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Insulin - producing cells derived from stem cells: recent progress and future directions

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Abstract

Type 1 diabetes is characterized by the selective destruction of pancreatic β-cells caused by an autoimmune attack. Type 2 diabetes is a more complex pathology which, in addition to β-cell loss caused by apoptotic programs, includes β-cell dedifferentiation and peripheric insulin resistance. β-Cells are responsible for insulin production, storage and secretion in accordance to the demanding concentrations of glucose and fatty acids. The absence of insulin results in death and therefore diabetic patients require daily injections of the hormone for survival. However, they cannot avoid the appearance of secondary complications affecting the peripheral nerves as well as the eyes, kidneys and cardiovascular system. These afflictions are caused by the fact that external insulin injection does not mimic the tight control that pancreatic-derived insulin secretion exerts on the body's glycemia. Restoration of damaged β-cells by transplantation from exogenous sources or by endocrine pancreas regeneration would be ideal therapeutic options. In this context, stem cells of both embryonic and adult origin (including β-cell/islet progenitors) offer some interesting alternatives, taking into

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account the recent data indicating that these cells could be the building blocks from which insulin secreting cells could be generated *in vitro* under appropriate culture conditions. Although in many cases insulin-producing cells derived from stem cells have been shown to reverse experimentally induced diabetes in animal models, several concerns need to be solved before finding a definite medical application. These refer mainly to the obtainment of a cell population as similar as possible to pancreatic β-cells, and to the problems related with the immune compatibility and tumor formation. This review will summarize the different approaches that have been used to obtain insulin-producing cells from embryonic and adult stem cells, and the main problems that hamper the clinical applications of this technology.

Keywords: embryonic stem cells • adult stem cells • cell therapy • β-cells • diabetes

Introduction

In the year 2000, 150 million people worldwide were found to be affected by diabetes mellitus, and this number is considered to double in 2025 [1]. Diabetic patients fall mainly into two categories: type 1 and type 2 diabetes [2]. Type 1 diabetes is caused by the autoimmune destruction of the insulin producing β-cells located in the endocrine pancreas. Type 2 diabetes presents a more complex etiology that affects 95% of the diabetic patients. The pathology occurs mainly at adult ages and is often associated with genetic predisposition as well as obesity due to an unbalanced diet and a sedentary lifestyle [1, 2]. The disease progresses from insulin resistance to glucose intolerance and subsequently β-cell death by apoptotic mechanisms. As opposed to type 1 diabetes, which shows a rapid and devastating evolution despite treatment, type 2 diabetes can be delayed by specific pharmacological agents and balanced diets [2].

Restoration of insulin production by β-cell surrogates, either by whole pancreas or isolated islets of Langerhans transplantation, is a therapeutic alternative to hormone injection for diabetes treatment. The mature pancreas has two functional compartments: the exocrine portion (99%), including acinar and duct cells, implicated in nutrient digestion to facilitate absorption in the gut, and the endocrine portion (1%), including the islets of Langerhans. Islets are composed of four cell types that synthesize and secrete distinct peptidic hormones: insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), and pancreatic polypeptide (PP-cells). β-cells represent approximately 60–80% of the whole islet, forming the central core from which the other cell types are arranged.

Although the whole pancreas transplantation procedure has undergone significant progress in the past years, this treatment has to still face technical obstacles such as immune rejection, appropriate blood supply to the allograft and the risk of activating the digestive enzymes of the exocrine portion. Interestingly, islet transplantation partially overcomes some of these problems, although this technology is still far to be a successful alternative. In this sense, several obstacles remain, such as the diabetogenic effects of some immunosuppressants [3], the establishment of an appropriate immunosuppressive therapy [4], and the scarcity of human donor pancreas [5]. Recently, the clinical trial known as the Edmonton Protocol tried to solve some of these issues by introducing key important variants [6], such as intraportal infusion of a correct number of freshly isolated islets, and the use of non-diabetogenic immunosuppressive agents. Although these changes have been instrumental, recipient immune response limits implant survival to 3–5 years, indicating that improvements are still necessary [4].

Thus, novel sources of β-cells are required to solve these general aspects in order to generate accurate insulin-producing cells for transplantation trials. Several approaches have been developed to differentiate insulin-producing cells from embryonic or adult stem cells. However, insulin presence in the final cell population does not mean that the differentiation protocol has been completed. In addition to hormone production, the resulting cell has to also express functional groups of proteins that are necessary to mimic correctly β-cell function and reverse diabetes in transplanted animal models. These groups of proteins include the glucose-sensing machinery, the exocytotic apparatus and the insulin processing pathway.

The glucose-sensing machinery is responsible for the detection of extracellular glucose changes and transmits this information to the secretory and insulin biosynthetic pathways [7]. This sensing sys-

tem uses key metabolic pathways that present some special features in pancreatic β-cells. Glucose enters in the β-cell through the glucose transporter GLUT1 (humans) or GLUT2 (rodents), and is quickly metabolized by glucokinase (GK), entering the glycolytic pathway and yielding pyruvate [7, 8]. This metabolite fuels mitochondria increasing the activity of the Krebs cycle and favoring the rise of ATP levels, which immediately produces the closure of the ATP-dependent potassium channels (K_{ATP}) located on the plasma membrane. The resulting depolarization contributes to the opening of voltage-dependent L-type calcium channels and allows extracellular Ca^{2+} to enter and activate specific sensors of the secretory vesicles [9, 10].

Lastly, insulin, as many secreted proteins in eukaryotic cells, results from a complex processing pathway which starts at the rough endoplasmic reticulum (RER) and ends at the Golgi complex. Translation of insulin mRNA yields preproinsulin, which is sequentially cleaved by endoproteinases PC1 and PC2 to give proinsulin first and mature insulin + C-peptide second, before packaging into secretory vesicles. In the secretory granule, 6 insulin molecules are coordinated by a Zn atom, which is evidenced under microscopy by dithizone staining.

Although substantial progress has been made in this field over the last 6 years, the definite protocol to in vitro production of functional β-cells is still to be found. Moreover, additional problems need to be solved before finding a clinical application of this technology, mainly concerning immune rejection and tumor formation. This review will summarize the different approaches that have been used to obtain insulin-producing cells from various embryonic and adult cell sources and will discuss some medical and ethical points that will be of interest in the future.

Insulin-producing cells from embryonic stem cells

Mouse embryonic stem cells (ESCs) are isolated from the inner cell mass of the blastocyst and maintained undifferentiated *in vitro* by culture over inactivated fibroblast feeder layers or by adding leukemia inhibitory factor (LIF) to the culture medium. In addition to their high proliferative capacity, ESCs can, under appropriate culture conditions, give rise to cell derivatives of all three embryonic layers (ectoderm, mesoderm and endoderm) as well as the germ line [11, 12]. To activate the differentiation programs, ESCs are forced to aggregate into spheroid structures called embryoid bodies (EBs) by culturing in suspension and in the absence of LIF. These unique properties make ESCs of great interest as a source to obtain insulin-producing cells for diabetes treatment. In this sense, several protocols reported to produce insulin-positive cells from mouse and human ESCs.

Spontaneous differentiation to insulin-positive cells

It has been noticed that insulin expression occurs spontaneously in EBs [13], and thereby insulin-positive clones can be specifically selected using cell-gating strategies [14]. This approach was used to isolate insulin-secreting cells that were further differentiated in the outgrowth phase in the presence of low glucose concentration, nicotinamide and forming three-dimensional islet-like structures. This strategy, however, yielded few clones (8 over 784) that contained adequate insulin levels capable of reversing hyperglycemia in streptozotocin-induced diabetic mice [15]. Although the strategy needed a number of improvements, this was the first report indicating that it was possible to derive insulin-producing cells from ESCs, albeit a low rate of success. At the same time, this protocol established the instrumental role of some extracellular determinants, such as nicotinamide, normoglycemia and cell aggregates, in the *in vitro* differentiation process. Subsequent protocols have exploited some of the selection and coaxial strategies developed in this report, however, the precise origin of the cells obtained by this approach has not been investigated in detail. Further experiments showed that the resulting clones may represent a mixture of all possible insulin-positive cells that have been reported to appear during embryonic development, such as neuroectoderm-derived cells, as well as primitive and definitive endoderm, evidenced by insulin I (pancreatic marker) and II (marker of extrapancreatic insulin-positive tissues)

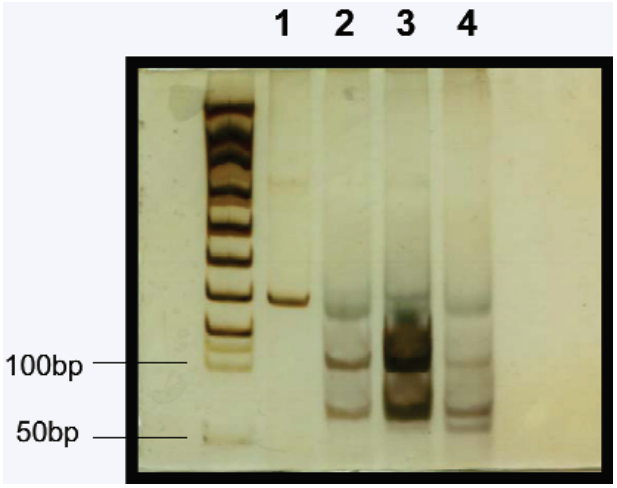

Fig. 1 Acrylamide gel showing the restriction pattern of RT-PCR amplified mouse insulin cDNA after silver staining. Digestion with MspI allows insulin I and II identification, generating fragments of 34 (not detected), 71 and 77 bp for insulin I and 76 and 112 for insulin II according to [107]. Gel samples correspond to non-digested insulin amplified from fetal brain (**1**) and MspI-digested insulin from fetal brain (**2**), yolk sac (**3**) and total pancreas (**4**). Insulin I is expressed only in pancreatic tissue, whereas insulin II is expressed in fetal brain, yolk sac and slightly in pancreas. Unpublished observations from our laboratory.

detection (Fig. 1). Altogether, this indicates that coaxial methodology by introducing new extracellular determinants and selection cassettes will help undoubtedly in the development of more precise differentiation protocols towards β-like cells.

Coaxial methodology: insulin-positive cells from nestin precursors

In this sense, a group of protocols have been developed based on the idea that the development of the pancreas shares many similarities with that of the nervous system [16, 17]. In addition, many phenotypic and functional traits between certain neurons and β-cells are very similar. Despite the similarities, it has been proven that an insulin-positive neuron displays marked differences when compared to a mature β-cell. The most important differences are the preproinsulin processing, the amount of protein produced and the physiological role exerted by the hormone itself [18]. Whereas insulin derived from the endocrine pancreas is a key factor in nutrient homeostasis, neuroectodermal insulin produced at very low amounts is considered as a growth factor in stages of nervous system development in which insulin-like growth factors are absent [19].

Despite these key differences, several *in vitro* strategies to obtain neurons have been redesigned to supposedly generate endocrine pancreatic cells from ESCs. Lumelsky *et al.* were the first to establish a protocol to obtain insulin-producing cells from ESCs through *in vitro* enrichment of nestin-positive cells [20]. Although nestin is an important marker of neuroectoderm-derived tissues, it was shown that the neurofilament protein nestin was present in rodent and human islet cells and ducts [21, 22]. The strategy included nestin-positive cell selection after EB formation, followed by culture in the presence of insulin-transferrine-selenium-fibronectin (ITSFn), expansion in the presence of basic fibroblast growth factor (bFGF/FGF-2) in N2 medium plus B27 supplement [20], and finally differentiation to insulin-positive cells by nicotinamide addition and bFGF withdrawal (Fig. 2). However, insulin-positive cells obtained in this protocol displayed low intracellular hormone content and were insufficient to correct hyperglycemia once transplanted into streptozotocin-induced diabetic mice.

On the basis of this study, subsequent protocols were developed in order to improve insulin content and secretion in bioengineered ESCs (Fig. 2). In this sense, Hori *et al.* [23] selected and expanded nestin positive cells and at the final stage added the phosphoinositide 3-kinase (PI3K) inhibitor LY294002. PI3K is a key intracellular kinase regulating cell proliferation events in many cell types. The final cells obtained by using this protocol produced higher insulin levels and displayed glucose-dependent hormone secretion *in vitro*. However, a subsequent study [24] showed that cells treated with PI3K inhibitors were not C-peptide immunoreactive, indicating that immunodetected insulin is taken in part from the culture medium, preferentially when cells enter into apoptosis.

Moritoh *et al.* [25] reported the differentiation of insulin-producing cells from an ES cell line transfected with the β-geo gene under the control of the mouse insulin II promoter. Although insulin II expression has been noticed mainly in extrapancreatic tissues, the obtained cells expressed not only

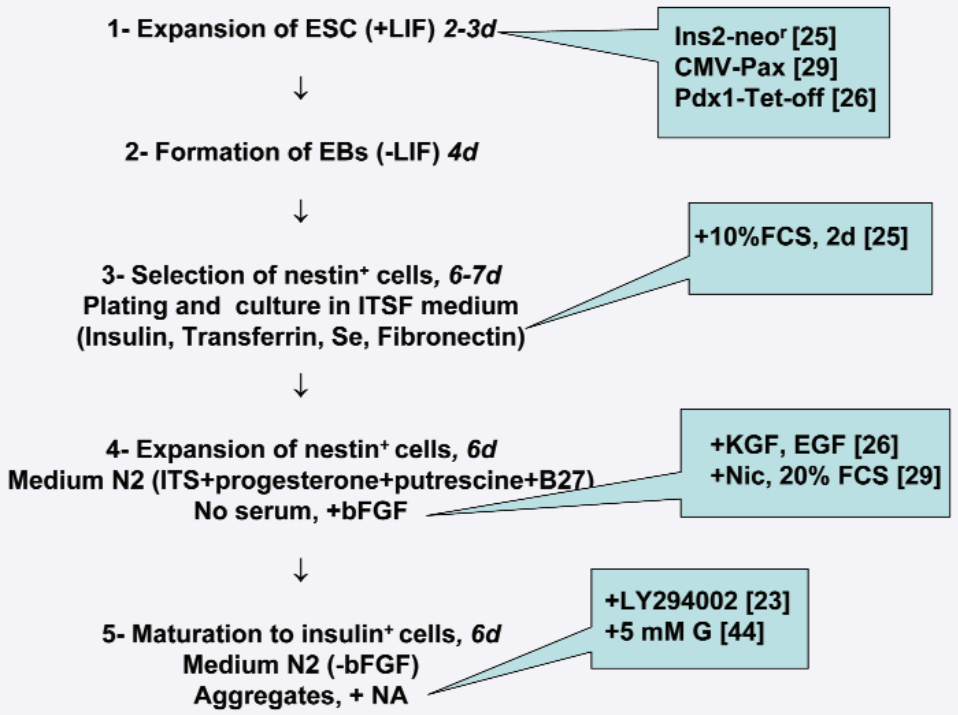

Fig. 2 Scheme comparing the different protocols based in selection of nestin-positive cells [20] and used to differentiate insulin-producing cells. See the text for more details.

the insulin II gene, but also glucagon, somatostatin, PP, p48, amylase, and carboxypeptidase A genes. This expression pattern may suggest a mixture of cell populations derived from ectoderm as well as definitive endoderm. It would be interesting to repeat this study by recombining a selection cassette in the insulin I locus.

Directional strategies applied to the nestin selection protocol

Miyazaki *et al.* [26] established a mouse ES cell line in which Pdx-1 expression was controlled by a tetracycline-switched vector. Pdx-1 is a homeodomain transcription factor which is instrumental during pancreas development [16, 27], and essential for insulin gene expression in adult β-cells [27]. Cells transfected with this construct were incubated according to the nestin protocol followed by the addition of keratinocyte and epidermal growth factors (KGF and EGF) at the expansion stage of nestin positive cells. The resulting cells displayed increased levels of insulin II expression and were immune-positive for C-peptide. However, the amount of insulin secreted was unable to re-establish euglycemia in transplanted streptozotocin-induced diabetic mice. These results could be explained in part because Pdx-1 is also expressed during neuronal development [28], suggesting that the strategy isolates as well a population of neuroectodermal precursors. In addition, this transcription factor seems to be insufficient to direct by itself this complex differentiation process.

In another strategy, ESCs expressing constitutively Pax4 (CMV-Pax4) and incubated according to the nestin expansion-selection protocol gave rise to aggregates containing cells positive for insulin, Isl-1, Ngn3, islet amyloid polypeptide, and the glucose transporter GLUT-2 [29]. Transplantation of these cells into the spleen restored normal blood glucose levels in diabetic mice. Surprisingly, transplantation of wild type ESCs also resulted in normoglycemic recovery, compared with non transplanted controls. This result may be because the wild type cells were already expressing specific β-cell transcription factors in the undifferentiated stage, and once transplanted they most likely underwent uncharacterized differentiation processes that lead to insulin gene expression. This report also demonstrated that the same strategy used for Pdx-1 (CMV-Pdx-1) did not result in insulin-positive cells as opposed to [26]. This may be due to the high expression levels of Pdx-1 obtained in this protocol, which were not following the appropriate pattern of expression that has been described during pancreas development.

Table 1 Positive characteristics that can be exploited (**a**) and aspects to improve (**b**) in insulin-secreting cells derived from neuroectodermal precursors

a) Positive characteristics	**References**
1 - Common set of transcription factors	[16, 95]
2 - Nutrient-sensing machinery	[8, 63, 96]
3 - Ion channels and Ca^{2+} responses	[97, 98]
4 - Response to non-nutrient secretagogues	[99–101]
5 - Components of the secretory pathway	[102–104]
b) Aspects to improve	
1 - Insulin gene regulation by glucose	[19, 105]
2 - Correct processing of proinsulin	[19, 106]
3 - Amount of insulin produced	[19]

All these coaxial/directional protocols based on expansion and selection of nestin-positive cells as precursors of insulin-producing cells need a number of improvements to be viable strategies. One of them is the low yield of endogenous insulin production and, as mentioned before, the insulin uptake when cells enter apoptosis. Insulin mRNA detection together with C-peptide immunostaining are solid evidences of intracellular insulin biosynthesis. In addition, the molecular ratio between insulin and C-peptide determined by radioimmunoassay has to be close to 1. A second key problem is the lineage from which insulin-producing cells derive. Many reports indicate [18, 30] that the main producers of insulin in the cultures are neuro-ectodermal precursors. In this sense, ESCs were committed to neuroectoderm by inserting the β-geo cassette into the Sox2 locus [31], a marker of neuroepithelial progenitors. Sox2-derived cells were differentiated into insulin-positive cells following the nestin selection protocol [24]. Other protocols have also demonstrated the possibility of deriving insulin-positive cells from neuronal precursors [32].

Alternative strategies to obtain insulin-producing cells

Novel strategies have been developed to balance the problems and advantages posed by neuroectodermal precursors (Table 1). In this context, neural commitment can be restricted by eliminating the use of ITSFn and bFGF during the selection and expansion of nestin positive cells [33]. This allowed the production of ecto-, meso- and endodermal precursors after EB formation that was followed by pancreatic differentiation using serum-free medium containing nicotinamide and laminin. The final cells obtained were positive for insulin, C-peptide and cytokeratin 19 (marker of pancreatic ductal epithelium), and displayed glucose-stimulated insulin secretion. Nestin expression was detected transiently at intermediate stages, and was completely absent in the final differentiated insulin-positive cells. Taking into account the gene expression profile and some functional properties, the authors concluded a close similarity of these cells to immature β-cells [34].

Another possible approach is to obtain islet precursors that could undergo maturation either *in vitro* or *in vivo* [35]. To this end, gating selection was performed by transfecting D3-ESCs with a construct containing the Nkx6.1 promoter driving the expression of a neomycin-resistance gene. The Nkx6.1 gene is detected in mice pancreatic precursors and its expression is restricted to the β-cell lineage after embryonic day 13 (e13). In addition to this selection strategy, cells were incubated at low serum concentrations (3%) and in the presence of different factors that include anti-sonic hedgehog, nicotinamide or co-culture with embryonic pancreatic buds from e17.5 fetal mice. In this last strategy, it was hypothesized that soluble factors secreted by the forming islets could drive the differentiation of committed ESCs. This protocol yielded a pure pop-

ulation of insulin-positive cells, which additionally expressed β-cell genes such as Pdx-1, Nkx6.1, glucokinase, GLUT-2 and Sur-1. Although the insulin content was low, transplantation in the kidney capsule of streptozotocin-induced diabetic mice normalized glycemia, suggesting that the implanted cells underwent *in vivo* maturation processes that need to be further characterized. A similar protocol, but using a selection cassette with the 1 Kb proximal human insulin promoter, gave rise to cells expressing consistent amounts of the hormone [36]. Selection was performed by adding 2.3 mg/ml G418 to the culture medium in order to select the most productive insulin-positive clones. Nevertheless, this high amount of added antibiotic may allow the selection of clones that contain several copies of the selection transgene, since, as we have demonstrated previously, a transgene is not regulated exactly as an endogenous gene. Furthermore, the amount of insulin released at 22 mM glucose was more than the 20% of the total insulin content, suggesting a mechanism of degranulation far from the regulated secretion that has been described in mature β-cells. This phenomenon could at long term compromise intracellular insulin storage, as we have noticed in our laboratory in very late passages of insulinoma cell lines (*i.e.* INS-1 cells). Further analyses are required to better characterize this particular cellular event.

Other protocols introduced new compounds at different stages and new strategies to differentiate ESCs to insulin-producing cells. In this sense, insulin-positive cells were obtained with a high efficiency rate by culturing EBs in the presence of monothioglycerol and serum-free conditions [37] and adding activinβ-B, nicotinamide and exendin-4 (a GLP-1 mimetic peptide) in the last phase of the culture. The differentiation capability of exendin-4 and GLP-1 (glucagon-like peptide-1) have been previously demonstrated in other protocols, where mouse and primate (rhesus) embryonic stem cells were differentiated into insulin-producing cells [22, 38, 39].

Recent evidence confers a role of retinoic acid (RA) signaling in pancreas differentiation from embryonic endoderm [40]. Based on this idea, incubating EBs with RA from day 4th favored the commitment to endoderm precursors [41], based on the expression pattern of early endoderm markers. However, there was no insulin expression, suggesting that additional maturation steps are necessary. Interestingly, the combination of RA with activin A in a simple three-step protocol allowed the development of insulin-positive cells [42]. Insulin release of these derived cells seems to be regulated by extracellular glucose concentration, and when transplanted to streptozotocin-induced diabetic mice, their glycemias were restored. However, incubation of EBs with RA from day 1st in protocols designed to obtain insulin-producing cells did not enhance insulin expression [43]. At this time of culture, EBs express preferentially ectoderm markers and it is very likely that RA is favoring ectodermal differentiation under these particular culture conditions [31, 43]. Endoderm commitment seems to occur late in EBs (around day 5–7) and this should be in theory the most appropriate period of time for RA addition [41].

Lastly, the legal restrictions imposed to use human ESCs in some countries have limited the amount of data generated with these cells. In this sense, insulin-producing cells were obtained from human ESCs using the nestin selection protocols [44] with certain modifications, such as bFGF withdrawal and lowering glucose concentration at the last stage. Immunodetection analysis revealed cells co-expressing insulin and glucagon, suggesting that the final cell product could correspond to immature islet precursors.

All protocols strongly demonstrate that ESCs have the ability to express insulin but the current methodology still needs key improvements. On one hand, gene expression must determine the exact origin of the insulin-positive cells obtained, *i.e.* ectoderm or endoderm. More functional tests should be introduced as routine test to further characterize the final cell product. These could include time-course and dose-dependent insulin secretion in response to different secretagogues, the study of electrical activity in membrane-specific channels, as well as intracellular Ca^{2+} oscillation patterns. Also, insulin staining alone can overestimate the number of differentiated insulin-positive cells. Complementary methods have to be adopted in order to ascertain precisely the number of cells that produce insulin *de novo*. These include insulin mRNA amplification by quantitative RT-PCR, C-peptide immunostaining, and secretory vesicle detection by electron microscopy. Finally, transplantation in diabetic animal models has to demonstrate a rescue from the pathology, ideally in the absence of immune rejection and tumor formation.

Insulin-producing cells from adult stem cells

Adult stem cells (ASCs) found within tissues of the adult organism could serve as an alternative to ESCs for the generation of insulin-producing cells. Although they possess a limited proliferation potential as well as commitment to specific cell fates, ASCs offer the advantage of autologous transplantation circumventing thereby the immune rejection dilemma. Recent data support the plasticity of these cells to differentiate to alternative cell fates beyond those derived from their natural body niches. This means that a broad spectrum of ASCs could be differentiated *in vitro* or *in vivo* to insulin-producing cells.

Insulin-producing cells from ectoderm precursors

As mentioned before, pancreatic β-cells of endodermal origin share many common features with ectoderm-derived neurons, including transcription factors, biosynthetic enzymes, as well as proteins of the secretory pathway and metabolism (Table 1). Although adult β-cells are phenotypically and functionally different from neurons, some common molecular mechanisms could be remodeled to bioengineer neuronal precursors to insulin-producing cells [32, 45]. Indeed, hypothalamic neurons display the ability to express insulin II gene, although molecular modifications will be required to increase the amount of insulin produced and to achieve correct pro-hormone processing. In this context, it has been reported that cultured neuronal stem cells can generate insulin-producing surrogates, expressing phenotypical markers and displaying functional responses typical of pancreatic β-cells [32, 45].

Although nestin expression has been considered a neuroectoderm marker, it has been previously proposed that nestin-positive cells in the adult pancreas could be endocrine precursors. However, recently transgenic mouse technology has evidenced that nestin is expressed in endothelial cells of islet vasculature [46]. Mesenchymal cells derived from islets *in vitro* have the ability of expressing nestin as well [47, 48]. It has also been shown that the replicating cells in expanded adult islets were mostly endocrine, displaying transient nestin expression and rapid de-differentiation in non-defined cell culture medium. The addition of LIF, bFGF and bone morphogenic protein-4 (BMP-4) in serum-free conditions maintained the pancreatic-derived progenitors for long periods of incubation [49]. Altogether, the data seem to indicate that nestin expression is not limited exclusively to ectodermal-derived tissues and could be a candidate marker for islet precursors.

Insulin-producing cells from mesoderm precursors: bone marrow and peripheral blood cells

The broad differentiation potential exhibited by bone marrow progenitors opens the possibility to generate insulin-producing cells. Many laboratories have explored this question, but with contradictory results. Ianus *et al.* [50] transfected bone marrow stem cells with a construct in which the insulin promoter drove the expression of enhanced green fluorescence protein (EGFP). The resulting cells were transplanted into irradiated recipient mice and 6 weeks later, EGFP-positive cells were detected in pancreatic islets, contributing to a 3% of the total cell number in this tissue. The authors supported a transdifferentiation mechanism to explain these results. In addition, sorted cells isolated from these pancreata displayed glucose- and incretin-stimulated insulin release. These results, however, have not been successfully reproduced in other laboratories, claiming that rare cell fusion events were most likely the explanation to these findings [51, 52]. Complementary information was provided by experiments from Hess *et al.* by using bone marrow cells expressing c-kit [53]. In this study, hyperglycemia amelioration in transplanted animals was accompanied by a very low contribution of donor-derived insulin-positive cells to recipient pancreata. The authors claimed that the transplanted bone marrow cells most likely stimulated endogenous pancreatic tissue regeneration rather than contribute directly to β-cell neogenesis. A similar mechanism was also suggested in subsequent studies [54], in which transplantation of wild-type bone marrow cells restored normoglycemia in E2f1-/-E2f2-/- double-mutant mice. In support of this hypothesis, multiple bone transplantations by regular injections over a 30

day period allowed normoglycemia recovery in diabetic mouse models [55]. In this sense, bone marrow-derived endothelial progenitor cells migrated to the pancreatic tissue in response to islet injury and stimulated neovascularization in order to improve the survival of the remaining β-cells [56]. Altogether, these data suggest that neogenesis of injured endogenous β-cell depends on restored hematopoiesis and/or formation of new vasculature by bone marrow-derived endothelial cells.

Mesenchymal stem cells isolated from bone marrow according to their adherent properties, or from adipose tissue can be alternative sources for differentiation towards insulin-positive cells [57–59]. In this sense, differentiation of rat marrow mesenchymal stem cells into insulin-secreting cells has been reported [57]. Interestingly, nestin was expressed in an intermediate stage of the differentiation process. In another study, human bone marrow mesenchymal stem cells infected with recombinant adenovirus coding for 3 specific early β-cell transcription factors (Foxa2, Hb9 and Pdx1) and co-incubated with islet tissue or islet-conditioned medium resulted in the differentiation to insulin-producing cells [59].

Human monocytes isolated from peripheral blood can be reprogrammed to endoderm precursors by exposure to interleukin-3 and macrophage-colony stimulating factor, and further differentiated to insulin-producing cells by the addition of EGF, hepatocyte growth factor (HGF) and nicotinamide [60]. Transplantation of resulting cells under the kidney capsule of experimental-diabetic mice led to restoration of normoglycaemia over a short period of time before immunological implant rejection. Although promising, the circulating pluripotential cell has not been identified, the C-peptide content is very low and, for unknown reasons this protocol seems to work only in 2/3 of the blood samples obtained. In any case, more solid animal models need to be developed in order to test the long term potential of these cells in correcting hyperglycemia.

Insulin-producing cells from endoderm precursors: intestine, liver and pancreas

During embryonic development, the pancreas initially is separated into two independent buds, the dorsal and ventral buds, which eventually fuse. The ventral primordium develops from the endoderm of the hepatic diverticulum, whereas the dorsal primordium derives from the duodenum. When the fusion process occurs, the ventral anlage will result in the head of the pancreas and the dorsal in the tail [61]. Thus, the pancreas, gut and fetal liver share a common embryonic origin, and most likely precursor cells of these organs share many phenotypical and functional traits that make them interesting candidates to generate insulin-secreting cells. This includes the possibility of pancreas/islet regeneration or identification/isolation of islet stem cells.

In this context, some laboratories have considered inducing gut stem cells to differentiate into β-cells. The intestinal epithelium contains active stem cells located in the crypts that allow gut renewal each 28–40 hrs. Among the 4 cell types present in the differentiated gut epithelium, the GLP-1 secreting L-cells have an endocrine phenotype, expressing molecules that are involved in glucose sensing and regulated secretion similar to β-cells [62, 63]. Therefore, these cells could be candidates that through minimal engineering can become β-cell surrogates. Intestinal crypts can be isolated from rodents and humans from a biopsy and cultured under specific conditions (Fig. 3). Cells derived from these cultures are capable of expressing insulin after transfection with Pdx-1 and exposed to betacellulin. Similar results have been obtained after a double transfection with the transcription factors Pdx-1 and Isl1 [64, 65]. Although the resulting cells displayed several pancreatic β-cell markers, they were unable to secrete insulin in a glucose-regulated manner.

Concerning the hepatic tissue, several reports indicate that the delivery of β-cell-specific transcription factors, such as Pdx-1 or Beta2/NeuroD, by helper-dependent adenoviral vectors resulted in insulin production [66, 67]. However, vectors with Pdx-1 resulted in high rates of hepatoxicity which was not due to the residual infective potential of the viral vector itself. Instead, it seemed that Pdx-1 expression was more likely implicated in the development of these hepatic alterations through the induction of differentiating exocrine tissue [67]. The presence of exocrine proteases, such as trypsin, could cause the self-destruction of hepatic cells, affecting at the same time newly-formed hepatic insulin-producing cells. These complications were not observed when cells were reprogrammed using NeuroD delivering vectors [67].

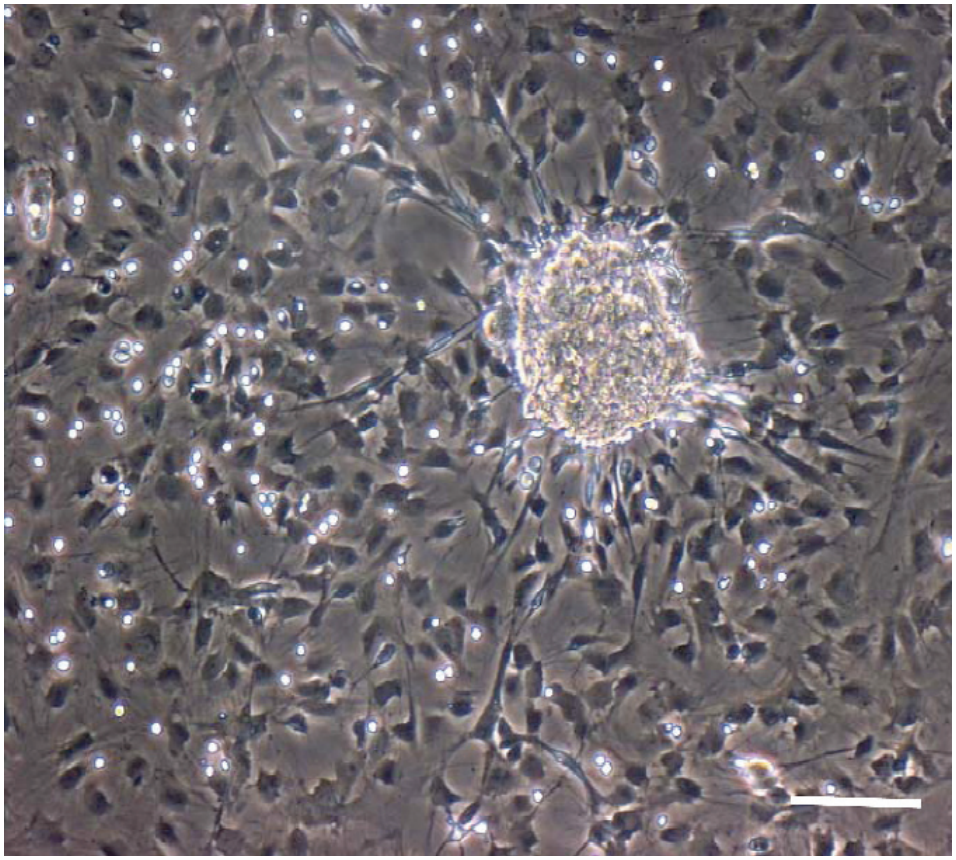

Fig. 3 Transmission image of a representative intestinal crypt isolated from a human donor and cultured in our laboratory. Images were captured at a magnification of X 20. Bar: 100 µm. Unpublished observation from our laboratory.

On the other hand, pancreatic tissue has been extensively studied in order to find regeneration pathways as well as precursors that could be managed *in vivo* and *in vitro* for tissue repair. In this context, several mechanisms have been proposed: (**a**) neogenesis from pancreatic ductal/islet stem cells, (**b**) replication of existing β-cells and (**c**) transdifferentiation of pancreatic exocrine cells/precursors. In this context, pancreas, as opposed to liver, does not present an apparent replicating or regenerating activity. Furthermore, markers indicating pancreatic cell divisions or the existence of cell precursors are still elusive. It seems obvious that a pool of β-cells may exist, because it is quite unlikely that insulin production in humans solely relies on the β-cells we were born with. The identification of this pool, which does not necessarily need to be the same as in rodents, is a very active area of scientific research.

Certain studies suggest that duct epithelium is a candidate niche for islet progenitors in the adult pancreas, and islet-like structures have been obtained from both mouse and human ducts [68, 69]. This would open interesting applications for duct cells isolated from cadaveric donors as a source for β-cell surrogates, although the low proliferation rates and insulin produced are important limiting factors. On the other hand, the presence of β-cell precursors outside of the ducts has been a more questioned matter. Lineage tracing for insulin positive cells has demonstrated that new β-cells in vivo derived from the replication of pre-existing β-cells, questioning the existence of an operating pool of pancreatic progenitors [70]. Aside from technical problems (*i.e.* Cre leakage), the experimental design of this study did not address the participation of other mechanisms that could contribute to β-cell neogenesis, such as reversable epithelial-to-mesenchymal transitions generated from pre-existing β-cells, which has been documented *in vitro* [71]. In addition, recent results from Susan Bonner-Weir´s laboratory have estimated that in 20–31 days-old rats, 30% of newly formed β-cells did not derive from pre-existing β-cells [72]. Also, positive bromodeoxyuridine incorporation was first detected in ductal cells after severe pancreatectomy. The controversy of all these studies could reside in the observation that *in vivo* β-cell renewal is difficult to ascertain *in vivo*, most likely due to the fact that the putative pancreatic stem cell population

displays a very limited proliferative or turnover capacity under normal conditions. Furthermore, this population is most likely not unique, where possibly different cell types including ductal cells, acinar cells and pre-existing β-cells, could work as progenitors of definitive β-cells. The identification of signals that maintain the population/s in a low replicative state could be extremely interesting in order to design pharmacological agents that could stimulate controlled islet divisions in pre-diabetic individuals.

In type 1 diabetic subjects, strategies for endocrine pancreas regeneration should be balanced with the extensive rate of β-cell death that occurs by designing drugs capable of inhibiting apoptosis and/or immune destruction [73]. In this context, diabetes was reverted in NOD mice by injection of complete Freund's adjuvant and allogenic splenocytes, resulting in new islet formation [74]. The adjuvant administration seemed to be involved in eliminating anti-islet autoimmunity. However, islet neogenesis did not occur in the injected spleen cells, but were rather of host origin, indicating the presence of pancreatic progenitors that can regenerate the β-cell population and restore euglycemia [75–77]. In this pathology, the rate of β-cell destruction is such that severely impairs the restoration of an adequate β-cell mass. Therefore, and as recently shown, the combination of pharmacological agents that interfere with autoimmunity (*i.e.* lisofylline), along with others that favour β-cell self-renewal (*i.e.* exendin-4), could allow diabetes reversal and re-establish normoglycemia in NOD mice for almost 5 months [78]. This combined therapy could also be very effective in prolonging the survival of transplanted islets.

In this context, the isolation and characterization of pancreas-derived multipotential precursor cells (PMPs) has been described [79]. PMPs isolated from islet and ductal tissues are present in a small proportion in the pancreas (around 0.03%), but can proliferate *in vitro* forming characteristic colonies. PMPs express neuronal and pancreatic precursor markers and display a wide differentiation potential, generating neurons, endocrine pancreatic cells (α-, β- and δ-cells), stellate and exocrine acinar cells. Interestingly, the *de novo* generated β-like cells contain insulin (30% of the amount estimated in mature β-cells) that can be secreted in response to extracellular concentrations of glucose. On the other hand, the surprising capability of PMPs to generate neuronal precursors could be explained by the coincident set of transcription factors, present both in neurons and endocrine pancreas, which upon activation under specific conditions might lead to the appearance of certain functional and phenotypic traits of these unrelated tissues. Therefore, and as mentioned before, this precursor cell population seems to remain quiescent in the adult tissue, but when cultured *in vitro* and possibly in the absence of these uncharacterized molecular "brakes", is capable of generating either neuronal or endocrine pancreatic precursors depending on the culture conditions.

Finally, *in vitro* generation of insulin-producing cells from exocrine pancreatic cells has been reported as another approximation. To this end, exocrine derived cultures, treated with alloxan in order to discard the presence of proliferating β-cells, were incubated in the presence of EGF and LIF. As a result, the obtained cells were functional in terms of insulin secretion in response to glucose. This finding opens interesting possibilities to generate β-cell surrogates from exocrine tissue of cadaveric pancreata. Nevertheless, it remains to be established whether parental cells that give rise to insulin-positive cells are deriving from transdifferentiation mechanisms or if a pool of undifferentiated cells located within the exocrine tissue exists [80].

Altogether, these reports underline the fact that β-cell progenitors or the β-cells themselves can produce new β-cells. Therefore, future research has to be focused in an improved characterization of the candidate progenitor pancreatic population and in finding the molecular factors that modulate replication and differentiation of these cells.

Medical and ethical concerns

Medical concerns: tumor formation

The potent self-renewal capacity of stem cells, particularly ESCs, poses the risk of tumor formation after transplantation due to the presence of undifferentiated cells remaining in the implant after differentiation processes. In fact, we have observed that pluripotential markers, such as Oct3/4, Nanog and Esg-1 are not completely down-regulated after EB formation, even at long-term incubations. We have

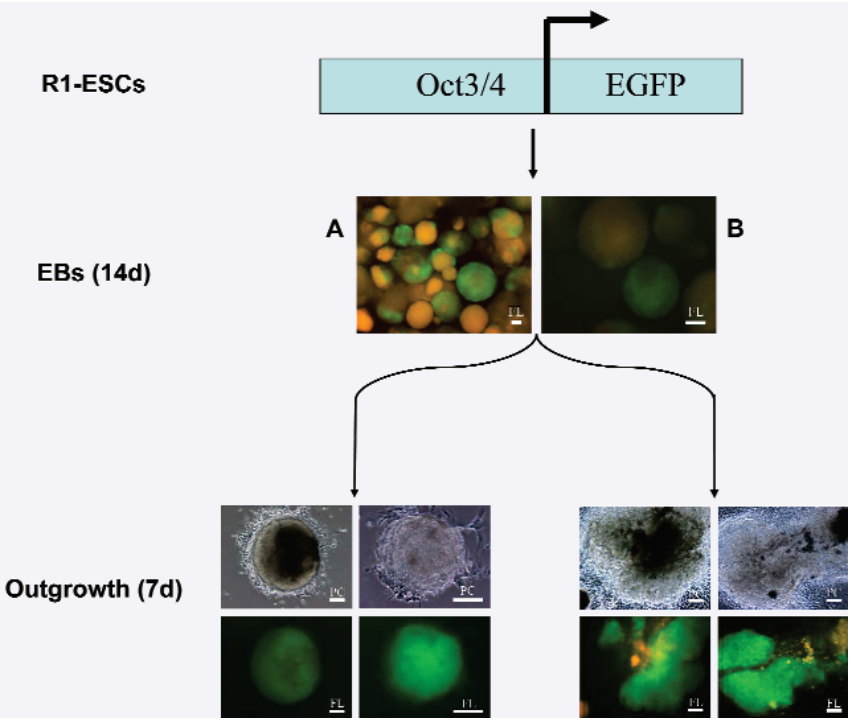

Fig. 4 Scheme showing the tracing of residual undifferentiated cells during *in vitro* differentiation protocols. R1-ESCs were transfected with a construct in which EGFP expression was under the control of Oct3/4 promoter. Cells were allowed to differentiated by EB formation for 14 d and then by plating in adherent conditions (outgrowth) for 7 d. Figure shows phase contrast (PC) and fluoresecence (FL) images of EBs and plated cells derived from different ESCs cultures (**A** and **B**). Note the areas that exhibit green fluoresecence, likely indicating residual undifferentiated cells. The orange areas correspond to cells displaying autofluorescence and likely committed to particular differentiation pathways. See reference [81] for more details. Bar: 100 µm.

traced these residual undifferentiated cells by transfecting mouse R1 and D3-ESCs with a construct containing the Oct3/4 promoter directing the EGFP expression (Fig. 4). After 30 days of culture in EBs, we have isolated a fluorescent cell population representing 15–20% of the total cell mass of the EB [81]. This cell population has interesting functional and phenotypic traits that allow its characterization.

These cells are expressing pluripotential markers as well as some markers found in the germ line. In addition, they display karyotypical alterations that have been reported as well in long-term cultures of human ESCs [82]. The recurrent gain of specific chromosomes (trisomy of chromosomes 8 and 9 in the cell line isolated in our laboratory) could explain the particular replicating behavior of these cells, most likely due to additional or unwanted expression of specific cell cycle genes located in these chromosomes. In addition, these cells can proliferate in the absence of LIF and can generate teratomas in immune-deficient mice after 3 months of transplantation. This is a key point, since many published protocols are claiming absence of tumor formation, although the implantation duration in recipient animals does not seem to be long enough to observe this phenomenon. High rates of BrdU incorporation should alert scientists that the final cell obtained could be closer to a tumoral cell line rather than cells designed for therapeutical purposes [36].

In the search for additional markers of these proliferative cells, we have observed increased expression of histone H2AX and a unique pattern of c-Myc phosphorylation (Fig. 5). Histone H2AX is involved, in association with other factors, in repairing DNA breaks. Therefore, its expression increases in cells undergoing a high proliferation rate and/or chromosomal rearrangements, such as lymphocytes and B-cell precursors [83, 84]. On the other hand, c-Myc is a transcription factor involved in the control of cell cycle events, such as proliferation, differentiation and apoptosis in many cell types, including β-cells [85–87]. Protein overexpression and de-regulation leads to the progression towards different types of cancer [85]. The stability of this transcription factor is controlled by its phosphorylation in specific residues (threonine-58 and serine-62) at the N-terminal region, determining or not its degradation by the ubiquitin-proteasome pathway [88]. We have observed changes in the phosphorylation pattern of c-Myc in these proliferative cells, but how this observation is related to the teratogenic potential of these cells is currently under investigation in our laboratory. In any case, these results need to be further analyzed since this onco-protein is expressed in both ESCs and ASCs,

where it seems to exert a crucial role in the control of their expansion *in vitro* [89] At the same time, c-Myc appears to be induced during the spontaneous transformation of human mesenchymal cells after long-term in vitro cultures [90]. Although not tested in our laboratory, we can hypothesize that telomerase activity should remain elevated in these cells, increasing thereby the list of markers that allow to better identify the presence of teratogenic residual cells in differentiation protocols.

Ethical concerns: ethical conduct for stem cell research

Stem cell research is certain to progress in basic science and to have key implications in medicine by investigating pathological mechanisms, allowing the design of new therapeutic drugs and providing functional cells for replacement trials, such as bioengineered insulin-secreting cells. These goals are well accepted by the majority of the scientific community. However, cultural, religious and legal particularities across different countries have questioned the necessity of this research, in particular for ESCs since they derive from a zygote that encloses the potential to generate a new human being. In this context, an international forum has to be opened to formulate guidelines that articulate uniform research practice respecting at the same time ethical and religious principles, and defining in this manner what is permissible and non-permissible research. These well-established regulations that have been articulated in international documents such as the International Ethical Guidelines for Biomedical Research Involving Human Subjects (2002) and the UNESCO Universal Declaration of Bioethics and Human Rights (2005), must adhere to transparent practices in performing and sharing results between laboratories and legal, religious and social institutions.

To this end, it is vital that stem cell research has to respect the laws of the country where it takes place. At the same time, clarity has to be a general principle for the open exchange of ideas and materials, in particular concerning human stem cells. International congress and bio-banks should facilitate clean and standard procedures to potentiate scientific collaborations. Along this line, national and international institutions have to coordinate efforts

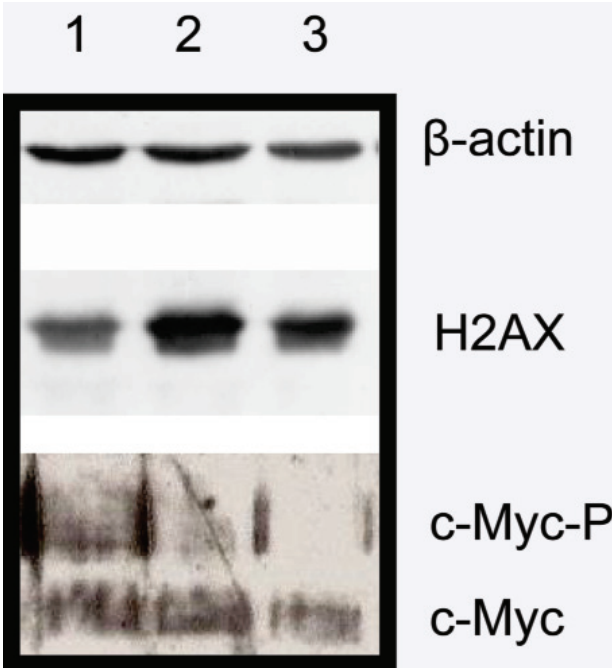

Fig. 5 Western blots showing the protein levels of histone H2AX, phosphorylated (c-Myc-P) and dephosphorylated c-Myc, and β-actin (un-variant control). (**1**) Mouse R1-ESCs transfected with the Oct3/4-EGFP construct, (**2**) Sorted Oct3/4-EGFP$^+$ cells (residual undifferentiated cells) from EBs after 30 d of culture, (**3**) Oct3/4-EGFP$^+$ cells isolated from 3 month teratomas after injection of these residual undifferentiated cells in animals. See the text for more details. Unpublished observations from our laboratory.

in order to rigorously review that the up-going research is accomplishing all scientific and ethical concerns. On the other hand, scientists must integrate a relevant expertise in the field together with an ethical behaviour in order to assure that the research responds to the principle of clarity within an ethical context.

Altogether, it is necessary to generate a consensus in managing the different investigations, which is not only a task for senior scientists or grant applicants, but also for Journal editors and political, religious and social representatives. In this sense, science and ethics can conciliate divergent points by creating a framework based on respect, clarity and relevant scientific proposals. This will generate a very positive athmosphere, not only for obtaining insulin-secreting cells from stem cells, but for all the research performed worldwide in which these cells are implicated.

Table 2 Criteria that human ESCs or ASCs-derived cells have to fulfill in order to obtain an effective cell therapy in diabetes

1 - Generate human ESC lines free from animal feeder layers and animal serum.
2 - Establish a list of gene markers and functional tests that identify properly the parental human ESCs and ASCs.
3 - Assess karyotypic stability in both human ESCs and ASCs.
4 - Possibility to generate large-scale cultures of human ESCs and ASCs.
5 - Differentiate human ESCs or ASCs towards the correct functional phenotype in the absence of immune rejection and teratome formation.
6 - Use appropriate extracellular components (extracellular matrix, encapsulation, etc) in order to obtain three-dimensional cell structures that facilitate integration of the implant in the recipient tissue.
7 - Solve all ethical, religious, and legal issues concerning the correct use of ESCs and ASCs in human cell therapy trials.

Concloding remarks

The main goal of tissue bioengineering for the treatment of diabetes is to obtain islet/β-cell surrogates that offer key improvements *versus* conventional therapies, such as insulin injections, insulin pumps and islets/pancreas heterologous transplantation. Studies in animal models have demonstrated the potential of ESCs and ASCs to treat not only diabetes, but many other degenerative disorders [91–94]. However, the transfer of this technology to human ESCs and ASCs has to fulfill a number of requirements in order to achieve an effective tissue repair (Table 2).

The main obstacle that needs to be resolved in a near future concerns the differentiation of a functional cell type displaying the ability of rescuing the organism from a diabetic pathology. Teratome formation and immune rejection are also main problems to take in account. To date, published protocols to obtain insulin-positive cells derived from ESCs have not yielded a fully differentiated cell or a 100% pure population. Therefore, new protocols to purify cell populations that can mimic β-cell function with no risk of teratome formation or immune rejection are required. In conclusion, the ASCs and ESCs potential in treating diabetes seems to be very promising. Nevertheless, it should be realized that we are still far from an applicable cell therapy in regular clinical trials. To this goal, we need to design new protocols respectful with ethical principles, and know much more about the basic biology of ESCs as well as ASCs.

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