glucose levels and secreting exacting amounts of insulin in response to increasing glucose concentrations. Without sufficient insulin secretion, diabetic patients are not able to metabolize glucose normally and control blood glucose levels. T1D is a chronic condition, usually diagnosed in children and young adults, in which insulin replacement is required for survival. There is no known way to prevent or cure T1D. Untreated T1D is a life-threatening issue due to ketoacidosis that can result in cerebral edema and coma, and children are at a higher risk than adults (Cryer 2013). The current T1D treatment protocol combines intensive attention to diet coupled with exogenous insulin administration, either using multiple daily injections or by insulin pumps. In addi-

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tion, there have been advances in the development of modified insulin analogs, which are fast and long acting, and can provide benefit (Pathak et al. 2019).

Despite diligent and intensive attention to blood glucose control with exogenous insulin, T1D patients are prone to severe episodes of hypoglycemia, insulin resistance, mild obesity, and other serious medical complications.

A SHORT HISTORY OF TREATING DIABETES

Diabetes (to flow or pass through in ancient Greek) mellitus is named after symptoms of the disease—frequent urination with a high content of glucose in the urine. Diabetes was lethal in ancient times; life expectancy of children with diabetes mellitus was short and the prognosis for adult-onset diabetes was very poor. In the 18th century, diabetes was treated with a meat-heavy diet (low in carbohydrates) and, during the early twentieth century before insulin was discovered, physicians Allen and Joslin recommended fasting and calorie-restricted diets. All diabetics were advised to reduce sugar and starch intake from their diet, and those who were obese were advised to lose weight. This resulted in some improvement of T1D symptoms, decreased coma, and delayed death among children.

The first understanding of a role for the pancreas in diabetes, in the late 19th century, is attributed to Joseph von Mering and Oskar Minkowski, who found that dogs whose pancreas was removed developed symptoms of diabetes. Insulin was discovered in 1921, when Banting and Best demonstrated a reversal of induced diabetes in dogs by injection of pancreatic islets extract (discovered in 1869 by Langerhans) of healthy dogs. Banting and Best were also able to purify insulin from bovine pancreases, which led to the first effective diabetes treatment in 1922 (Banting et al. 1922). Animal insulin derived from cows and pigs was the first type of insulin to be administered to humans to control diabetes. In 1923, Eli Lilly and Company, with the University of Toronto, began to produce commercial animal insulin, supplying treatment for thousands of patients in Canada and the United States. Banting and Best made the patent available without charge and did not try to control commercial production. Rapidly, insulin treatment for diabetes became affordable and accessible around the world. Insulin produced from large animals was the only available insulin until the 1980s when the first recombinant human insulin was prepared and mass-produced in bacteria (Quianzon and Cheikh 2012).

While lifesaving, insulin treatment is complicated and does not prevent long-term complications that result from chronic increase in blood glucose levels, including damage to the microvasculature causing retinopathy, nephropathy, and neuropathy, as well as macrovascular disease. Furthermore, treatment with excessive insulin injections can cause hypoglycemia, which is still a common and dangerous complication of the disease. In short, insulin injection is onerous, complicated, and it is not a cure.

Cell-based therapy with cadaveric islet transplantation can provide what is essentially a cure for diabetes (Scharp et al. 1990; Socci et al. 1991). In 2000, Shapiro et al. reported a year of follow-up in seven subjects with transplanted islets prepared from pancreases from deceased donors into their liver, a treatment known as the Edmonton protocol. Using this approach, years of complete insulin independence have been achieved in patients with T1D (Shapiro et al. 2003, 2006; Bellin et al. 2008; Berney et al. 2009; Langer 2010).

While islet transplantation can provide a very significant improvement, there is a severely limited availability and quality of donor islets, and the need for chronic immunosuppression, which increases the risk of infections and malignancies, is an undesirable requirement. At present, a small proportion of T1D patients, those who suffer from repeating or severe hypoglycemia events, are eligible for cadaveric islet transplants.

UNDERSTANDING β-CELL DEVELOPMENT

Extensive developmental research over the last decades has provided a deep understanding of the mechanisms that coordinate pancreas and β-cell development (Gittes 2009; Larsen and Grapin-Botton 2017; Sharon et al. 2019a). The

pancreas consists of two compartments: an exocrine compartment that participates in digestion of macronutrients through the production and release of digestive enzymes to the intestine and the endocrine compartment. The pancreatic endocrine cells (mainly glucagon-secreting α cells, β cells, somatostatin-secreting δ cells, and pancreatic polypeptide-secreting cells) form spherical clusters, the islets of Langerhans, within the larger exocrine tissue (Jennings et al. 2013, 2015).

As in many major organs, including the liver, lung, and the intestines, the development of the pancreas initiates from patterning and differentiation of the definitive endoderm (Wells and Melton 1999). In the embryo, specification of the pancreatic domain is mediated by a combination of signals from the mesoderm, including transforming growth factor β (TGF-β), retinoic acid (RA), and fibroblast growth factor (FGF) (Henry et al. 1996; Apelqvist et al. 1997, 1999; Kim et al. 1997a,b, 2000; Kim and Melton 1998; Hart et al. 2003; Rankin et al. 2018). Pancreas specification becomes evident with the expression of pancreatic duodenal homeobox 1 (PDX1) in ventral and dorsal domains within the gut endoderm (Jonsson et al. 1994, 1995; Offield et al. 1996). Epithelial budding of the foregut endoderm generates two pancreatic buds (dorsal and ventral buds) that later fuse to form the pancreas. The ventral and dorsal pancreatic buds are marked by the transcription factors SOX9, PDX1, and GATA4, which are required for pancreatic growth (Fukuda et al. 2008; Wandzioch and Zaret 2009; Carrasco et al. 2012). The fused ventral and dorsal buds form a multilayered epithelium consisting of multipotent pancreatic progenitor cells, which give rise to ductal, endocrine, and exocrine cell lineages. Lineage-tracing studies reveal that PDX1-expressing cells are pancreatic progenitor cells and PDX1-null mice display pancreatic agenesis (Jonsson et al. 1994). Similarly, human congenital pancreatic agenesis is caused by homozygous deletion of IPF1, the human ortholog of PDX1 (Stoffers et al. 1997). The early pancreas undergoes a large expansion through proliferation of the progenitor cells. By receiving appropriate niche signals from their microenvironment, the progenitors differentiate into endocrine cell types and leave the epithelial cords while maintaining cell-to-cell contacts, thus creating a cohesive bud-like structure (Pan and Wright 2011; Sharon et al. 2019a). Endocrine differentiation from epithelial cords is first marked by expression of the bHLH transcription factor Neurogenin3 (NGN3) (Gradwohl et al. 2000; Gu et al. 2002, 2003; Gouzi et al. 2011). Upon transient increase in the expression levels of NGN3, these progenitors stop proliferating and differentiate into endocrine cells. The expression of NGN3 is required for the commitment of progenitor cells to an endocrine cell fate as NGN3-null mice are completely lacking both intestinal and pancreatic endocrine lineages. The mechanisms by which $NGN3^+$ cells give rise to the different endocrine lineages are not completely understood; however, lineage-tracing experiments and conditional gene-ablation studies has revealed that many transcription factors, including PAX4, HNF4a, FOXA2, NKX6.1, and MAFA, have important roles in β-cell formation, differentiation, and function (Sosa-Pineda et al. 1997; Gupta et al. 2005; Henseleit et al. 2005; Zhang et al. 2005; Gao et al. 2008; Taylor et al. 2013; Röder et al. 2016; Villamayor et al. 2018; Lee et al. 2019).

Importantly, endocrine progenitors or pancreatic adult stem cells do not exist in the mature pancreas. Therefore, β-cell maintenance and regeneration depend, perhaps entirely, on β-cell replication (Dor et al. 2004). However, baseline and compensatory β-cell replication decline sharply with age, in both rodents and humans, as a mechanism for improving the function of differentiated β cells while sacrificing the ability to mount a regenerative response (Helman et al. 2016; Puri et al. 2018). Consequently, if β cells are destroyed, there is no significant replacement or regeneration, unlike other tissues.

STEM CELL THERAPY FOR DIABETES

The discovery of pluripotent embryonic stem cells (ESCs) and engineered induced pluripotent stem cells (iPSCs) that have a potential to differentiate, in vitro, into any cell type, created a new promising strategy for β-cell replacement therapy (Takahashi and Yamanaka 2006; Okita et al.

2007; Takahashi et al. 2007; Wernig et al. 2007; Latres et al. 2019).

Studies over the past decade have clearly demonstrated that the most effective way to generate specific cell types from iPSCs in vitro is to recapitulate embryonic development (Murry and Keller 2008). This approach has been successful and revealed that many of the signaling pathways and transcription factors regulate embryonic development of the pancreas. However, because of their high potency, directing stem cells to generate a specific differentiated cell type is difficult to control, and years of research were needed to succeed in directing human stem cells toward a functional β-like cell fate.

Induction of endoderm from pluripotent cells, which is the first step of this process, had been studied in frog and fish and murine models and identified molecular signals that induce differentiation into endoderm, including the TGF-β superfamily members (Henry et al. 1996; Wells and Melton 2000; Brennan et al. 2001; Tremblay 2010; Nostro and Keller 2012). Another TGF-β family member, Activin A, can signal via similar downstream pathways as Nodal but is easier to produce as a recombinant protein (Chen et al. 2013), enabling the first protocol for generating definitive endoderm in vitro from human pluripotent stem cells (D'Amour et al. 2005). This was followed by differentiation of ESCs into progenitors that could further differentiate into mature pancreatic cells after transplantation into immune-deficient mice (Kroon et al. 2008). To advance regenerative medicine for diabetes with stem cells, a major challenge in the field was to make functional human β cells in vitro. These required discoveries using different inducing factors and genetic markers for the in vitro differentiation of stem cells and was achieved in 2014 by development of a protocol that produced stem cell–derived (SC) β cells that secreted insulin in response to successive glucose challenges in vitro (Pagliuca et al. 2014). The fact that these β-like cells performed glucose-responsive insulin secretion in response to multiple glucose stimulations, like primary human islets, showed that it is possible to make physiologically functional cells entirely in vitro in the absence of signals from other mesenchymal cells and a natural blood supply. This opened up the possibility of producing islets for further physiological studies and drug studies in vitro as well as transplantation to directly treat the disease.

Subsequent modification of differentiation protocols has enhanced the quality of β-like cells generated in vitro from polyhormonal, glucose-unresponsive cells to a high percentage of monohormonal, insulin-expressing β-like cells (Rezania et al. 2014; Nostro et al. 2015; Russ et al. 2015; Nair et al. 2019; Rosado-Olivieri et al. 2019; Sharon et al. 2019b; Velazco-Cruz et al. 2019; Veres et al. 2019). The common underlying principle for the different protocols is that millions of human pluripotent stem cells grown in 3D clusters are differentiated in a stepwise manner by exposing them to various growth factors and small molecules to activate or inhibit embryonic signaling pathways, such as Nodal, WNT, RA, FGF, bone morphogenetic protein (BMP), and Notch. For most protocols, it takes between five and seven stages that last 20–30 days in total to transform human stem cells into β-like cells. The terminally differentiated cells maintain their identity for long periods in culture and can rescue diabetes after transplantation in animal models.

The first three stages of differentiation generate a nearly homogenous (about 90%) population of pancreatic progenitors that express the master transcription factor PDX1. Thereafter, distinct populations are identified by staining for C-peptide (processed insulin fragment), the pan-endocrine marker CHGA, and the β-cell transcription factor NKX6.1. Single-cell analysis of the different populations in SC islets identifies three types of endocrine cells: SC β cells that express β-cell markers including INS and NKX6.1, α-like cells that express GCG and ARX, and nonpancreatic endocrine cells that mostly resemble intestinal endocrine cells. There are also remaining nonendocrine progenitor cells in the final SC islets clusters. In addition to β-like cells, these protocols also generate α-like cells that may contribute to proper islet function after transplantation. The undesired cells such as nonendocrine progenitors can be reduced by different reaggregation methods (Nair et al. 2019; Veres et al. 2019).

The progress that has been made in generation of an unlimited supply of functional, insulin-secreting cells from human ESCs and iPSCs holds the most promise for transplantation therapy to treat T1D. Moreover, the ability to generate functional β cells in large quantities makes a novel tool for studying mechanisms of human β-cell formation and maturation and would establish a platform for modeling disease in vitro and for screening drugs and molecules that could impact proliferation and/or function of β cells and to identify new targets for diabetes therapy (Huch and Koo 2015; Dutta et al. 2017; Bakhti et al. 2019).

REMAINING CHALLENGES

Improvements in the efficiency of generating functional SC β cells depend on understanding the extracellular signals that control cell fate choices during embryonic and postnatal development. There is still a lack of complete understanding of the cues required to fully control ESC differentiation toward all the endocrine cells found in islets. A typical SC islet cluster contains 40%–50% of the desired endocrine cells, while the rest of the cluster contains progenitor cells that fail to reach terminal differentiation and other undesired nonpancreatic endocrine cells. Methods for β- and α-cell enrichment, using FACS and magnetic beads sorting, are currently used to reduce the numbers of the undesired cells within the final SC islet clusters. But the overall efficiency of β-like cell production is greatly reduced during the sorting process because of high cell loss (Veres et al. 2019).

It will be important to control the ratio between β and non-β endocrine cells in the clusters to best mimic human islets function. Differentiation protocols are directed toward the generation of β-like cells and generate fewer α- and δ-like cells. It is an open question whether sufficient insulin secretion by β-like cells will be achieved by a pure population or whether the presence of other endocrine cells will be beneficial in achieving optimal β-cell function.

SC β cells made in vitro are highly similar to mature human β cells but lack expression of the maturation markers UCN3, MAFA, and SIX3, and glucose-stimulated insulin secretion capacity does not meet the magnitude of that found in fully mature human β cells (Blum et al. 2012; Hrvatin et al. 2014; Arda et al. 2016). The inability to achieve the same functionality in vitro reflects the lack of knowledge of the factors that normally regulate β-cell maturation during neonatal development in vivo. For example, the drastic nutritional and metabolic changes that might affect the β-cell niche are not completely known and not recapitulated into SC β-cell differentiation protocols. In agreement with the effects of in vivo environment on SC β-cell maturation, in vivo maturation markers are expressed after transplantation. Functionally, β-cell maturation is characterized by secreting insulin in a glucose-dependent manner; in general, mature β cells secrete similar levels of insulin in response to glucose and to potassium (that induces membrane polarization and maximal insulin secretion), while in fetal β cells and SC β cells glucose induces much less than maximal insulin secretion. In addition, SC β cells secrete insulin in response to amino acids that are normally insufficient to induce insulin secretion in mature β cells. These functional differences may mirror the role of fetal β cells in mediating embryonic growth, which requires continuous insulin secretion, as opposed to mature β cells that secrete insulin only after meals. Recent work highlights the differences between fetal β cells to mature β cells in terms of glucose metabolism, mitochondrial activity, and nutrient sensing. It was recently shown that endocrine cell clustering or expression of mitochondrial activity regulators induces metabolic maturation by driving mitochondrial oxidative respiration, a process central to insulin secretion in mature β cells (Yoshihara et al. 2016). These findings are encouraging, as generating cells that function more robustly could reduce the number of transplanted cells in diabetic patients and/or reverse diabetes more quickly.

It may be that the most significant challenge remaining for transplanting SC β cells to T1D patients is protecting the cells from immune rejection. Physical protection, by encapsulating the SC β cells in alginate microspheres or macro-

devices, has shown promise (Sneddon et al. 2018). Macroencapsulation devices have been used with SC progenitor cells to demonstrate blood glucose control in immunocompromised mice (Motté et al. 2014; Robert et al. 2018).

Modified alginate spheres containing SC β cells have been transplanted into diabetic rodents, without immunosuppressants, and achieved long-term control of blood glucose levels with human insulin (Bruin et al. 2013; Agulnick et al. 2015; Vegas et al. 2016; Chang et al. 2017; An et al. 2018; Alagpulinsa et al. 2019).

One of the challenges with physical protection by alginates or macrodevices is providing an adequate nutrient and oxygen supply without direct contact with blood oxygen. In all, encapsulation is a promising approach that could provide long-term glucose control with SC β cells.

A complementary approach is to use engineering strategies to provide immunity protection. Suggested gene modifications in the transplanted cells that could be used to induce immune tolerance include tolerogenic cytokines and immunomodulatory proteins such as HLA-G, PD-L1, and CTLA-4 as well as reducing HLA expression (El Khatib et al. 2015; Figueiredo and Blasczyk 2015; Li et al. 2015; Gornalusse et al. 2017). In this manner, expression may be able to increase survival also after allogeneic transplantation and could be used as universal donor β cells that would match all patients, independent of the genetic background and HLA type of the recipient. These modifications could enhance tolerance to β-cell antigens without affecting the patient's immune system by applying immunosuppressive drugs. One could also take the complementary approach of trying to blunt the T-cell-specific attack on β cells, perhaps by modulating Treg cells (Bluestone and Tang 2018).

Importantly, approaches to induce immune protection to SC cells has the potential concern of creating rogue cells that cannot be eliminated by the immune system. These contaminating or rogue cells might proliferate and cause harm. Using inducible suicide genes may provide protection against this happenstance (Liang et al. 2018). Transplanting cells within a protective device in an accessible location where they can be readily removed will reduce this risk.

SUMMARY

Tremendous progress has been made in the last decades toward understanding how β cells are formed during normal development and how to generate functional β-like cells through stem-cell differentiation. Yet additional challenges remain for this field. The immune system problem needs to be addressed, whether through systemic immunomodulation or by using immunoprotective devices. Moreover, clinical trials should be executed to address safety and efficiency questions including optimal implant size and whether other endocrine cells may be required for the best metabolic control. The clinical need for an unlimited source of β-like cells will require methods to generate homogenous cell populations on a very large scale by moving toward industrial production. In summary, 100 years after the discovery of insulin, we have the possibility of more breakthroughs and a meaningful cure for insulin-dependent diabetics.

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