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Generation of islets of Langerhans from adult pancreatic stem cells

Ammon B. Peck[1,3], Janet G. Cornelius[1], Desmond Schatz[2], and Vijayakumar K. Ramiya[3]

[1] Department of Pathology, Immunology and Laboratory Medicine, PO Box 100275 JHMHSC, University of Florida, Gainesville, Florida 32610, USA
[2] Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, USA
[3] Ixion Biotechnology, Alachua, Florida, USA

Abstract Ductal structures of the adult pancreas contain multipotent stem cells that, under controlled in vitro conditions, are able to self-renew and differentiate into functional islets of Langerhans. In vitro-generated islets, whether derived from stem cells of human, porcine, or mouse origin, exhibit temporal changes in mRNA transcripts for islet-associated markers as well as regulated insulin responses following glucose challenge. When in vitro-generated mouse islets were implanted into diabetic mice, neovascularization of the implant material occurred, followed by reversal of insulin-dependent diabetes. The possibility of growing functional islets from adult stem cells provides new opportunities to produce large numbers of islets, even autologous islets, for use as implants.

Key words Adult stem cells · Islets of Langerhans · Type 1 diabetes · Regenerative medicine · In vitro culture

Introduction

During embryogenesis, the pancreas forms from a fusion of the dorsal and ventral primordia. These two primitive glands evaginate from the gut, develop independently of one another, then merge during midgestation. Islet development within the pancreas appears to initiate from a pool of undifferentiated precursor cells associated with the ductal epithelium.[1–3] As a result, the mature islets of Langerhans appear to be derived from stem cells, and these stem cells possess the capacity to differentiate into the four distinct islet-associated endocrine cell populations: glucagon-producing alpha cells, insulin-producing beta cells, somatostain-producing gamma cells, and pancreatic polypeptide-producing delta cells.[4] During differentiation of the endocrine tissue, the progenitor cells co-express various endocrine hormones prior to final maturation into cells expressing a single hormone. Once islets have formed from the proliferating precursor cell populations, the islets migrate into the surrounding exocrine tissue, but remain associated with the pancreatic ductal structures. Angiogenesis results in vascularization that leads to direct arteriolar blood flow to mature islets. Vascularization has the effect of increasing further the number of beta cells, most likely because blood glucose stimulates beta cell mass expansion. Lastly, neurogenesis leads to the innervation of the islets with sympathetic, parasympathetic, and peptidergic neurons;[5–7] however, the role of neural innervation in the function of the islets remains unknown.

Pancreatic islets of Langerhans reveal a cellular organization that optimizes rapid and highly regulated responses to changes in blood glucose levels. In individuals predisposed to type I diabetes, this organization can be lost due to beta cell destruction caused by a progressive autoimmune attack. Because islet beta cells per se have a limited capacity to proliferate after postfetal development, especially in the face of an active autoimmune response, beta cell mass is rapidly lost in diabetic patients. Thus, most diabetic patients today require lifelong insulin therapy. While insulin therapy offers a means to achieve normoglycemia, only ecto-pancreas transplants or islet implants represent a real "cure" for the insulin-dependent diabetic patient. Unfortunately, availability of donor pancreata for transplantation or as a source of islets from implantation is acutely limited.

Recently, attention has focused on the use of stem cells to generate specialized cells for treating a variety of diseases, including diabetes. In this report, we update our recent evidence that immature, yet functional endocrine pancreas can be grown in vitro from stem cells isolated from pancreatic ductal epithelial cells. Using the NOD mouse as a model for human type I diabetes, we have shown: (a) the feasibility of obtaining such stem

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cells from prediabetic, postdiabetic, or normal adult mice, (b) the potential for establishing stem cell cultures for the growth of immature functional islet-like structures, and (c) the efficacy of the in vitro-generated islets to reverse insulin-dependent diabetes when implanted into diabetic animals. The final result is the controlled growth of mature functional islets of Langerhans that have the necessary cellular organization ideal for rapid, yet finely controlled responses to changes in blood glucose levels. Although the ability to control the growth and differentiation of islet stem cells potentially provides an abundant source for beta cell reconstitution in type I and specific forms of type II diabetes, obtaining pancreatic tissue for autologous implants poses additional and interesting challenges, especially for the physician.

In vitro organogenesis of islets of Langerhans from stem cells

As early as 1995, Peck and co-workers[8,9] reported the successful generation of functional islets using long-term cultures of islet-producing stem cells (referred to as IPSCs) established from ductal epithelial cells of digested pancreatic tissue freshly explanted from either human organ donors or prediabetic NOD/Uf mice. The established protocol, summarized in Table 1, is divided into four distinct steps. Step 1: ductal epithelial cells, isolated from digested pancreas, are cultured to form monolayers. Step 2: islet progenitor cells (IPCs) are induced to bud from the epithelial-like monolayers, which proliferate into spheroid structures. Step 3: cells of the spheroid structures are stimulated with glucose, forcing the development of well-organized islet-like structures containing differentiated cells, the vast majority of which stain weakly for insulin and/or glucagon. Step 4: the islet-like structures are implanted to an in vivo environment to promote final maturation of the beta cells. Differentiation of IPSCs to immature (preimplanted) islets is presented in Fig. 1. The immature islets maintain their structural integrity anywhere from a few days to several weeks, but can be dissociated into new IPSCs/IPCs to start the process again (self-renewal). We have maintained mouse IPSC cultures for nearly 3 years with constant expansion via serial transfers. Each subculture retains the ability to produce increasing numbers of islet-like structures, provided that culture expansion is not pushed too rapidly.

During differentiation of ductal epithelial stem cells to islet-like clusters, the IPSC and IPC-derived cells exhibit temporal changes in the expression of various endocrine hormones and islet cell associated factors, as shown in Table 2. These include insulin-I and II, insulin receptor, hepatocyte growth factor and its receptor C-MET, glucagon, somatostatin, glutathione-2 receptor, GAD-67, and insulin-like growth factors I and II.[9,10] In addition, genes related to development and differentiation were also detected, including those for REG-1, PDX-1, beta-galactosidase, tyrosine hydroxylase, and beta2/neuroD. Despite expression of these islet-associated genes, the cells within the islet structures remain mostly immature. The finding that islets generated in vitro fail to achieve full maturation prior to implantation into an in vivo environment is supported by the observations that: (1) many of the cells continue to express, during the culture phases, more than a single endocrine hormone, and (2) insulin secretion in response to glucose challenge remains minimal. When nicotinamide, a reagent known to enhance maturation of mouse beta cells, was added to cultures of mouse IPSCs, the resulting islet cells exhibited both increased insulin synthesis (Fig. 2) and increased insulin secretion following glucose challenge (Table 3). Whether other factors, such as islet neogenesis-associated protein (INGAP),[11] might prove efficacious in promoting enhanced proliferation and differentiation of beta cells in vitro would be of interest. To date, however, only combinations of epidermal growth factor plus fibroblast growth factor appear to enhance growth, mostly proliferation and not differentiation.

Table 1. Protocol for the production of functional islets of Langerhans from pancreatic ductal epithelial stem cells

Step 1:	Isolation of pancreatic ducts from partially digested pancreas and growth of stem cells in cultures of serum-free EHAA medium
Step 2:	Induction of islet progenitor cells from established stem cell cultures by addition of serum from diabetic individuals
Step 3:	Enhancement of cell proliferation and differentiation, especially beta cells, by addition of glucose
Step 4:	Implantation to an in vivo environment to complete beta cell maturation

EHAA, Eagle Hanks high amino acid

Table 2. Gene expression during the differentiation of IPSC and IPC-derived islets of Langerhans in long-term cultures

Early markers of endocrine pancreas differentiation — IPSC and IPC cultures
Reg-1, IPF-1 (PDX-1), tyrosine hydroxylase, Isl-1, Pax-4, Pax-6, Nkx6.1
Late markers of endocrine pancreas differentiation — IPC cultures
GLUT-2, β2/neuroD t-factor, β-galactosidase, HGF, c-MET
Endocrine hormones and their receptors — IPC cultures
insulin-I, insulin-II, glucagon, somatostatin, insulin receptor

IPSC, Islet-producing stem cell; IPC, islet progenitor cell; HGF, hepatocyte growth factor; c-MET, HGF receptor

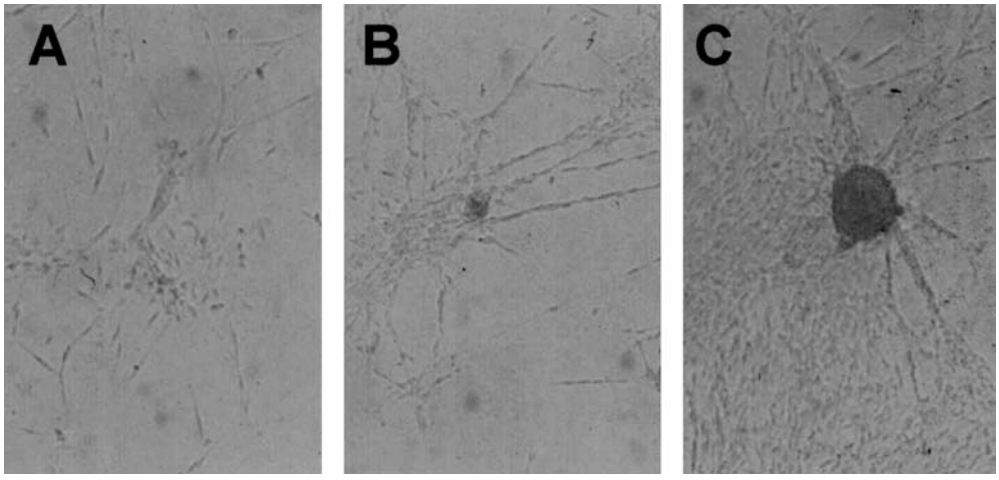

Fig. 1A–C. Differentiation of human ductal epithelial stem cells into immature, functional islet-like structures. **A** Single-cell suspensions of adult human pancreatic ducts were cultured in glucose-reduced medium until primary cultures were established. **B** After foci of epithelioid cells appeared (islet-producing stem cells [IPSCs]), the cells were induced into production of small rounded cells (islet progenitor cells [IPCs]), the progenitors of islet formation, that underwent rapid proliferation to form organized clusters of cells. **C** The cell clusters expanded to form tightly grouped glucagon-producing, insulin-producing, and somatostatin-producing cells, but the beta cells remained immature

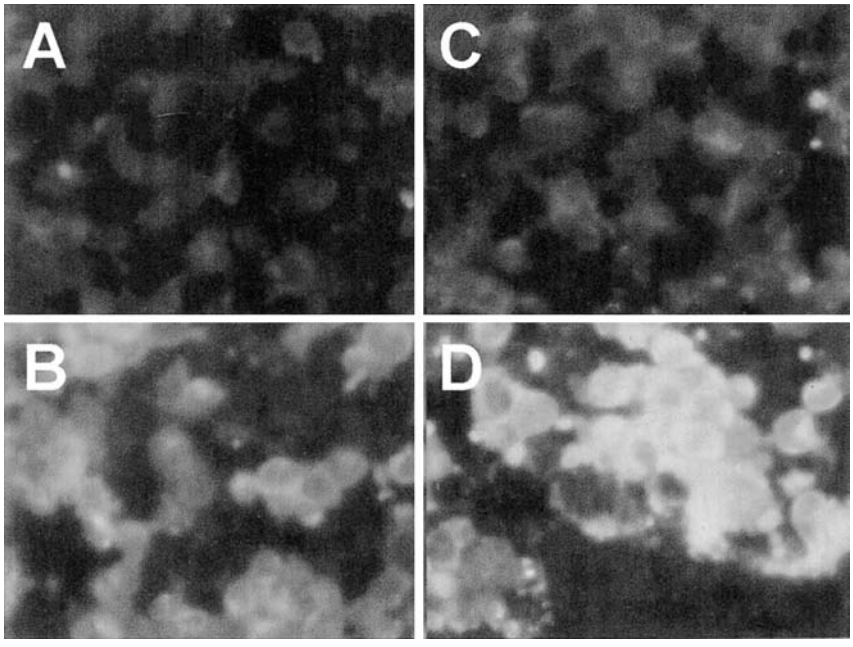

Fig. 2A–D. Synthesis of insulin by cells present in vitro-generated islets. Islets isolated from untreated (**A** and **B**) or nicotinamide-treated (**C** and **D**) in vitro cultures were collected, the clustered cells dispersed by gentle pipetting, and the dispersed cells cytocentrifuged onto glass slides. The slides were then stained with either fluorescein isothiocyanate (FITC)-conjugated anti-insulin antibody (**B** and **D**) or FITC-conjugated anti-albumin antibody (**A** and **C**)

Functional efficacy of stem cell-derived, in vitro-generated islets

The functional capacity of in vitro-grown, stem cell-derived mouse islets has been investigated in a set of implantation experiments.[10] In the first set of experiments, female diabetic NOD mice that had been maintained on daily insulin injections for more than 3 weeks were each implanted with 300 IPSC-derived islets into the subcapsular region of the left kidney, then weaned from their insulin injections. In addition, other mice received implants to other sites, including the spleen and leg muscle. Within 1 week, mice implanted with islets to the kidney or the spleen exhibited stable blood glucose levels of 180–220 mg/dl and remained insulin-independent until euthanized for analysis of the implants. Diabetic mice that had islets implanted into the muscle or had not received any islet implants exhibited

Table 3. Increased synthesis and release of insulin following treatment of in vitro-generated islets with nicotinamide

Treatment	Insulin synthesis[b]	Increase	Insulin secretion[b]	Increase
No treatment	42 ± 2	—	8.7 ± 6	—
Nicotinamide (10 mM)[a]	146 ± 60	248%	152 ± 57	1647%

[a] IPC-derived cells were incubated for 5 days with or without nicotinamide
[b] Cells stimulated with 17.5 mM glucose; insulin measured by enzyme-linked immunosorbent assay (ELISA; pg/300 islets)

severe wasting syndrome when weaned from their insulin and had to be euthanized. In a second experiment, three female diabetic NOD mice maintained on insulin for 4 weeks were implanted subcutaneously with 1000 in vitro-grown, stem cell-derived mouse islets with similar results, except that the blood glucose stabilized at more normal levels (100–150 mg/dl). Islets placed subcutaneously required about 2–3 weeks to achieve a homeostatic state with the recipient. We have speculated[10] that this might be the time required for the islets to become fully vascularized and establish the necessary glucose-sensing for rapid responses.

One of the more interesting observations from the implant studies was the fact that none of the implants of the in vitro-grown islets reactivated the autoimmune response, as evidenced by a lack of immune cells in histological examinations of implant sites at the time of euthanization (Fig. 3). We have speculated that this may be due to: (1) a loss of expression of the beta cell autoantigen(s) due to in vitro culturing, (2) development of peripheral tolerance following the restimulation of the autoimmune response by the newly implanted islets, (3) an insufficient time allowed for the reactivation of an autoimmune response, or (4) implant site specificity. In addition, lack of reactivation of the autoimmune response in the implanted diabetic mice might be related to the derivation of the islets, i.e., ductal epithelial stem cells. Regardless, understanding why the autoimmune response is not reactivated in this system could prove valuable for islet transplantation in general.

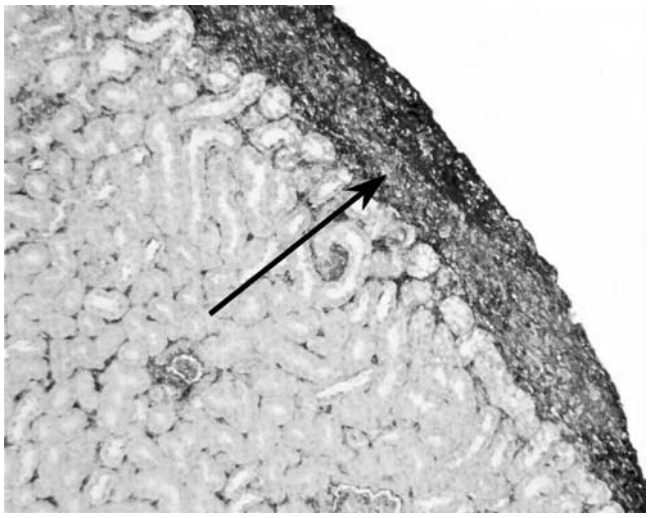

Fig. 3. Histological characteristic of the intrarenal implant site. A diabetic mouse implanted with 300 in vitro-grown islets in the subcapsular region of the kidney was taken off daily insulin injections. At 55 days post-implantation, the mouse was euthanized. The implanted kidney was explanted, fixed in formalin, embedded in paraffin, and sectioned (0.4 μm). Sections were stained with anti-insulin antibody, then counterstained with H&E dye. While the islets lost their structure, forming a more continuous mass, the implant site showed no signs of leukocyte infiltrates. Intense insulin staining of the implant cell mass can be seen (*arrow*)

Conclusions

Despite the high profile of type I diabetes and the implementation of new genetic screening programs for families and newborns to identify "high-risk" individuals, the incidence of type I diabetes is increasing worldwide. Type I diabetes is an especially insidious disease, with clinical symptoms usually not being detected until after the patient's own immune system has destroyed more than 90% of the total insulin-producing beta cells of the endocrine pancreas.[12,13] While routine insulin injections can provide diabetic patients with their daily insulin requirements, blood glucose excursions are common, resulting in hyperglycemic episodes. Hyperglycemia represents the major health problem for the diabetic patient, especially long-term. When inadequately controlled, chronic hyperglycemia can lead to microvascular complications (for example, retinopathy and blindness, nephropathy an renal failure, neuropathy, foot ulcers and amputation), and macrovascular complications (for example, atherosclerotic cardiovascular, peripheral vascular, and cerebrovascular disease). Both the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated a strong relationship between good metabolic control and the rate/progression of complications.[14,15] Unfortunately, adequate control of hyperglycemic excursions cannot be attained by most patients, and attempts at maintaining euglycemia through intensive insulin treatment lead to increased incidences of hypoglycemia.

A "cure" for type 1 diabetes relies on replacement of the beta cell mass. Currently, this is accomplished either by ecto-pancreas or islets of Langerhans transplantation.[16,17] Pancreas organ transplantation often results in normalization of fasting and postprandial blood glucose levels and the normalization of hemaglobin A1C

(HbA1C), as well as secretion of insulin and C-peptide in response to glucose. However, this procedure requires long-term immunosuppression, thereby restricting it to patients who have end-stage renal disease listed to receive a kidney transplant, or to those with longstanding type 1 diabetes who have failed insulin therapy due to extremely poor compliance.[18] Islet implantation, while historically being less efficacious, possesses several notable advantages over pancreatic organ transplantation: (1) the islets can be isolated and delivered by percutaneous catheterization of the portal vein under local anesthesia, thus relieving the patient from undergoing general anesthesia; (2) genetically, islets can be manipulated in vitro to resist immune attack — either by reducing potential islet immunogens, or by expressing immunomodulators; and (3) the islets can be encapsulated in order to be protected, theoretically, from the immune system, but still release insulin.[18–22] Drawbacks to islet cell transplantation have included the inability to obtain sufficient viable numbers of islets and the need for immunosuppressive agents.[21–25] Recent success in islet implantation protocols has brought this intervention to the forefront; however, such success portends an even greater shortage of implantable islets.

In recent years, there has been an increasing interest in the possible use of cell implants derived from stem cells to treat a variety of diseases.[26] Pluripotent epidermal, hematopoietic, pancreatic islet, and mesenchymal stem cell populations have all been shown to possess the capacity for both self-renewal and differentiation along multiple cell lineages. Such stem cells have been isolated from fetal and adult tissues and propagated in vitro. Use of epithelial skin grafts[27] and hematopoietic cell transplants[28] are already well-established clinical procedures, thus showing the utility of stem cell therapies. The possibility that stem cell-derived, in vitro-generated islets may soon be an alternative to cadaver-derived islets for treating diabetic patients is gaining credibility, now that others have started to reproduce this work. For example, Bonner-Weir and co-workers[29] have now shown that adult human ductal tissue can be expanded and differentiated in vitro to form islet clusters. The ability of this group to achieve differentiation of ductal cells to endocrine pancreas also appears to be dependent on the sequential stimulation of growth and differentiation through the use of specific media, growth factors (for example, keratinocyte growth factor), and a Matrigel environment.[29] Although insulin, glucagon, pancreatic polypeptide, and somatostatin were expressed and the islets were responsive to glucose challenge, cytokeratin 19, pancytokeratin, and islet-promoter factor (IPF-1) expression remained. Insulin-positive cells were widely scattered within the islet structures and IPF-1-positive cells without insulin staining were present, while some cells were double-stained for insulin and non-beta cell hormones. Similar results have been previously reported by Pattou and co-workers.[30,31] Considered together, these studies confirm the fact that in vitro-grown islets only achieve an immature beta cell phenotype, indicating that much more work is essential to fully understand the differentiation process.

Although the presence of stem cells associated with the pancreatic ducts has been known to exist in both healthy and diabetic individuals, the ability to stimulate these stem cells in vitro into functional islets represents a major breakthrough that has unlimited potential for therapeutic intervention in type I diabetes. However, results from a number of laboratories indicate that, while we are able to initiate expansion and differentiation of such stem cells, we still lack the basic understanding to control fully the process. Identification of the temporal changes in gene expressions during the developmental stages of islets may provide the information necessary to sequentially stimulate stem cells to mature to endstage islets using cocktails of appropriate growth factors. Alternatively, the immature islets may represent the ideal reagent for implantation, because they appear to have the potential to respond to the in vivo environment, thereby establishing homeostasis with the recipient through vascularization, expansion of the beta cell mass, and differentiation to insulin-secreting cells. Understanding fully each of these stages will require further investigations.

A recurrent question is: "How and when can diabetic patients expect to benefit from this new stem cell technology?" Except for ethical issues, implantation of in vitro-grown allogeneic islets derived from cadaveric donors could be performed now. While the use of allogeneic islets is most expedient, autologous islets, if available, would no doubt be preferred by the patient over either allogeneic or xenogeneic islets. Thus, the ideal situation will be to isolate the recipient's own stem cells and grow autologous islets in order to provide autologous implants. With the advent of new technology, e.g., transesophageal endosonographically monitored fine-needle tissue sampling of the pancreas,[32] it may someday be possible to grow autologous islets from overtly diabetic patients. In addition, genetic modification of the isolated endocrine pancreas stem cells may allow us the opportunity to build even healthier and stronger islets. Thus, although several major hurdles remain before stem cell therapy is a routine procedure, we are rapidly nearing that time.

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