Pluripotent stem cells: a new strategy for the treatment of diabetes

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ABSTRACT

Diabetes is now one of the most prevalent diseases in the world. Over the next twenty years, the number of affected individuals could reach 693 million. Diabetes is a group of chronic carbohydrate metabolism disorders responsible for increased blood glucose levels. It has several effects on the human body, and over the years can even become disabling, greatly impacting the patient's quality of life. The most common therapy is based on daily and chronic administration of exogenous insulin, but this is associated with a series of dangerous and potentially fatal complications. Unsurprisingly therefore, over time, studies have explored various alternatives for the treatment of diabetes, not involving the use of insulin, primarily transplantation of the whole pancreas and of the islets of Langerhans. However, neither of these solutions can be applied on a large scale, mainly due to the lack of donors. In recent years, a viable alternative to insulin and to transplantation has emerged through exploration of the use of pluripotent stem cells and their properties. In this review we analyze the state of the art in this field, and the advantages and disadvantages of cell therapies used for reconstruction of the functional unit of the pancreas, the islets of Langerhans.

KEYWORDS

Diabetes, stem cells, β cells, stem cell therapy.

Diabetes: classification, effects and therapy

The pancreas is an abdominal organ consisting of two portions (endocrine and exocrine) with different functions and characteristics. The exocrine portion, which makes up approximately 95-98% of the entire pancreas, produces the enzymes involved in the digestion of food and is composed of three different cell types: acinar, centroacinar and ductal [1]. The endocrine portion is responsible for controlling blood glucose homeostasis and is constituted by islets of Langerhans (which we will refer to simply as "islets" from here on), each of which is made up of five different cell types. The most abundant are β cells (60-80%), which secrete insulin, and α cells (15-20%), which secrete glucagon. The others, less abundant, are δ cells, ε cells, and γ or pancreatic polypeptide-secreting cells, which produce somatostatin, ghrelin, and pancreatic polypeptide, respectively [2].

Each islet can be considered a highly vascularized mini-organ, characterized by a large number of connections between different cell types and innervated by the sympathetic and parasympathetic systems. The sympathetic nervous system inhibits the secretion of hormones, while the parasympathetic system stimulates their release [3]. This fine regulation is important because, in humans, insulin is the only hormone produced and secreted according to changes in blood glucose levels, in order to keep these levels, always, within a precise range [4]. In fact, in the presence of elevated blood glucose levels (a condition known as hyperglycemia), insulin restores normoglycemia by promoting glucose uptake by liver, fat, and muscle cells [5].

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Diabetes mellitus (DM) is the main consequence of impaired pancreatic function. DM affects more than 450 million people worldwide, and the World Health Organization (WHO) predicts that this number will rise to 693 million by 2045 [6]. DM is a group of chronic disorders of carbohydrate metabolism, characterized by a pathological condition of hyperglycemia, which is the normal condition of a patient with DM.

In 2019, the WHO classified DM into Type 1 diabetes (T1DM), Type 2 diabetes (T2DM), hyperglycemia during pregnancy, hybrid forms, other specific types (which include monogenic DM, among others), and unclassified forms [7]. The most common forms are T1DM (or insulin-dependent DM) and T2DM (or adult-onset diabetes, characterized by insulin resistance).

What are the causes of DM? T1DM is caused by autoimmune mechanisms that result in β-cell destruction, resulting in insufficient insulin production to maintain normoglycemia. T2DM, on the other hand, is due to insufficient insulin release related to insulin resistance and it accounts for approximately 90% of total cases [8]. Although the causes of these disorders are

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different, their common feature is loss of β-cell function. The etiology of DM is still unclear. It is thought to be due to genetic and environmental factors [9]. There are several complications associated with DM, including cardiovascular disease, cerebral vascular damage, retinopathy and peripheral neuropathy [10].

Given the increasing number of T1DM cases over the years, this form has become the focus of growing scientific attention. Pharmacological therapy of T1DM is based on repeated (chronic daily) administration of exogenous insulin, in order to ensure blood glucose homeostasis. Instead, patients with T2DM resort to exogenous insulin when they cannot adequately control glucose levels with oral medication. The most serious complication associated with daily insulin administration is hypoglycemia. In fact, patients have to monitor their glucose levels continuously, and this affects their quality of life. In addition, severe hypoglycemia can result in cognitive impairment, cardiovascular problems, and even death [11].

Although the discovery of insulin in 1921 has allowed a great revolution in DM therapy, making the condition treatable and thus, for many people, ensuring survival, it should still be emphasized that while it is a life-saving therapy, it does not eliminate either the underlying cause of the disease or the possibility of developing the complications associated with DM itself [9].

To try to overcome the problems associated with both hypoglycemia and suboptimal control of glucose levels, recent decades have seen the development of new insulin formulations with different onset and duration of action, so as to meet the different needs of patients [5]. Furthermore, in order to compensate for the lack or absence of β cells, insulin pumps have been produced, which allow the continuous infusion of the hormone into the subcutaneous tissue, [12,13] removing the need for injection, which has a strong psychological impact on patients. However, problems have also been encountered with these devices, such as the development of dermatitis in the area of application and the onset of hypoglycemia or hyperglycemia phenomena following delays in the release of insulin from the device itself [14]. Indeed, while β cells manage to measure blood glucose concentration every millisecond, insulin pumps take much longer [13]. Moreover, despite these improvements, other aspects remain: the considerable economic impact on the lives of patients, the possibility of developing the aforementioned disease-related complications, and the need for exercise and diet [15].

In this regard, new strategies have been implemented, aimed ultimately at finding a therapy for T1DM which is not based on daily insulin administration. In this review we discuss these new strategies. These include cell therapy and, in particular, whole pancreas transplantation, but also islet transplantation. Here, however, we focus mainly on the use of stem cells and the differentiation protocols to which they are submitted, as this seems to be the most promising avenue to date.

Transplantation: organ and islets

Initially, the main idea behind transplantation was to increase the amount of functioning β cells. An early clinical strategy was whole cadaver-derived pancreas transplantation. This achieved insulin independence in many patients, improving their quality of life.

However, several complications are associated with whole pancreas transplantation. First, it is a very complex type of surgery, which can lead to postoperative difficulties, including acute graft rejection, development of infections, and portal vein thrombosis [7,16,17]. In addition, this operation requires lifelong immunosuppressive therapy [18], and is not suitable for all types of patients. This type of therapeutic strategy is generally considered for young patients who do not have cardiac problems [19].

The aforementioned problems prompted the development of islet transplantation, an innovative clinical strategy that consists of obtaining islets from donor patients, separating them from other pancreatic cell types, purifying them, and then infusing them into the patient via the portal vein of the liver, which, due to its ease of access, is the route through which most transplants have been performed in the clinic [20,21]. It has been shown that islet transplantation, in addition to freeing the subject from insulin use for a few years, allows better metabolic control and a slower onset of DM-related problems compared with daily administration of insulin [16,22,23].

Despite these important successes, islet transplantation is associated with problems such as lack of donors, insufficient revascularization and immunosuppression. The lack of donors is certainly the most difficult obstacle to overcome. It has been seen that two or more donors are needed for each recipient, as less than half of the islets present in the healthy pancreas can be isolated and some of those are lost in the purification processes [24-26]. There is also a loss of islets in the days immediately following transplantation due to poor revascularization, which, by creating a hypoxic environment, [27,28] hinders islet survival [29,30]. As with whole pancreas transplantation, immunosuppressive therapy is also required, and this, while necessary to defend the transplanted islets from the immune system, may result in damage to other tissues or organs [31,32].

Until these obstacles are overcome, particularly the lack of donors, neither whole pancreas nor islet transplantation can be considered a viable therapeutic alternative to insulin administration for DM. For this reason, as mentioned above, great attention has been paid in recent years to stem cells and the possibility of using them.

Pluripotent stem cells in DM

Stem cells are undifferentiated but capable of self-renewal and differentiation into various somatic cell types [33]. Stem cells have found application in drug development, disease modeling and regenerative medicine [34]; in particular, they have been used in the treatment of several diseases, including diabetes, cancer, neurological and cardiovascular disorders [35]. They can be classified according to their potency (totipotent, pluripotent, multipotent, oligopotent, and unipotent) or their origin (embryonic, adult, tissue-resident, and induced pluripotent) [35].

Human pluripotent stem cells include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Human ESCs (hESCs) are derived from the inner cell mass of the blastocyst. Although they are capable of differentiating into any cell type, and may therefore represent a valuable opportunity in

regenerative medicine, their origin has raised ethical issues [36]. These have been overcome by the use of human iPSCs (hiPSCs), which are obtained from terminally differentiated cells with the subject's genetic profile. This means that adult somatic cells taken from the subject can be reprogrammed into iPSCs and, after the differentiation process into the desired cell type, re-implanted without the need for immunosuppression [37].

For the therapeutic use of hESCs and hiPSCs in T1DM, the cells must undergo a precise and reproducible *in vitro* differentiation protocol leading to the production of pancreatic β cells that are capable of releasing insulin (and not the other hormones) and characterized by typical human β-cell markers. Cells derived from this differentiation are capable of secreting insulin in amounts necessary to respond to rapid changes in blood glucose levels. Thus, in this way, it is possible to replace insulin-secreting β cells both in DM patients in whom these cells are dysfunctional, and in those who lack these cells.

Such protocols were performed by reproducing *in vitro* the different stages that lead, in a stepwise fashion, to the *in vivo* development of the human and murine pancreas. Accordingly, stem cells were differentiated in succession into the definitive endoderm, the multipotent pancreatic progenitor, the endocrine progenitor, and finally into hormone-secreting endocrine cells [38].

For hESCs and hiPSCs to transform into insulin-secreting endocrine cells, it is necessary to add small molecules and growth factors to the culture medium, to momentarily allow activation or inhibition of different signaling pathways. It is equally important to define which combination of small molecules or growth factors to use for each step of the differentiation process, as well as their concentration and exposure time. Activin A (mimics the proendodermic action of Nodal), NOGGIN (inhibits liver lineage differentiation), thyroid hormone tri-iodothyronine (T3) (promotes the expression of neurogenin 3 (NEUROG3), which is essential for β-cell differentiation and maturation), and vitamin E (an inducer of avian musculoaponeurotic fibrosarcoma oncogene homolog-A (MAFA)) are some of the factors used, respectively, in the steps of the differentiation protocol [38].

Differentiation protocols also include analysis of key markers of differentiation at the end of each stage. The definitive endoderm is characterized by the expression of forkhead box (FOX) A2, sex-determining region Y box (SOX) 17, and chemokine receptor 4. The multipotent pancreatic progenitors express pancreatic and duodenal homeobox 1 transcription factor (PDX1), NK6 homeobox protein 1 (NKX6.1), and SOX9. Endocrine progenitors are characterized by the expression of PDX1, NKX6.1, NEUROG3, and neurogenic differentiation factor 1 (NEUROD1). Finally, insulin, PDX1, NKX6.1, paired box homeodomain transcription factor (PAX) 6, NEUROD1, MAFA, and motor neuron and pancreatic homeobox 1 (MNX1) expression characterizes mature β cells [19]. So, analysis of specific markers is necessary to be sure that the differentiation process has started, and is proceeding correctly, step by step, and also to remove any undifferentiated stem cells [4].

In 2009, Zhang *et al*. performed the first protocol, which allowed them to obtain mature insulin-secreting pancreatic cells from hESCs and iPSCs in four steps, namely a) endoderm induction, b) pancreatic specialization, c) progenitor expansion, and d) maturation [17]. As the first step, they induced stem cells to differentiate into the endoderm lineage using Activin A and Wortmannin, and confirmed the successful differentiation through analysis of the expression of SOX17 and FOXA2, two markers that characterize the endoderm lineage (Table I). The cells obtained were treated with retinoic acid (RA), NOGGIN and Fibroblast Growth Factor (FGF) 7 and the markers analyzed were PDX1, PAX4, PAX6 and hepatocyte nuclear factor (Hnf) 6. The third phase is characterized by the presence of Epidermal Growth Factor (EGF) and the differentiation markers analyzed were PDX1, FOXA2, SOX9, and Hnf1B. At this point, progenitor cells were induced to mature through the use of a mix of factors (basic FGF (bFGF), Exendin-4, nicotinamide and bone morphogenetic protein 4). After 20 days, expression of MAFA, insulin, Glut2, NKX6-1, glucokinase, and transcription factor 12 was observed, indicating the obtaining of insulin-positive cells. Through flow cytometry, it was seen that 25% of the cultured cells were insulin-positive.

In 2014, Rezania *et al*., refining some steps of their previous protocols, developed one using hESCs and consisting of seven steps, namely 1) definitive endoderm, 2) primitive gut tube, 3) posterior foregut, 4) pancreatic endoderm, 5) pancreatic endocrine precursors, 6) immature β cells, and 7) maturing β cells [39]. For each step, they used a different combination of small molecules and growth factors (i.e., GDF8, GSK3β inh, FGF7, vitamin C, RA, SANT, TPB, LDN193189 (LDN), activin receptor-like kinase 5 II inhibitor (Alk5 inh), T3, GS inh XX, N-acetyl cysteine (NAC), AXL inh) (Table I). This protocol differs from the previous ones in that, previously, polyhormonal cells with fetal endocrine-like characters were obtained, whereas with the protocol of Rezania *et al*., about 50% of mature β cells were found to be insulin-positive and expressing typical β-cell markers (i.e., PDX1 and NKX6.1). Furthermore, the mature β cells thus generated had an insulin content similar to that of human islets and, like these, were able to secrete insulin in response to glucose. Although these cells were also able to reverse DM in mice, differences with human islet cells emerged: insulin was released from the mature β cells more slowly, and in smaller amounts.

In 2014, another research group developed a six-step protocol that, in five weeks, allowed a large-scale, three-dimensional culture system to obtain β cells from hPSCs with 30% efficiency [40]. This protocol was innovative in that gamma secretase inhibitor (XXI), Alk5 inh, and T3 were used in the final steps. Other substances used were Activin A, CHIR9901 (CHIR), Keratinocyte Growth Factor (KGF), RA, SANT1, LDN, phorbol 12,13-dibutyrate (PdbU), heparin, betacelluin (Table I). *In vivo* studies reported that differentiated β cells were monohormonal, capable of secreting insulin after glucose stimulation, and of decreasing hyperglycemia in mice, and that their gene expression was similar to that of human islet cells.

Instead, Russ *et al*., in 2015, attempted to draft a simplified protocol that traces the various stages of human endocrine development and leads hESCs to differentiate into human pancreatic progenitors and endocrine cells. This protocol involves treating hESCs with WNT3a, Activin A, TGFb inh, KGF, RA, cyclopamine, NOGGIN, EGF, TBP, and Alk5 inh (Table I) [41]. β cells derived from hESCs were able to release insulin follow-

Table I Summary of key features of differentiation protocols.

REPORT	NUMBER OF STAGES	NAME OF STAGES	SMALL MOLECULES AND GROWTH FACTORS	REFERENCE
Zhang *et al.*	4	Endoderm induction, pancreatic specialization, progenitor expansion, maturation	Activin A, Wortmannin, RA, NOGGIN, FGF7, EGF, bFGF, Exendin-4, nicotinamide, bone morphogenetic protein 4	17
Rezania *et al.*	7	Definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, pancreatic endocrine precursors, immature β cells, maturing β cells	GDF8, GSK3β inh, FGF7, vitamin C, RA, SANT, TPB, LDN, Alk5 inh, T3, GS inh XX, NAC, AXL inh	39
Pagliuca *et al.*	6	Definitive endoderm, primitive gut tube, pancreatic progenitors 1, pancreatic progenitors 2, endocrine cells, stem cell derived β cells	Activin A, CHIR, KGF, RA, SANT1, LDN, PdbU, T3, XXI, Alk5 inh, heparin, betacellulin	40
Russ *et al.*	5	Gut tube, PDX1+ progenitor, PDX1/NKX6.1+ progenitor, endocrine progenitor	WNT3a, Activin A, TGFb inh, KGF, RA, cyclopamine, NOGGIN, EGF, TBP, Alk5 inh	41
Nair *et al.*	8	Gut tube, pancreatic foregut, pancreatic progenitors, immature β-like cells, immature β-like cell selection, reaggregation, enriched β-cell clusters	WNT3a, Activin A, TGB inh, KGF, RA, EGF, Alk inh, T3, XXI, LDN, vitamin C	42
Velazco-Cruz *et al.*	6	Definitive endoderm, primitive gut tube, pancreatic progenitors 1, pancreatic progenitors 2, endocrine cells, stem cell derived β cells	Activin A, CHIR, KGF, RA, SANT1, Y, LDN, PdbU, T3, XXI, Alk5 inh, betacellulin	45
Yoshihara *et al.*	5	Definitive endoderm, pancreatic progenitors, endocrine progenitor, islet-like organoids	Activin A, GSK3β inh, FGF7, vitamin C, RA, TGFβ inh, BMPR inh, Hedgehog inh, PKC, T3, Alk5 inh, Notch inh, vitamin E, cAMP, WNT4	46
Li *et al.*	6	Stage 1-6	Activin A, CHIR, bFGF, KGF, vitamin C, NOGGIN, SANT1, RA, EGF, nicotinamide, RepSox, GC1, LDN, XXI, Y, R428, Trolox, NAC	49

ing an increase in glucose and expressed key factors of human β cells. Furthermore, these differentiated cells remained glucose-sensitive after short-term transplantation.

A step forward in the study of pancreatic differentiation was achieved by Nair *et al*., who developed a protocol that reproduced not only pancreatic organogenesis but also the subsequent clustering of endocrine cells, which is a necessary condition for cell maturation [42]. Their protocol was structured into ESCs, gut tube, pancreatic foregut, pancreatic progenitors, immature β-like cells, selection of immature β-like cells, reaggregation and obtaining of enriched β-cell clusters by treating the cells with several pancreatic differentiation protocols (i.e., WNT3a, Activin A, TGB inh, KGF, RA, EGF, Alk inh, T3, XXI, LDN and vitamin C) (Table I). β-like cells were selected by fluorescence-activated cell sorting and then re-aggregated into clusters, which were cultured for five to seven days. Hence, they obtained "pancreatic clusters" similar to human islets, but with higher expression of β cell markers. They compared β cells before and after clustering and showed not only that those constituting clusters were more mature, but also that these cells had physiological properties corresponding to those of human islet cells, like dynamic insulin release. This is an important aspect to confirm the development of an improved differentiation protocol. In recent years, several studies have in fact attempted to find the best differentiation protocol. For example, Wang *et al*. and Sharon *et al*. differentiated stem cells into insulin-secreting cells through protocols created by modifying existing and cited ones [43,44]. The modifications concern the number of steps or the concentrations of the substances used.

Recently, Velazco-Cruz *et al*. implemented a six-step differentiation protocol, improving on a number of other existing protocols [45]. The steps of their protocol were definitive endoderm, primitive gut tube, pancreatic progenitors 1 and 2, endocrine cells, and stem cell-derived β cells, and the substances added to the culture medium were Activin A, CHIR, KGF, RA, SANT1, Y27632 (Y), LDN, PdbU, T3, XXI, Alk5 inh, and betacellulin, while the last step involves the use of serum-free medium (Table I). With this protocol, they were able to obtain colonies of endocrine cells that were almost monohormonal and expressed β-cell markers. The changes they made were the use, in the last step, of substances different from those commonly used in other protocols (such as T3 and NAC) and of serum-free medium, the decrease in cluster size, and the modulation of the transforming growth factor-β pathway. Certainly, the most important result of this protocol was the demonstration that stem cell-derived β cells showed a dynamic response to glucose. This is something that is typical of healthy β cells but is impaired in DM. The authors also demonstrated that stem cells differentiated in this way began to control DM in streptozotocin-treated mice as early as 10 days.

Recently, Yoshihara *et al*. [46], echoing their own previous work and that of other groups [39-42,47], developed a protocol consisting of five steps and using Activin A, GSK3β inh, FGF7, vitamin C, RA, TGFβ inh, BMPR inh, Hedgehog inh, PKC, T3, Alk5 inh, Notch inh, vitamin E, cAMP, and WNT4, which allowed them to differentiate pluripotent stem cells into insulin-positive and glucose-sensitive β-like cells and thus obtain human islet-like organoids (Table I). They demonstrated that these organoids are able to restore normoglycemia in immunocompetent diabetic mice and that the non-canonical WNT4 pathway is critical for metabolic maturation of β-like cells so that they release insulin in response to glucose.

Finally, in 2020, Li *et al*., on the basis of the studies of Pagliuca *et al*. [40], Rezania *et al*. [39], and Nostro *et al*. [48], generated an efficient six-step protocol using Activin A, CHIR, bFGF, KGF, vitamin C, NOGGIN, SANT1, RA, EGF, nicotinamide, RepSox, GC1, LDN, XXI, Y, R428, Trolox, and NAC (Table I) [49]. They were able to reproduce the structure of human cadaveric islets with the presence of all cell types, particularly β cells. In addition, these β-like cells, upon depolarization with KCl, were able to secrete a higher amount of insulin than the cells released in previous protocols, demonstrating the improvement of the protocol. The authors also observed that CD9 is a negative marker of β-like cells and that $CD9^-$ β-like cells showed a better response to glucose *in vitro* than $CD9^+$ β-like cells.

Table I lists the main steps characterizing the analyzed differentiation protocols.

As the above studies show, differentiation protocols have changed over the years, making it possible to obtain, from stem cells, β-like cells with characteristics similar to those of human islets. However, before they can be clinically applied in humans, these protocols require further improvements to ensure that these β-like cells really are mature, monohormonal, and able to respond to glucose.

Conclusions

Over the years several complications related to the therapeutic use of insulin have been identified, leading researchers to start to evaluate whether transplantation of both whole pancreas and islets can be considered a good therapeutic alternative. As we have seen, this route does not constitute a definitive solution. In recent years there has been considerable progress in the study of the properties of stem cells and their possible therapeutic application in DM. It is clear that there remain obstacles to overcome, including safety issues, variations in differentiation efficiency, polyhormonal cell formation, lack of maturation, low survival rate and immunogenicity, and heterogeneity of islet cells [50]. Despite these objective issues, it is undeniable that, to date, pluripotent stem cells are among the most advanced cell therapies both in terms of study and feasibility, as confirmed by the optimization of various differentiation protocols over the years. In fact, there has been a progressive improvement of protocols that, with different approaches, have managed to obtain, with increasing efficiency, insulin-positive β-like cells capable of reversing DM in mice. Further *in vivo* studies in patients are needed to understand the cellular mechanisms of β-like cells differentiated from stem cells and thus allow even better insights into the mechanisms of DM itself.

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