



Beyond Islet Transplantation in Diabetes Cell Therapy: From Embryonic Stem Cells to Transdifferentiation of Adult Cells

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ABSTRACT

Exogenous insulin is, at the moment, the therapy of choice of diabetes, but does not allow tight regulation of glucose leading to long-term complications. Recently, pancreatic islet transplantation to reconstitute insulin-producing β cells, has emerged as an alternative promising therapeutic approach. Unfortunately, the number of donor islets is too low compared with the high number of patients needing a transplantation leading to a search for renewable sources of high-quality β -cells. This review, summarizes more recent promising approaches to the generation of new β -cells from embryonic stem cells for transdifferentiation of adult cells, particularly a critical examination of the seminal work by Lumelsky et al.

THE GOAL OF therapy of type 1 diabetes (DM-1) is to restore a glycometabolic picture as close as possible to normal. Because the cause of DM-1 is failure to produce insulin owing to destruction of β -pancreatic cells, the therapy is replacement of lost endocrine function. Since 1921, when Nicolae Constantin Paulescu, was able to cure diabetes with insulin, which he called Pancreina, most patients undergo replacement therapy with exogenous insulin. There has been a search for a permanent diabetes ‘cure’ that replaces β -pancreatic cells, freeing patients from insulin requirements.

The radical solution is whole pancreas transplantation, a surgical procedure developed in the last 10 years. It has success rates comparable to those of other organ transplantations: 80% of patients achieve and maintained insulin independence for >6 to 8 years.¹ However, pancreas transplantation is a challenging procedure, requires a significant immunosuppressive therapy to prevent organ rejection,^{2,3} and therefore, is performed only in combination with a kidney transplantation in diabetic dialysis patients with end-stage renal disease.⁴ Unfortunately, this intervention is not without complications.⁵ Also, xenotransplantation has received attention in the past, but implies serious problems of health surveillance.^{6,7} In the last few years islet transplantation has proffered an alternative promising therapy. Recently, experimental approaches using stem cell administration, transdifferentiation of ductal cells, or genetic reprogramming seem the future of diabetes therapy.

PANCREATIC ISLET TRANSPLANTATION

General Considerations

The islets of Langerhans are clusters of endocrine cells that constitute about 1% of pancreatic tissue where they sense blood glucose levels, secreting hormones to regulate them. Pancreatic islets for transplantation have been prepared by an isolation procedure, developed by Ricordi, which combines mechanical disruption and enzymatic digestion, followed by purification through gradients of varying density (Ricordi Chamber) (Table 1).⁸

The possibility to culture pancreatic islets offers a range of therapeutic opportunities to improve efficiency and graft survival. This procedure, which is much simpler than vascularized pancreas transplantation, is associated with a low morbidity and can be repeated to infuse additional islet doses to achieve adequate metabolic control and insulin independence.

The first islet transplantations performed in immunosuppressed patients with DM-1 were characterized by a low

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Table 1. Procedure for Isolation of Pancreatic Islets

Pancreatic islet cells are obtained from multi-organ donors after separation from the tissue surrounding the gland.

The Wirsung duct is cannulated to allow injection of a solution containing collagenase in order to relax the organ and enable the achievement of an effective enzyme activity during the dissociation.

The gland is then divided into several parts and transferred to the dissociation chamber, consisting of a lower portion, where the pancreas is inserted together with metal balls, and an upper portion consisting of an inverted funnel, separated by a porous metal filter with a specific porosity.

The digestion process is the combination of the enzymatic effect (obtained by increasing the temperature) and mechanical (generated by the action of the ball during the stirring of the room) that results in the release of fragments of the pancreas that can pass through the filter metal due to the unidirectional flow of liquid dissociation.

The digested pancreas is then purified by using different density gradients (continuous and discontinuous) to enrich the endocrine component.

The islets that make up only 1% of pancreatic tissue can be viewed using a dye that gives it a distinctive red color.

After the purification, enriched fractions containing levels of purity can be obtained.

success rate, both in terms of insulin independence and recovery of C-peptide secretion, owing to the inconsistent quality of the transplanted islets and the inadequate immunosuppression.⁹ The revival of this procedure occurred with the development of an innovative immunosuppressive scheme, the 'Edmonton Protocol', which was applied in selected centers worldwide to reproducibly obtain high-quality islets.^{10,11} Although initial results were excellent with a 1-year success rate of almost 100%, continued experience revealed them to decrease dramatically at 2 to 3 years. However the extraordinary initial data stimulated research in the field of β -cell replacement.

STEM CELLS: A SOURCE FOR NEW β -CELLS Pancreatic Stem Cells

The postnatal pancreatic duct may harbor islet precursor/stem cells because of specific cellular migration during embryonic development.¹² Islet neogenesis, the generation of new islets from pancreatic stem/progenitor cells located in the ducts, could be an active process in the postnatal pancreas. Interestingly insulin-producing cells have been generated from adult pancreatic ductal tissues *in vitro*.^{13–18} Fluorescent activated cell sorting, which characterizes human β -cells through detection of CD95,^{19,20} may be a promising technique to isolate multipotent pancreatic progenitors from neonatal and adult pancreata. By combining flow cytometry and clonal analysis,^{21,22} some authors have described molecular markers including hepatocyte growth factor (HGF) c-Met expressed specifically on pancreatic stem/progenitor cells candidate. Moreover, they have identified a new specific marker for ductal cells CD133.²²

The use of adult stem cells isolated from patients could solve immunologic problems associated with cell transplantation. Unfortunately, adult stem cells are rare and difficult to expand in culture. In contrast, new generation of β -cells after birth, has been reported from existing cells, and not putative pancreatic stem cells.²¹ Insulin-producing β -cells have also been produced from endogenous endocrine progenitors after injury.²² The existence of adult pancreatic stem/progenitor cells and their ability to proliferate in response to particular stimuli requires more investigations.

Stem Cells of Non-pancreatic Origin: Is Lumelsky's Protocol Free From Inconsistencies?

Recent studies have demonstrated that embryonic stem cells (ESCs)^{23–25} induced pluripotent stem cells,^{26,27} and adult stem cells from bone marrow (BM),²⁸ pancreas,^{29,30} liver,³¹ umbilical cord blood,³² Wharton's jelly,³³ or placenta,³⁴ can differentiate into insulin-producing elements. Because of their high pluripotency, ESCs may be ideal for islet regeneration; obviously, their use is under debate for ethical and legal issues as well as the risks of teratoma formation.³⁵

The seminal work regarding the potentiality of ESCs to differentiate into insulin-secreting structures similar to pancreatic islets was published by Lumelsky et al,²³ who observed pancreatic islet generation from an highly enriched population of nestin-positive cells from embryoid bodies (EBs). EBs plated into serum-free medium showed negative selection for all types other than nestin-positive cells. These cells were expanded in a mitogen, basic fibroblast growth factor, in N2 serum-free medium. Mitogen withdrawal thereafter promoted cessation of cell division and differentiation. Using reverse transcription polymerase chain reaction (RT-PCR), the authors showed that these ESC expressed markers GATA-4 for visceral endoderm, HNF3b for definitive endoderm, as well as for pancreatic cells, including murine insulin I, insulin II, islet amyloid polypeptide, and the glucose transporter-2. Glucagon, a marker for the pancreatic α cells, was also induced in the differentiated cells. The acquisition of a pancreatic fate was confirmed by immunocytochemical analysis, which showed many strongly insulin-positive cells by the end of the processing.

The ability of ESC-derived islet-like cell clusters to survive and function *in vivo* was tested by grafting clusters subcutaneously into the shoulders of streptozotocin-diabetic mice. Implanted cells were vascularized, forming aggregates morphologically similar to normal pancreatic islets and remaining immunoreactive to insulin. There was no sustained correction of hyperglycemia, although the grafted animals maintained their body weights and survived longer than hyperglycemic sham-grafted controls.

Boyd et al³⁶ raised doubts about the Lumelsky protocol. The first concern was about the interpretation of the gene expression data regarding the pancreatic markers. In fact, Boyd et al assert that it must be defined whether the gene expression was attributable to a single cell within the

isolated cluster or whether all cells in the cluster had transcribed the genes. Furthermore, Lumelsky's data showed that "insulin-producing cell clusters" (IPCCs) had a mixed pancreatic phenotype because they contained not only β -cell but also α -cell markers.

Another weakness in Lumelsky's protocol emerged from the quantitative PCR data,³⁶ which demonstrated that expression of insulin-1 and insulin-2 mRNAs was consistently higher in IPCCs obtained by Blyszczuk et al³⁷ compared with Lumelsky-generated clusters, implying that the Blyszczuk protocol produced IPCCs with superior de novo insulin-producing activity. The amylase-2 expression in IPCCs obtained by other groups following Lumelsky's protocol³⁸ suggested that it may also promote exocrine differentiation; evidence not in favor of a unique β -cell phenotype acquired by ESCs. Using immunofluorescence, Rajagopal et al³⁹ observed an attenuated relative ratio of C-peptide to insulin, suggesting that the insulin content by IPCCs was an unequal combination of de novo synthesis and adsorption from the culture medium. Most notably, however, the Blyszczuk protocol³⁷ consistently produced the highest level of C-peptide, indicating that it achieved superior de novo insulin synthesis. Furthermore, cell clusters obtained by Lumelsky and Blyszczuk showed prominent glucagon costaining with insulin,³⁶ indicating that the IPCCs derived from ESCs may not represent surrogate β -cells but rather more closely resemble an α and β cell hybrid. Boyd et al⁴⁰ proposed that glucagon/insulin costaining could alternatively indicate that IPCCs derived from ESCs were developmentally immature endocrine cells as suggested by the high expression of neurogenin-3 which, instead, is downregulated in fully mature islets. Furthermore, IPCCs seem to be defective in glucose-sensing capacity in vivo, supported by glucose stimulation assays that showed that IPCC insulin released in response to minimal glucose stimulation (3.3 mmol/L glucose) but not significantly by greater glucose stimulation (25 mmol/L glucose). In light of these issues, it seems that ESCs-based strategies to obtain pancreatic islet-like cells require further investigation.

Mesenchymal Stem Cells From Adipose Tissue or Umbilical Cord: Is It a Valid Alternative to ESCs?

An alternative to ESCs could be adipose-derived stem cells (ADSCs) because of their abundance, availability and possibility for autologous use.⁴¹ Three research groups have shown that under specific culture conditions, ADSCs differentiate into insulin, somatostatin, and glucagon-expressing or C-peptide positive cells.^{42,43} Packing these cells into immunoisolation capsules to test their in vivo/in vitro functionality, Chandra et al⁴⁴ reported their ability to restore normoglycemic conditions in streptozotocin-induced diabetic mice. Interestingly for the first time, this study showed that undifferentiated ADSCs exerted moderate control of blood glucose levels, leading to the speculation that the autocrine and paracrine factors of a regenerating pancreas and an hyperglycemic local diabetic micro-environment can contribute to ADSC differen-

tiation. This phenomenon has also been shown for bone marrow-derived stem cells.⁴⁵

Islet neogenesis has also been reported from constitutively nestin-expressing human umbilical cord matrix-derived mesenchymal stem cells. Kadam et al⁴⁶ reported that human placenta-derived mesenchymal stem cells (hPDMSCs) could differentiate into islet-like cells able to restore normoglycemia when transplanted under the kidney capsules of streptozotocin-induced diabetic mice. Interestingly unlike human cord blood or amnion-derived mesenchymal stem cells, the gene expression profile of undifferentiated hPDMSCs showed that they expressed mRNA for insulin, glucagon, somatostatin, Ngn3, and Isl1.

Cooperation Between β -Cells and Bone Marrow: Stem Cells as a Support for Islet Function and Regeneration

Vascularization of pancreatic islets is important for their ability to secrete insulin. Obviously, islet isolation⁴⁷ breaks vascularization, which can compromise irremediably the endocrine function; a preservation method needs to counteract this deleterious effect.^{48,49} Endothelial cells deliver oxygen and nutrients to endocrine cells, contributing to create a microenvironment for β -cell function. In particular, they can induce insulin gene expression during islet development, stimulate β -cell proliferation, and produce a number of vasoactive, angiogenic substances, and growth factors.⁵⁰⁻⁵²

Hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitor cells contribute to islet revascularization through vessel formation by differentiation into mature endothelial cells (EPCs) or release of pro-angiogenic factors such as HGF and vascular endothelial growth factor A.⁵³⁻⁵⁵ In this regard, Mathew et al⁵⁶ provided evidence that bone marrow-derived EPCs, transplanted after a pancreatic injury migrate to the site of damage helping injured β -cells to recover even if they did not differentiate into insulin-producing cells.

Two other papers stress the synergism between β -cells and BM-derived cells. Sakata et al⁵⁷ showed that only a combined transplantation under the kidney capsule of total BM-derived cells (not only the mesenchymal cellular fraction) with islets was able to significantly lower blood glucose levels in a streptozotocin-induced diabetes murine model. Interestingly BM-cell transplantation alone did not produce normoglycemic mice or insulin-positive cells, suggesting that direct differentiation of BM cells into β -cells was unlikely. Ito et al⁵⁸ provided similar evidence but in this case the site of combined BM cells/islet transplantation was the liver not the kidney. BM-derived stem cells may also have a role to stimulate β -cell regeneration.⁵⁹⁻⁶¹ For example, infusion of mesenchymal stem cells into NOD/SCID mice increased the number of endogenous β -cells improving hyperglycemia after streptozotocin-induced islet destruction.

BEYOND STEM CELL-BASED THERAPY:
TRANSDIFFERENTIATION OF ADULT CELLS TOWARD
AN ISLET-LIKE PHENOTYPE
Biologic Premises

Pancreas development begins with dorsal and ventral protrusions of the primitive gut epithelium that fuse later to form the definitive organ characterized by the appearance of glucagon-producing cells. Thereafter a few insulin-producing cells appear, often co-expressing glucagon. A later step consists of a peak of endocrine cell genesis leading to numerous fully differentiated insulin-expressing β -cells and glucagon-producing α -cells. At the end of pancreas development, endocrine cells begin to form well-organized islets of Langerhans.

The mechanisms responsible for the development of these endocrine cell types are not fully understood, but experiments consisting of generation of mice deficient for a number of pancreatic transcription factors have helped to shed more light about them by identifying crucial mediators of organ development: Sox9, Pdx1, Ngn3, IA1, Pax4, Arx, Nkx2.2, Nkx6.1, Nkx6.2, Pax6, and MafA.⁶²

Transdifferentiation Achieved by Genetic Reprogramming
Using Transcription Factors Carrying Vectors

All pancreatic cells derive from Pdx1-expressing progenitors,⁶³ the basis of experiments to obtain pancreatic elements from other cell types.^{64–66} In all of these cases, adenoviral-mediated misexpression of Pdx1 in mouse liver prevented streptozotocin-induced hyperglycemia in animal models. Kaneto et al⁶⁷ showed that concomitant adenoviral application of 2 factors, Pdx1 and Ngn3 or NeuroD in the livers of mice produced transdifferentiation of hepatic cells into insulin-producing elements associated with significant amelioration of glucose tolerance.

Transdifferentiation Achieved Modifying Cellular Micro-
environment In Vitro

Intriguingly, exocrine pancreatic cells have the capacity to generate their endocrine counterparts when exposed in vitro to a particular microenvironment, consisting of epidermal growth factor and leukemia inhibitory factor, agonists of the JAK2/STAT3 signalling pathway.⁶⁸ We have reported our own experience in a porcine model concerning the isolation by surgical microdissection of Wirsung duct cells that, upon in vitro exposure to glucose, acquired the ability to secrete insulin and glucagon.¹⁸ Finally, it seems note worthy that human monocytes treated with macrophage colony-stimulating factor and interleukin 3 transdifferentiated into pancreatic islet-like cells in a glucose-dependent manner.⁶⁹

In conclusion, regenerative medicine has undergone great development in the last few years. Due to its enormous social and economic implications, diabetes cell therapy is among the most investigated branches of this field. Islet transplantation has shown significant limitations. Alternative approaches based on a full knowledge of cellular

and developmental biology of pancreatic cells have been under investigation. Transdifferentiation, the process that leads an adult cell to change its phenotype, seems to be a promising approach given that adult cells are autologous, not dangerous or potentially tumorigenic, and free of the ethical implications associated with embryonic stem cells.

The transfer of the experimental results on experimental approach to increase availability of β -cells to the clinic still seems distant.

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