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PhD Thesis

"Innovative Strategies to Improve Pancreatic Islet Transplantation"

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Abstract

In spite of the great promise of pancreatic islet transplantation in the clinical management of type 1 diabetes, several technical problems related to the transplated tissue's viability have yet to be solved before the technique can be used as a routine therapeutic tool. The aim of this work is to explore new strategies to improve pancreatic islet transplantation, exploiting combined cell and gene therapy approaches.

We have established optimized lentiviral-mediated gene transfer protocols into islet beta cells and endothelial progenitors, the two cellular components playing a major role in determining engraftment efficiency and ultimately clinical benefit for the patient. Both islets and endothelial progenitor cells could be efficiently transduced by lentiviral vectors carrying expression cassettes for the green fluorescent protein (GFP), used as a genetic marker.

The viability of engineered cells was documented *in vitro* in microchimerism experiments, where lentiviral-transduced cells were able to participate in the formation of complex structures (micro-organoids) that recapitulate the initial phases of engraftment and neovascularization *in vivo*.

The combination of these techniques opens the way to the enhancement of islet transplantation procedures by *ex vivo* approaches based on the modulation of tissue viability, host immune responses, and neovascularization of the grafted tissue.

Introduction

Clinical islet transplantation has recently received a strong impulse due to the introduction of new glucocorticoid-free immunosuppressive regimens and low doses of calcineurin with rapamycin as central immunosuppressant drugs. This therapeutic scheme, called Edmonton's Protocol, allows to maintain insulin independence in 90% of patients one year after transplant (1).

In spite of the advantages derived from this approach, some crucial problems related to the procedure remain to be solved. In most cases, it is still necessary to use multiple donor pancreas to obtain a sufficient islet mass, because a lot of islets will not engraft in the liver or will generate a poorly vascularized graft.

The low rate of vascularization is a limiting factor in islet transplantation because native islets are more vascularized than the transplanted ones: indeed, although islet mass is only the 1% in pancreatic tissue, they receive 10% of the entire blood rate directed to the pancreas.

Soon after transplantation, islets form a cell conglomerate without vascularization, and they progressively receive new capillaries both from donor and host endothelial cells. Experimental data demonstrate that islet vasculature after transplantation is strongly reduced compared to native islets (2,3); moreover, transplanted islets receive relatively poorly oxygenated blood supply within the liver. In addition, transplanted islets have a low revascularization rate: the first signs of angiogenesis (formation of sprouts and vessels buds, new capillaries) start after about 2 days and the entire process takes about 10-14 days to complete. These problems account for the failure of transplantations in other anatomical sites, such as subcutaneous implants. Due to all these difficulties, pancreata from multiple donors are still needed to guarantee a sufficient islet mass since a substantial number of transplanted islets fail to engraft into the liver, as a result of their destruction by thrombotic/inflammatory mechanisms triggered by introducing the islets into the blood stream (3).

Furthermore, activation of auto- and allo-immunity makes treatment with immunosuppressant drugs necessary. All this phenomena contribute to further tissue loss during infusion and islet engraftment.

Type 1 Diabetes Mellitus

Diabetes mellitus type 1 (juvenile diabetes) is an autoimmune disease that results in destruction of the insulin-producing beta cells of the pancreas.

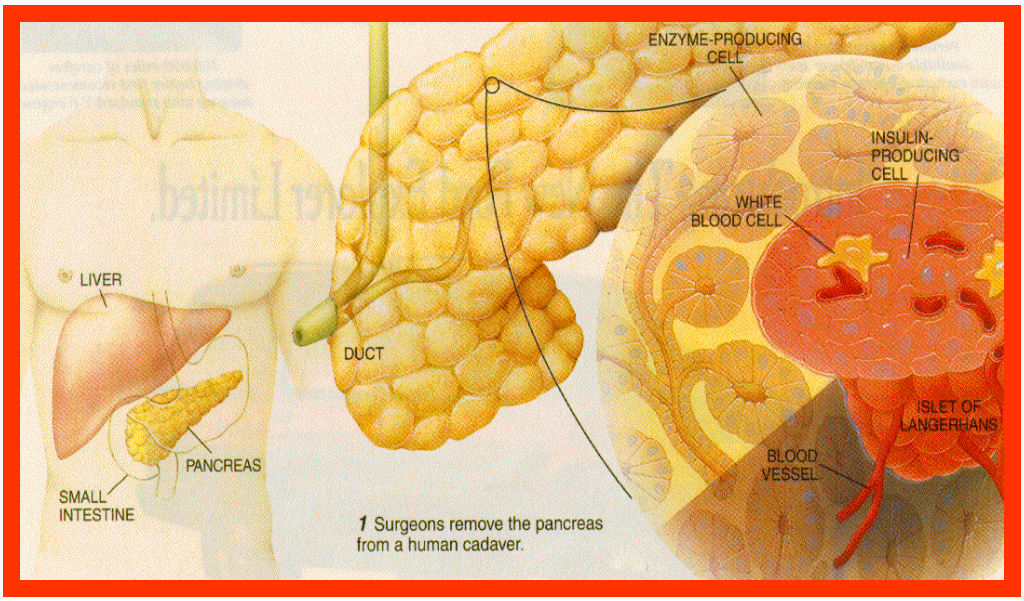

Figure 1. Representative picture of pancreas structure. Pancreatic islets are embedded in exocrine tissue.

Lack of insulin causes an increase of fasting blood glucose levels (around 70-120 mg/dL in non diabetic people) that begins to appear in the urine above the renal threshold (about 190-200 mg/dl in most people); hence the symptom by which the disease was identified in antiquity, i.e. sweet urine. Glycosuria causes patients to urinate more frequently (pollakiuria), and drink more than normal (polydipsia). Classically, these were the characteristic symptoms which prompted discovery of the disease.

Type 1 is lethal unless treated with exogenous insulin. Injection is the traditional and still most common method for administering insulin; jet injection, indwelling catheters, and

inhaled insulin has also been available at various times, and there are several experimental methods as well. All replace the missing hormone formerly produced by the now non-functional beta cells in the pancreas. In recent years, pancreas transplants have also been used to treat type 1 diabetes. Islet cell transplant is also being investigated and has been achieved in mice and rats, and in experimental trials in humans as well. The use of stem cells to produce a new population of functioning beta cells is actively being explored as a therapeutic option, but it is still at the experimental stage.

The cause of type 1 diabetes is still not fully understood. Some theorize that type 1 diabetes is generally a virally triggered autoimmune response in which the immune system's attack on virus infected cells is also directed against the beta cells in the pancreas. The autoimmune attack may be triggered by reaction to an infection, for example by one of the viruses of the Coxsackie virus family or German measles. In type 1, pancreatic beta cells in the islets of Langerhans are destroyed or damaged sufficiently to effectively abolish endogenous insulin production.

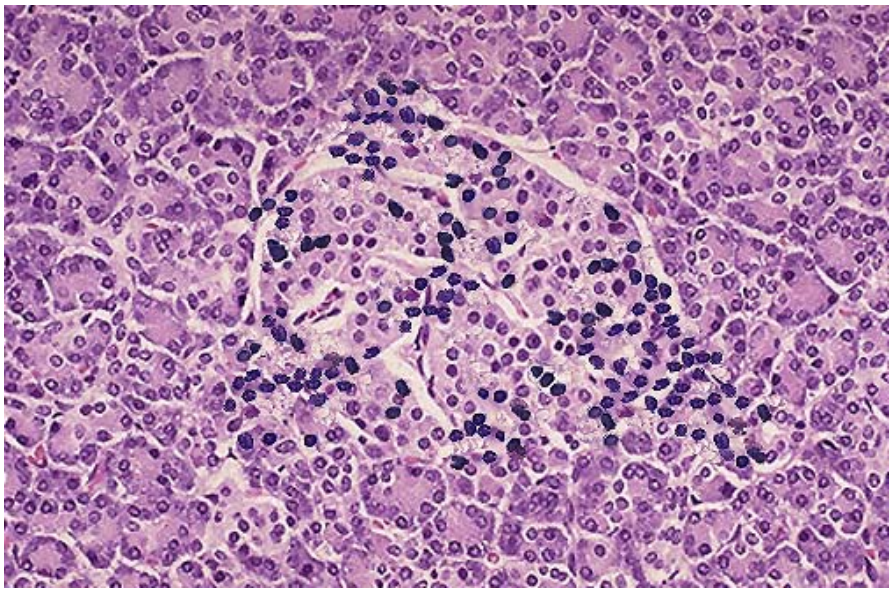

Figure 2. Micrograph of Pancreatic islets showing strong lymphocyte infiltrate.

This etiology distinguishes type 1 from type 2. It should also be noted that the use of insulin in treating a patient does not mean that patient has type 1 diabetes; the type of diabetes a patient has is determined only by the cause—fundamentally by whether the patient is insulin resistant (type 2) or insulin deficient without insulin resistance (type 1).

Type 1 Diabetes is a polygenic disease, meaning many different genes contribute to its development. Depending on locus or combination of loci, it can be dominant, recessive, or somewhere in between. The strongest gene, IDDM1, is located in the MHC Class II region on chromosome 6, at staining region 6p21. This is believed to be responsible for the histocompatibility disorder characteristic of type 1: insulin-producing pancreas cells (beta cells) display improper antigens to T Cells. This eventually leads to the production of antibodies that attack these beta cells. Weaker genes are also located on chromosomes 11 and 18.

Environmental factors can strongly influence expression of type 1. A study showed that for identical twins, when one twin had type 1 diabetes, the other twin only had type 1 30-50% of the times. Despite having the exact same genome, one twin had the disease, where the other did not; this shows that environmental factors, in addition to genetic factors, can influence disease prevalence.

This vulnerability is not shared by everyone, for not everyone infected by the suspected organisms develops type 1 diabetes. This has suggested presence of a genetic vulnerability and there is indeed an observed inherited tendency to develop type 1. It has been traced to particular HLA genotypes, though the connection between them and the triggering of an auto-immune reaction is still poorly understood.

The mechanism is not fully understood. No connection has been established between autoantibodies and type 1 diabetes. A subtype of type 1 (identifiable by the presence of antibodies against beta cells) typically develops slowly and so is often confused with type 2. In addition, a small proportion of type 2 cases manifest a genetic form of the disease called maturity onset diabetes of the young.

Vitamin D in doses of 2000 IU per day given during the first year of a child's life has been connected in one study in Northern Finland (where intrinsic production of Vitamin D is low due to low natural light levels) with an 80% reduction in the risk of getting type 1 diabetes later in life. The causal connection, if any, is obscure.

In type 1 diabetes, the body does not produce insulin. Insulin is a hormone that is needed to convert sugar (glucose), starches and other food into energy needed for daily life.

Type 1 is treated with insulin replacement therapy—usually by insulin injection or insulin pump, along with attention to dietary management, typically including carbohydrate tracking, and careful monitoring of blood glucose levels using glucose meters. Today the most common insulins are biosynthetic products produced using genetic recombination techniques; formerly, cattle or pig insulins were used, and even sometimes insulin from fish.

Untreated type 1 diabetes commonly leads to coma, often from diabetic ketoacidosis, which is fatal if untreated. Continuous glucose monitors have been developed and marketed which can alert patients to the presence of dangerously high or low blood sugar levels, but technical limitations have thus far limited the impact of such devices on clinical practice.

In more extreme cases, a pancreas transplant can restore proper glucose regulation. However, the surgery and accompanying immunosuppression required is considered by many physicians to be more dangerous than continued insulin replacement therapy, and is therefore often used only as a last resort (such as when a kidney must also be transplanted, or in cases where the patient's blood glucose levels are extremely volatile). Experimental replacement of beta cells (by transplant or from stem cells) is being investigated in several research programs. Thus far, beta cell replacement has only been performed on patients over age 18, and with tantalizing successes amidst nearly universal failure.

Pancreas transplants are generally performed together with or sometime after a kidney transplant. One reason for this is that introducing a new kidney requires taking immunosuppressive drugs such as cyclosporine. Nevertheless this allows the introduction of a new, functioning pancreas to a patient with diabetes without any additional immunosuppressive therapy. However, pancreas transplants alone can be wise in patients with extremely labile type 1 diabetes mellitus. Scientists have found another alternative mode of pancreas transplantation through the use of xenografts especially from pigs. This alternative mode of transplantation from animals, provides an alternative therapy for the treatment of Type 1 diabetes.

Islet cell transplantation is expected to be less invasive than a pancreas transplant which is currently the most commonly used approach in humans. In one variant of this procedure, islet cells are injected into the patient's liver, where they take up residence and begin to produce insulin. The liver is expected to be the most reasonable choice because it is more accessible than the pancreas, and islet cells seem to produce insulin well in that

environment. The patient's body, however, will treat the new cells just as it would any other introduction of foreign tissue, unless a method is developed to produce them from the patient's own stem cells or there is an identical twin available who can donate stem cells. The immune system will attack the cells as it would a bacterial infection or a skin graft. Thus, patients now also need to undergo treatment involving immunosuppressants, which reduce immune system activity.

Islet Transplantation

The concept of islet transplantation is not new. Investigators as early as the English surgeon Charles Pybus (1882–1975) attempted to graft pancreatic tissue to cure diabetes. Most, however, credit the recent era of islet transplantation research to Paul Lacy's studies dating back more than three decades. In 1967, Lacy's group described a novel collagenase-based method (later modified by Dr. Camillo Ricordi) to isolate islets (27).

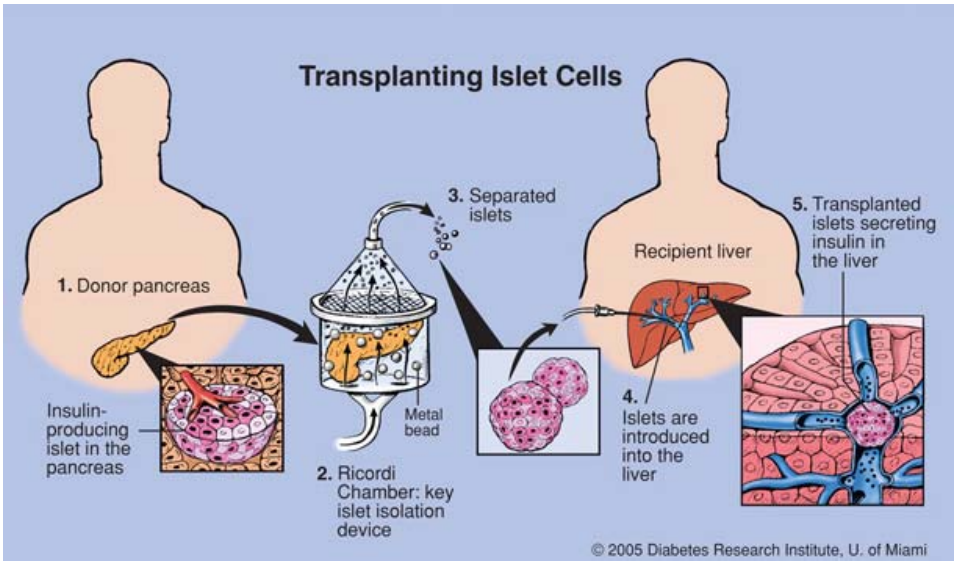

Figure 3. Ricordi's procedure for islet transplantation (Diabetes Research Institute, University of Miami, 2005).

Subsequent studies showed that transplanted islets could reverse diabetes in both rodents and non-human primates (28,29). Improvements in isolation techniques and immunosuppressive regimens ushered in the first human islet transplantation clinical trials in the mid-1980s. The first successful trial of human islet allotransplantation resulting in long-term reversal of diabetes was performed at the University of Pittsburgh in 1990. Yet

despite continued procedural improvements, only about 10% of islet recipients in the late 1990s achieved euglycemia (normal blood glucose) (30). In 2000, Dr. James Shapiro and colleagues published a report describing seven consecutive patients who achieved euglycemia following islet transplantation using a steroid-free protocol and large numbers of donor islets, since referred to as the Edmonton protocol. This protocol has been adapted by islet transplant centers around the world and has greatly increased islet transplant success (1).

Researchers use a mixture of highly purified enzymes (Collagenase) to isolate islets from the pancreas of a deceased donor. Collagenase solution is injected into the pancreatic duct which runs through the head, body and tail of the pancreas. Delivered this way, the enzyme solution causes distension of the pancreas, which is subsequently cut into small chunks and transferred into so-called Ricordi's chamber, where digestion takes place until the islets are liberated and removed from the solution. Isolated islets are then separated from the exocrine tissue and debris in a process called purification.

During the transplant, a radiologist uses ultrasound and radiography to guide placement of a catheter through the upper abdomen and into the portal vein of the liver. The islets are then infused through the catheter into the liver. The patient will receive a local anesthetic. If a patient cannot tolerate local anesthesia, the surgeon may use general anesthesia and do the transplant through a small incision. Possible risks of the procedure include bleeding or blood clots.

However, several problems with the current procedure still need to be addressed to improve certain aspects, such as organ procurement and preservation, islet isolation and culture, modality of transplant and immunosuppression. Pancreata from multiple donors are still needed to guarantee a sufficient islet mass since a substantial number of transplanted islets fails to engraft into the liver or suffers from poor vascular engraftment. Indeed, native islets *in vivo* are richly vascularized and even though islets make up only 1% of the pancreatic mass, they receive about 10% of the blood flow (2). After transplantation, islets are revascularized most likely by both the host and the recipient endothelial cells that form a chimeric vascular tree (3). However, revascularization process is not immediate and transplanted islets show the first signs of angiogenesis (i.e. capillary sprout formation and protrusion) no earlier than 2 days after transplantation, and the entire process is completed

after 10–14 days. In addition, the vascular density of vascularized transplanted islets is markedly reduced in comparison to native islets (4,5). Based on these considerations, the identification and consequent removal of factors that may impair the angiogenic processes after islet transplantation may likely increase the success of this procedure.

Rapamycin is widely used as central immunosuppressant for islet transplantation as part of the original Edmonton protocol (1). The immunosuppressive mechanism of rapamycin is based on the selective blockade of the mammalian target of rapamycin (mTOR) activation, a molecule known to play a pivotal role in cell cycle progression from late G1 into S phase in response to T-cell growth factor stimulation (20). Unfortunately, considering the ubiquitous expression of mTOR in different cell types, the effects of rapamycin are not restricted to the immune system but affect different physiopathological processes involved in cell survival and proliferation, inducing leucopenia (21), thrombocytopenia (21), delays in wound repair (22) and tubular regeneration after acute ischemic injury (23). Moreover, it has been recently shown that rapamycin inhibited metastatic tumor growth and angiogenesis in an *in vivo* mouse model (24). The dissection of this phenomenon revealed that rapamycin exerted antiangiogenic activities linked to a decrease in the production of vascular endothelial growth factor (VEGF) and to a markedly inhibited response of tumor endothelial cells to stimulation by VEGF itself (24).

Islet transplantation-related complications

Islet transplantation related complication must be divided in two main classes: procedure related complications and detrimental effects derived from immunosuppressive regimen.

Particularly, the most commons troubles derived from islet infusion in the bloodstream are:

- Rise of liver enzyme levels (54%)(31);
- Abdominal pain and nausea (50%);
- Liver steatosis (20%)(32);
- Intra-peritoneal bleeding (10%). Cause of this effects is not well understood but it seems derived from pre-operating treatment with heparin to avoid thrombosis of portal vein;

- Portal vein thrombosis in the 4% of patients. Although risk to have this complication can't be avoided completely it seems that to use large amount of high purity preparation of islets, good experience in catheterization of portal vein and anticoagulant use can contribute to reduce significantly thrombosis;
- Portal vein high blood pressure derived from infusion and it can increase in following infusion.

Immunosuppression derived detrimental effects are: mucosal ulcers (96%), augmentation of LDL levels (this effect often makes necessary use of statins), reduction of glomerular filtration rate (50%), rising of proteinuria (50%), high blood pressure (50%) and neutropenia.

Instant Blood Mediated Inflammatory Reaction (IBMIR)

It has been recently observed that islets, soon after transplantation, undergo to inflammatory/thrombotic reaction mediated by tissue factors present on islet surface following digestion and during culture (33, 34, 35, 36).

Indeed exposure of basal membrane fragment activates reaction that leads to thrombotic process activating platelets. Two pathways are involved in thrombotic reaction: intrinsic and extrinsic.

This pathway begins with trauma to the blood vessel, exposure of blood to collagen in a damaged vascular wall, or exposure of the blood to a wettable surface such as glass. In response to these stimuli, two events occur. First, Factor XII (aka Hageman Factor) is converted from its inactive form (zymogen) form to an active form Factor XIIa. Second, platelets are activated (again to be discussed later). Activated Factor XII is actually a protease which enzymatically activates Factor XI to Factor XIa ('a' at the end of factor name denotes an activated enzymatic factor). This reaction requires the presence of High Molecular Weight Kininogen and Prekallekrein. Activated Factor XI is also a protease, but its function is to convert Factor IX to Factor IXa. Also a protease, Factor IXa then converts Factor X to Factor Xa. This activation of Factor X is also greatly accelerated by Factor VIIIa. Deficiencies in either Factor VIII or Factor IX lead to bleeding diatheses known as Hemophilia A and Hemophilia B, respectively. Activated Factor X functions as a protease to converts the inactive molecule prothrombin to the active thrombin. This step requires the presence of

Factor Va. Thrombin then cleaves fibrinogen to fibrin, which then polymerizes to form fibrin strands.

In this pathway, the initial step is a traumatized vascular wall or extravascular tissue. Non-vascular tissue cells contain an integral membrane protein called tissue factor. Damage to the vessel wall or extravascular tissue exposes the plasma to tissue factor. Factor VII is a circulating plasma protein that then binds to tissue factor, creating a complex. In doing so, Factor VII is activated to Factor VIIa. This complex, in the presence of Ca^{++} and phospholipids, activates Factor X to Factor Xa. Once Factor Xa is generated, the remainder of the cascade is similar to the intrinsic pathway.

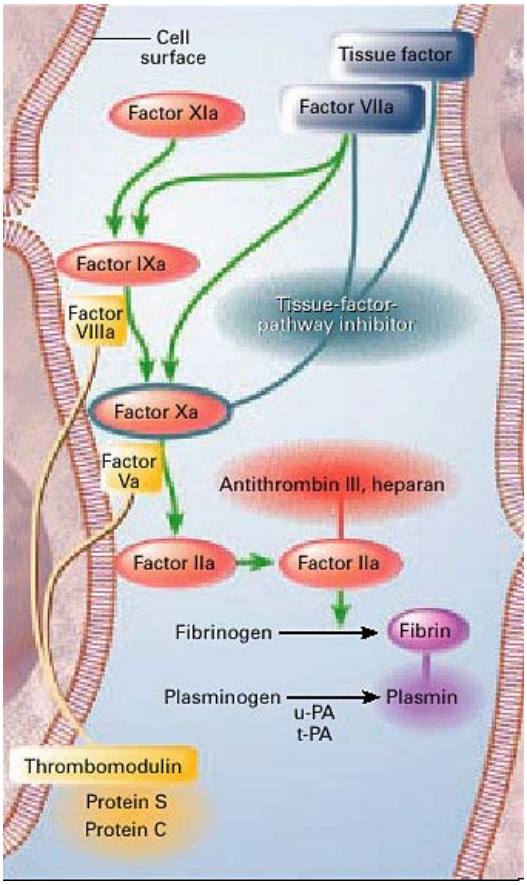

Figure 4. Schematic representation of inflammatory/thrombotic reaction activation. *Rosenberg RD, Aird WC. N Engl J Med. 1999;340:1555-1564.*

Following these events there is concomitant binding of platelets to the islet surface, rapid loss of platelets from the blood, and fibrin formation that generated a capsule surrounding the islets. Simultaneous secretion of b-TG and upregulation of P-selectin indicated platelet activation.

The platelet-binding ligand on the islet surface has not been identified; however, type I, III, IV, and V collagens are found within and surrounding human islets, and it is known that collagens mediate platelet binding and activation. The integrins α2β1, CD36, P65, and GPVI have all been proposed as platelet collagen receptors; thus, the extracellular matrix proteins are likely to be ligands of platelet binding and activation when isolated islets are exposed to blood (37).

Moreover, recent studies demonstrate that cultured islets expressed several inflammatory mediators. Indeed, it was showed high mRNA expression of MCP-1, MIF, VEGF, and thymosin beta-10 was detected in all islet samples. IL-8, IL-1-beta, IL-5R, and INF-gamma antagonist were expressed in islets cultured for 2 days. IL-2R was expressed in islets cultured for more than 6 days.

Indeed, up to 60% of the β cell mass undergoes apoptosis in experimental models of syngeneic PIT, and half of this loss occurs within the first 3 days after transplantation. This rate of apoptosis following PIT is 10 times higher than the rate seen in the native pancreatic islet. Furthermore, the molecules and cells that are active following PIT are suggestive of an alloantigen-nonspecific, inflammatory process. In particular, the inflammatory cytokines interleukin-1β (IL-1β), interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) are elevated following PIT, and tissue macrophages also appear involved in mediating cellular injury to the recently transplanted pancreatic islets. Administration of drugs that inhibit cytokine actions or macrophage function has been found to improve function of the pancreatic islets after transplantation (36).

Immunological problems related to islet transplantation

Similarly to other forms of transplantation, islet transplantation is not only troubled by alloimmunological anti-graft reactions. Indeed, there is a possibility of autoimmunity-related complications.

Autoimmunity - Autoimmunity derives from immune response against *self* antigen as consequence of tolerance loss. This process is derived from loss of deletion of auto-reactive cells during lymphocyte maturation (38).

Indeed, during maturation process all auto-reactive B lymphocyte were deleted with a process called clone deletion in bone marrow and auto-reactive cells in spleen and lymph node. T-cells tolerance is divided in peripheral and central tolerance. Peripheral tolerance derives from deletion in thymus of auto-reactive T-cells. Central tolerance is deletion of T-cells with mechanisms of ignorance (loss of the cell ability to react with *self* antigens present in low doses and physical separation between lymphocyte and antigen), deletion (apoptosis of T-cells induced from loss of costimulatory signals or activation of FAS/FAS-L pathway), inhibition (costimulation blocks CTLA-4 mediated) and suppression (T-regulatory lymphocyte mediated).

Type I Diabetes Mellitus represent an auto-immune disease and its pathogenesis can be related with environmental factors. It seems that there is a genetically based disease and involved genes are probably bound to MHC (major histocompatibility complex) I and II. 95% of patients affected from type I diabetes mellitus seem to have HLA allels codifying for DR3, DR4 and some DQ locus (DQb1 *0301 e DQb1 *0602) with high affinity for diabetogenic peptides.

Dependence from environmental factors is demonstrated from diabetes insurgence after viral infections like parotitis, hepatitis, mononucleosis, german measles, Coxsackie and from evidence of the presence of cytomegalovirus genes integrated in 1/5 patients affected from diabetes in their genome.

It well evaluable from this tables that in diabetes pathogenesis besides environmental and genetic factor, immunological events play a key role in and they result equally involved in as in humoral than in cell-mediated immunity.

Anomalous activation of humoral immunity determine production of immune-antibody directed against pancreatic cells.

- “Islet Cell Cytoplasmic Antibodies” (ICCA) are presents in about 0,5-4% of healthy people and 90% of diabetic patients. These antibodies are not specific for beta cells but it seems they preferentially recognize that cells.
- “Islet Surface Antibodies” (ICSA) are present in the 80% of diabetic patients at the moment of diagnosis. In presence of complement factors they can produce cell lysis.
- Antibodies against glutamic acid decarbossilase (GAD) are present in about 2% of healthy people and 80% of diabetics patients.
- Antibodies anti-insulin and pro-insulin.

Cell mediated activation immunity starts in aberrant way; NK (Natural Killer) cells and CD8+ T cells (cytotoxic) become responsive to self antigen on defective MHC I on beta cells surface. So, beta cell mass were attacked and destroyed in selective manner.

Alloimmunity - One of the main problems in transplantation is the induction of allo-immunity directed against transplanted tissue. Indeed, immune system recognize self (everything that belong to the same organism) from not self (everything come in from other way, like pathogens and virus for example). Following transplantation immune system of the host recognize transplanted tissue *not self* starting to destroy donor derived organ and cells, this process is called rejection (39,40).

This phenomenon make necessary to inhibits immune system in the way to promote graft survival and improve transplantation. Indeed, transplanted tissue have on the surface of its cells donor MHC that can lead to the activation of different pathways for immune response by antigenic recognition:

- In the direct pathway, T-cells recognize MHC antigen expressed on donor cell surface as foreign molecules. Because T-cells recognize antigen bound on MHC cells, it is possible that this effect is derived from molecular mimicry between *self* and *not self*.
- In the second pathway catabolytes derived from digestion of MHC expressed from donor can be presented onto the complexes in recipient cell surface and the recognized as *not self*.

T-cells are responsible for the activation of rejection in transplanted tissue. This cells presents on their surface a glycoprotein called CD4 (T-helper) and they secreted cytokines

responsible to start rejection processes. Cytokine (the main cytokine in this process is interleukin-2,IL-2) secreted from T-helper acts by two principal pathway: they stimulated the same cell by autocrine signaling and by paracrine control on cytotoxic T cells, B-lymphocyte and macrophage. Cytotoxic T cells are e class f T lymphocyte that presents on their surface CD8. The action of cytotoxic T cells is to lyse directly donor cells during rejection. All classes of T-cells presents on their surface kind of receptor, called T-cells receptor (TCR), able to bind antigen-MHC complex on the antigen presenting cells (APCs). APCs are a cell class able to present fragment of protein bound on the MHC class II in their membrane to immune cells to stimulate the activation of immune reaction. Binding between TCR and MCH is not sufficient to start immune reaction, activation of T-cells needs another stimulus. The second stimulus was leaded by costimulatory signals, that mean e series of molecules that acts like receptor-ligand activating the immune response. Costimulatory signals are necessary in the immune activation pathway, indeed, lack of costimulation lead to a low cytokines secretion followed by immune cells anergy and apoptosis.

Costimulatory Molecules

The recent discovery of lymphocyte costimulatory signals has opened the way to the design of strategies for local immunosuppression, to create immunoprivileged sites in the areas of islet engraftment. Lymphocyte costimulatory signals derive from cell_surface receptor_ligand interactions that are necessary to start and control immune cell activation during antigen recognition (6).

Absence of these signals results in abortion of lymphocyte activation, anergy or even apoptosis. The main co-stimulatory pathways are represented by the B7 family members and by the CD40-CD154 receptor-ligand pair. B7-1 and B7-2 co-stimulates T cells through binding to CD28. Their binding is prevented by the neoexpression CTLA-4, a homologous of CD28 able to deliver a negative signal. The CD40-CD154 interaction works as a bidirectional co-stimulatory system by triggering activation signals to both T cell and APCs. Beside these, other receptor-ligands pairs relevant during antigen-presentation process were identified and are currently under evaluation.

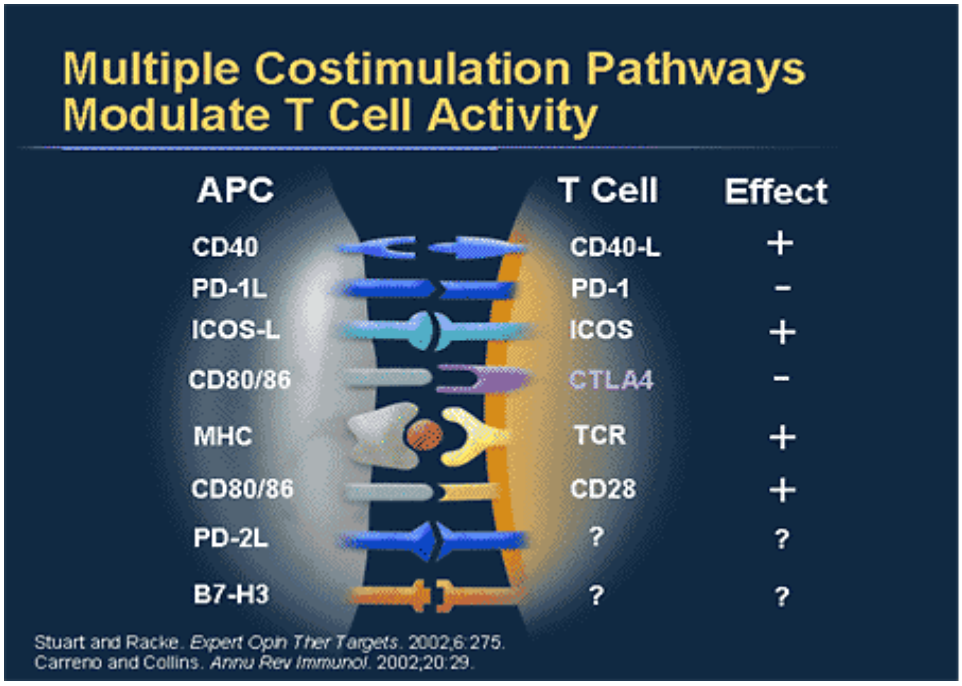

Figure 5. Costimulatory Pathways.

Among these, several studies indicated that CD5 may provide accessory signals in concomitance to TCR or BCR activation, by binding to different ligands (7, 8, 11, 12, 13, 14). Studies from a number of groups, (7, 8, 9), demonstrated that disruption of natural costimulatory interaction by means of mAbs or soluble receptor-IgG fusion proteins were highly effective in several experimental models of autoimmune disease and transplant rejection, thus proposing costimulatory mechanisms as the main target for molecular-based therapy.

Indeed, costimulation blockade has thus been developed as a specific field of interest towards achieving improved antigen specific control over transplant rejection while minimizing broad attenuation of protective immunity seen with conventional immunosuppressives. This field has grown rapidly in the past decade and is now poised to become a valuable therapeutic option for transplant clinicians. This review will outline the basic premise of costimulation biology, review the seminal experimental basis for its use in preventing organ rejection, and discuss the relevant data derived from its initial use in clinical transplant trials. Specific attention will be focused on two major costimulatory

pathways, the CD28/CD80-CD86 and the CD40-CD154 pathways, and the clinically applicable data supporting their validity as therapeutic targets. Newly discovered costimulatory pathways will also be discussed as potential therapeutic targets for future clinical drugs.

Immunosuppression in Islet Transplantation

All this observation underlined why an efficient protocol for immunesuppression (Edmonton Protocol) in islet transplantation needed 36 year to be found. Previous immunesuppressive regimen were based on two principal drugs categories: corticosteroids and calcineurin inhibitors.

Corticosteroids exerted their immune-suppressive effect blocking cytokine expression from T-Lymphocytes and APC like dendritic cells. This hydrophobic molecules can come into the cells through plasma membrane and interact directly with cytoplasmatic receptor to produce complexes.

Steroid-receptor complex go into the nucleus binding specific DNA sequences and inhibiting gene transcription for cytokines like IL-1, IL-2, IL-3, IL-6, TNFα and IFNγ. Through this mechanism, steroids lead to the blocking of T-lymphocyte activation at different levels.

Side effects derived from these drugs are bone-necrosis, cataract, iperlipidemia but in particular a strong diabetogenic effect that leads to leave this drugs from immunosuppressive protocols in islet transplantation.

Calcineurin inhibitors, for example Cyclosporine and Tacrolimus (FK506), are different from other immune-suppressant for their ability to inhibits in a selective way immune-response. Their immunosuppressive effect is exerted by binding with cytoplasmatic receptor, cyclophyllin for cyclosporine and FK binding protein (FKBP) for tacrolimus.

These drugs-receptor complex exerted their function by binding calcineurin, that act dephosphorylating some nuclear factor with regulatory functions as nuclear factor of T-activated cells (NF-AT) facilitating their escape from nucleus. Moreover, calcineurin inhibition affect transcription of genes for cytokines implied in T-cells activation like IL-2, IL-3, TNFα and IFNγ. Further, it result inhibited transcription for genes codifying CD40L and

protooncogenes c-myc and H-ras. Final effect of calcineurin inhibitors is to block lymphocyte proliferation.

Main effects of these drugs are nephrotoxicity (reduction of glomerular filtration rate, interstitial fibrosis, alteration in electrolytic balance), gastrointestinal diseases, neurotoxicity, cardiotoxicity, thromboembolic events, iperlipidemia and glucose intolerance (demonstrated toxicity for pancreatic islet).

The Edmonton Protocol

Although isolation method is well known from 1960 following studies of Paul E. Lacy, technological innovation in 1990 by Camillo Ricordi with semi-automated method give the possibility to obtain a better biological product derived from introduction of the use of Ricordi's Chamber for islet isolation (62, 63).

In spite of these progresses in 2000 only 35% of transplanted islet were viable at one year from transplantation with a rate of insulin-independence of about 8-10% of patients.

Islet transplantation become from experimental method to clinic after the year 2000, following publication by James Shapiro group of seven patients that reached insulin-independence with a new transplantation scheme. In this new therapy, they used an immune-depressive protocol without steroids (1).

The main waypoint of Edmonton protocols are:

- Cold ischemia reduced with immediate transplantation of purified islets;
- Elimination of animal protein (for example fetal calf serum) from cell culture medium to reduce immunogenicity;
- Transplantation of more than one preparation of islets derived from more patients to reach value of cells >10,000 islets/Kg body mass;
- Elimination of glucocorticoids from immune-suppressive therapy;
- Administration of low doses of calcineurins inhibitor (FK506).

Therapeutic scheme represents the main innovation proposed by Edmonton protocol and it introduces in islet transplantation of a new immune-suppressant drug: Rapamycin or Sirolimus.

This protocol counts association of Sirolimus with low doses of Tacrolimus (to reduce diabetogenic effect induced from calcineurin inhibitors) and monoclonal antibody daclizumab. They acts in the following way:

- Rapamicyn, binding to a cytoplasmic protein (*Mammalian Target of Rapamicyn, mTOR*) with regulatory function on cell cycle, inhibits cell proliferation by blocking G1-S phase transition in activated T-Cells. Side effects associated to Rapamicyn therapy there are insurgence of anemia, thrombocytopenia, leucopenia, alteration of hepatic function indexes, increased viral, mushroom, bacterial infections and myeloma risk. Furthermore, rapamicyn, although it does not affect kidney function, delays function recovering after acute renal failure (ARF) slowing proliferative rate on tubular cells (44).
- Tacrolimus inhibits cytokine synthesis that are a critical for T-cell activation and proliferation.
- Daclizumab is a chimeric monoclonal IgG1 antibody composed by 90% of human sequences and 10% of mouse sequences localized in variable regions involved in antigen recognition. This antibody bind in specific way α subunits of IL-2 receptor acting as inhibitor for its ligand on activated lymphocytes. Administration of this molecule did not produce relevant side effects.

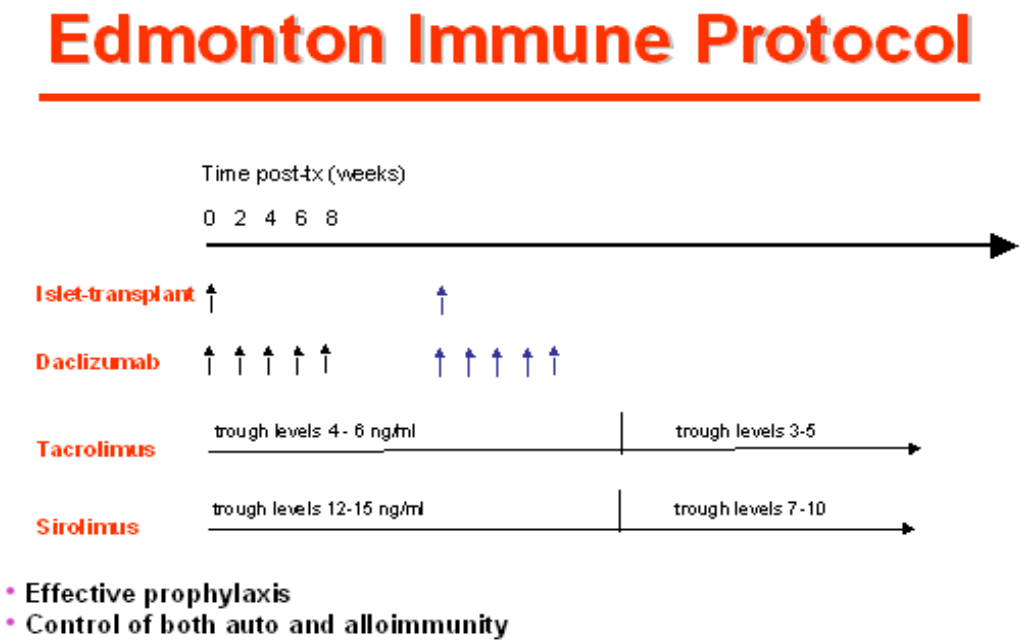

Figure 6. Immunosuppressive therapy of Edmonton protocol. *Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV, N Engl J Med 2000; 343:230-8.*

Results obtained from this study on the seven diabetic patients (age from 29 to 54 years) are:

- All seven patients at 12 months from transplantation results insulin-independent;
- No episodes of severe hypo-glycemia were observed differently from patients that underwent to insulin therapy;
- All patients presents normal value of glycated haemoglobin;
- After transplantation rate of antibody anti-insulin results reduce drastically;
- No infection of Cytomegalovirus were observed;
- There are not evident episodes of rejection against transplanted islets.

This work lead to an international multicenter clinical trial called ITN (Immune Tolerance Network) that confirmed results derived from the Edmonton Protocol in other centers, reaching better results in centers with more extensive experience in islet isolation. Particularly, data derived from 75 islet transplantation recipients in Miami, Edmonton and

Minneapolis demonstrated a functionality of transplanted tissue of 96% after one year and 85% of insulin independence and about 70% after two years. On these basis many other centers around the world started with clinical islet transplantation.

In 2005 were published on *Diabetes* (45) results of the first five years *follow up* of patients that underwent to islet transplantation treated with Edmonton Protocol. In this publication, authors underlined that in the 80% of transplanted patients (N=44) viability of transplanted tissue were preserved (Presence of plasmatic C-peptide), although only 10% remain insulin-independent (15 month) and at the same time with good control of glycated haemoglobin.

Patients that returned to insulin therapy can receive half dose of insulin than before transplantation with substantial improvement of metabolic complications.

These encouraging results underlined that this pharmacologic association, without corticoids, can give a fundamental support to transplanted patients both for the ability of minimizing beta cells damage and to reduce acute rejection.

Islet Vascularization Pre- and Post- Transplantation

Another critical passage in islet transplantation is represented from grafting and revascularization of transplanted tissue.

Islet were one of the most vascularized tissue present in the organism. Indeed, they receive about 10% of blood directed to entire pancreas although they represents only 1% of entire organs.

Islet vasculature presents structure glomerular like and it is organized in a network of strongly fenestrated capillaries (46, 47).

This organization is fundamental for function of because it ensures a quickly trans-endothelial transport of secreted hormones.

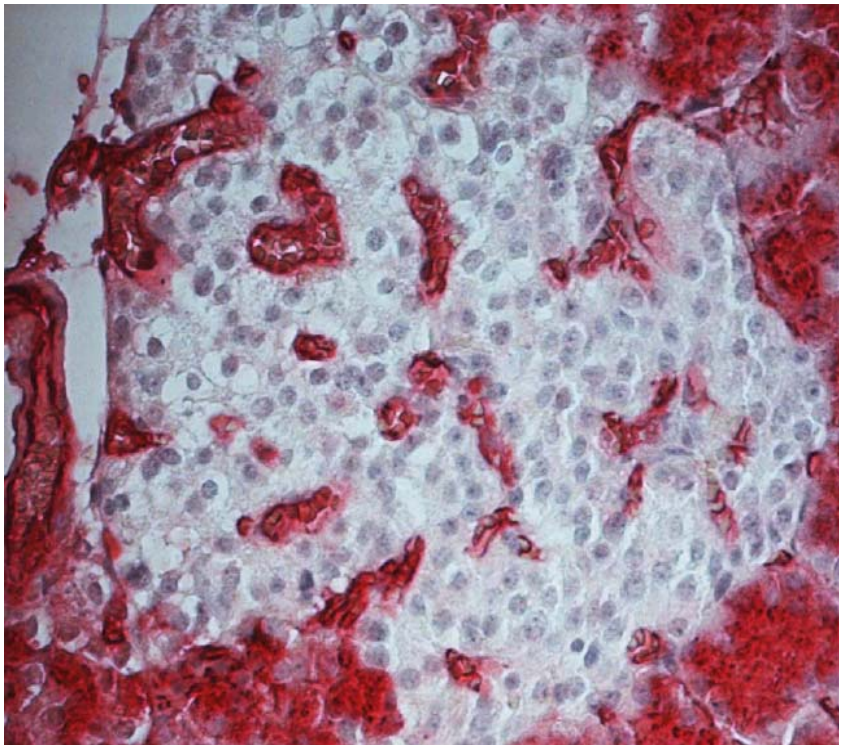

Figure 7. Micrograph of pancreatic islet. Endothelium was stained with Bandeiraea simplicifolia lectin. *The International Journal of Biochemistry & Cell Biology 38, 492–497 , 2006.*

Blood perfusion of pancreatic islets is regulated above all at pre-capillary level through combined action derived from factors locally produced, gastrointestinal hormones e nervous system. This mechanism gives to islet a suitable contribution of nutrients and at the same time facilitate a quick diffusion of secreted hormones in the blood-stream.

Purified islets were separated from vascular network and then their survival after transplantation is strictly related to the reconstitution of neo-formed vessels in the graft to be connected to the host vascular system.

It was estimated that less than 30% of transplanted islet implants in recipient and, although it was infused a large amount of islets in diabetic patient. The main factor that limits islet graft is represented from vascularization.

This process begin in the first 2-3 days after islet transplantation and it is completed in about two weeks generally. Moreover, Mattsson (3) and colleagues results demonstrated that vascular rate is lower in transplanted islets than native one at about one month after transplant, when tissue must be fully revascularized. Furthermore, transplanted islets received blood both from portal vein and epatic artery, than, poorly oxygenated.

Another main topic about revascularization of transplanted islets is the origin of cells that give their contribution in neo-vessels formation after transplantation. For many years it was commonly thought that islets were an avascular conglomerate of cells and revascularization is derived from host vessels infiltration.

Studies of Linn (48) and colleagues demonstrated on freshly purified islets that intra islet endothelial cells (IECs) *in vitro* show a strong angiogenic behavior and that they are able to survive into the transplanted tissue. On these bases, a Swedish (49) team of researchers studied the role of IECs in islet revascularization after transplantation. They compared, using mouse models, behavior of IECs purified and presents in native vasculature. After this analysis they concluded that:

- Endothelial cells remain into the islets after purification in a high quantity;
- Intra islet endothelium viability in culture decreased in time dependent mode (80% after 2 days to 4-8% after 4 days);
- Donor endothelial cells contributed in neo vessel formation for about 40% during revascularization process of transplanted islets, producing a chimerical network with recipient endothelial cells.

These results confirmed data derived from studies conducted on mouse and rat islets, published by Brissova in 2004 on *Diabetes* (50).

Many experimental evidences induced researchers to transplant freshly purified in order to promote a more rapid and efficient revascularization process. Moreover, recent work demonstrated that Rapamicyn, the main immunosuppressant in islet transplantation, exerted a detrimental role in islet revascularization by acting on IECs. Indeed, it is seen that Rapamicyn inhibits *in vitro* and *in vivo* angiogenic behaviour of IECs exerting its action on cell motility and proliferation. Moreover, treatment with Rapamicyn on freshly purified islets reduce outgrow of IECs from islets *in vitro* and revascularization *in vivo* in experimental transplantation models in SCID mice (59).

Based on these relevant evidence my work was focused on the possibility to introduce innovative transplant method directed to reduce immunosuppression and to improve revascularization in islets after infusion in portal blood flow. Results of this study can be useful to maximize transplantation result by using less amount of tissue to reach insulin independence.

Aim of the Study

The aim of this study is to explore new therapeutic approaches by combined cell and gene therapy to improve islet graft after transplantation and to minimize immunosuppressant detrimental effects in the process of engraftment. Combined gene therapy and tissue−engineering to form endothelial−islet micro−organoids may also be potentially beneficial in accelerating islet revascularization after transplantation.

In this study we generated chimaeras between islets and EPCs with the aim to analyze efficiency off cells transduction with lentiviral vectors. Moreover, we will analyze if the process affects islet viability.

In particular, in this preliminary study we evaluated:

- whether EPCs may represent an more suitable target for gene transfer rather than beta-cells, based on the following rationale: (a) transducibility of EPCs compared to beta-cells; (b) avoidance of the risk of beta-cell function alterations when undergoing transduction procedures; (c) better quality control of the transduced cells, since the procedure can be performed in advance and EPCs are relatively not a limited resource of material such as beta-cells.
- the development of microchimeric “organoids” composed by pancreatic islets coated with *ex vivo*-expanded blood-derived EPCs. The perspective is a scenario where EPCs are isolated from patient blood, *ex vivo* expanded, cryopreserved and, when needed, implanted in revascularization sites as autologous transplantation. Co-implanting islets microchimerized with previously prepared autologous EPCs may potentially favor rapid and abundant revascularization after transplantation.

Materials and Methods

Islet Isolation

Human pancreatic islets were isolated from organs derived from cadaver donors.

- Pancreas was cleaned from fat. Pancreatic duct were recovered and clamped with two clamps for every duct and then spleen and duodenum were detached.
- After cleaning pancreas were rinsed in DIP solution (solution containing antibiotics) and betadine. Soon after were washed with two passages in HBSS (Hanks Balanced Salt Solution).
- Pancreas were perfused with a solution of collagenase through pancreatic duct. Briefly pancreas were cut in two pieces and then pancreatic duct were retrieved and cannulated with two catheters. Catheters were then fixed through suture to the pancreas.
- Collagenase were injected in pancreas using siringe for ten minutes and organ distension were evaluated. If there were some cut on the organ they were closed using fibrin glue or clamps.
- After perfusion, organ were cut in 6-7 pieces and were located in Ricordi's Chamber were mechanical/enzymatic digestion started. Sample were collected during process to evaluate when islets started to be free from exocrine tissue. Then started dilution of the digested tissue to collect isolated pancreatic islets.
- Tissue were washed to eliminate collagenase and the rinsed in belzer.
- Using a COBE, islets were purified trough continuous ficoll gradient and then washed. In this step were also evaluated islet purity to obtain layers of purity.
- Islets were counted and then cultured.

EPC Isolation

EPCs derived from mononuclear component of peripheral blood were isolated as following:

- Blood were retrieved from voluntary healthy patients.
- Sample of blood were stratified on ficoll. In a falcon 6 ml of blood were added to 3 ml of ficoll gradients. All falcon were centrifuged at 2000g for 30 minutes.
- Serum was eliminated and PBMCs were collected from the ring of cells on the ficoll surface.
- Retrieved cells were washed in M199.
- Cells were then resuspended RPMI containing 5% FBS, VEGF, FGF−2, EGF, and insulin−like growth factor−1(EndoGF) and cultured in fibronectin coated culture flasks.
- After 4 days medium were changed and supernatant were collected in culture flask coated with fibronectin coated flasks.
- Obtained cells were then tested for endothelial (vWF, CD31,CD105,KDR) and stem (CD34,CD133) cells markers by fluorescence microscopy and flow cytometry.

Lentiviral Vector Production

To obtain were used a self-inactivating vector design and a third-generation, 4-plasmid, packaging system containing a CMV-expression cassette for Green Fluorescent Protein (GFP).

Briefly, 293T cells were transfected with a 4-plasmid lentiviral system by the $CaCl_2$ precipitation method. Supernatants were collected 48 and 72 hours after transfection, filtered, and concentrated by 2 successive ultracentrifugations at 19400 rpm in a Beckman SW55 rotor. Pellet containing virus was resuspended in Hanks balanced salt solution (HBSS) and viral preparation titers were determined by p24 enzyme-linked immunoabsorbent assay (ELISA), by GFP titer determination on 293T cells, and by TaqMan real-time PCR determination of transduced proviral genomes.

Vector batches were tested for the absence of replication-competent virus by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for at least 3 weeks (70,71).

EPC Transduction

EPCs were plated on tissue culture plates in RPMI containing 5% FBS and EndoGF. Lentiviral vectors carrying CMV-GFP expression cassette were added to the medium with Multiplicty of infection (MOI) about 20. Cells were transuced overnight. After transduction, medium was removed and fresh medium was added to the culture. Cells were tested with fluorescence analysis and flow cytometry for GFP staining.

Generation of Chimeric Organoids EPCs/GFP-Islets

About $1*10^6$ EPCs GPF were co-cultured in a rotating system in micro-gravity with islets in RPMI containing 5% FBS and EndoGF for about 36 h. After co-culture micro-organoids EPCs GFP-islets were retrieved and coating degree of islets were evaluated by fluorescence microscopy.

Micro-organoids Islets-EPCs (MOIE) were evaluated also for islet integrity and viability. Islets were stained with ditizone to evaluate integrity briefly:

- Islets and MOIEs were seeded in a tissue culture plate,
- Ditizone was added to culture medium and islets were analyzed under the microscope (ditizone stains zinc contained in insulin granules).
- Islet viability was evaluated by differential staining with propiodium iodide (PI) and fluorescein diacetate (FDA) briefly:
- Islets and MOIEs were seeded in a tissue culture plate,
- 20 µl of PI were added to the plates,
- 20 µl of FDA were added to the plates,
- Plate was shaken and then analyzed under a fluorescence microscope.

With this kind of staining live cells are green fluorescent while death cells are stained in red.

Neo-angiogenesis In Vitro

Islets and MOIES were seeded on 24 wells tissue culture plate for 24 hours to evaluate neo vessel formation *in vitro*. Briefly, tissue culture plates were coated with matrigel basement membrane growth factor reduced. When matrigel became solid, islets and MOIEs were seeded in RPMI 5% FBS supplemented with EndoGF. After 24 hours samples were analyzed by inverted microscope to evaluate tubular like structure formation. Image analysis was performed with the MicroImage analysis system (Casti Imaging, Venice, Italy).

Islet Transduction

Freshly purified islets were seeded on 24 wells tissue culture plates. Lentiviral vector carrying a CMV-GFP expression cassette were added to the medium to transduce cells overnight. After incubation islets were washed and analyzed to a fluorescence microscope to evaluate GFP expression.

Islets were digested with a proteolytic and collagenolytic solution called ACCUTASE:

- Islets were washed from culture medium by centrifugation at 1000 rpm for 1 minute
- Pellet were resuspended in accutase solution and then incubated for 15 minutes. During incubation, islets were frequently resuspended to facilitate islet disaggregation.
- After 15 minutes, cells obtained were centrifuged to remove solution.
- Cells were washed three times with RPMI.
- Cells were plated on 24 wells tissue plates and analyzed to flow cytometry and fluorescence microscope to evaluate GFP espression

Flow Cytometry Analysis

Cells were detached with non enzymatic solution. Reaction was blocked with FBS and cells were centrifuged at 1500 rpm for 5 minutes. Then cells were incubated with antibodies appropriate for 30 minutes at 4°C. After incubation cells were washed two times to remove antibody and then analyzed by FACScan (Becton Dickinson Biosciences, San Jose, CA). The instrument was rinsed with particle-free rinse solution for 15 minutes to eliminate the background.

Results

Efficient EPC Transduction by Lentiviral Vectors

Gene therapy has received a lot of attention in the field of cell transplantation, due to its tremendous potential in modifying the biological properties of transplanted cells and/or modifying the responses of the host. However, commonly used vectors are still unfit for the task of safely and efficiently transferring genes into the appropriate target cells. High efficiency gene delivery into non-dividing cells is a major requirement for gene transfer into islet transplantation because beta cells are fragile, do not proliferate, and are therefore refractory to transduction by retroviral vectors. Lentiviral vectors are promising tools in this field, due to their well documented ability to transduce efficiently both quiescent and dividing cells (53).

Starting from these hypotheses we transduced whole islets to evaluate the efficiency of transduction using a lentiviral vector carrying expression cassette for green fluorescent protein (GFP), with the aim to make transduced cells visible. Islets transduced with lentiviral vector showed staining for GFP only into the cells located on islet surface (Figure 8).

This result confirmed that islets are sensitive to vector transduction but the transduction was limited to a small subpopulation of cells. Indeed, digestion with a enzymatic solution of transduced islets, showed that only a few number of cells are positive for GFP (Figure 9). All these data were demonstrated by flow cytometry analysis for GFP. Moreover the GFP staining disappeared after few day of cell culture of these cells.

Starting from these preliminary observation it seemed necessary to analyze new strategies with the aim of carrying genes of interest.

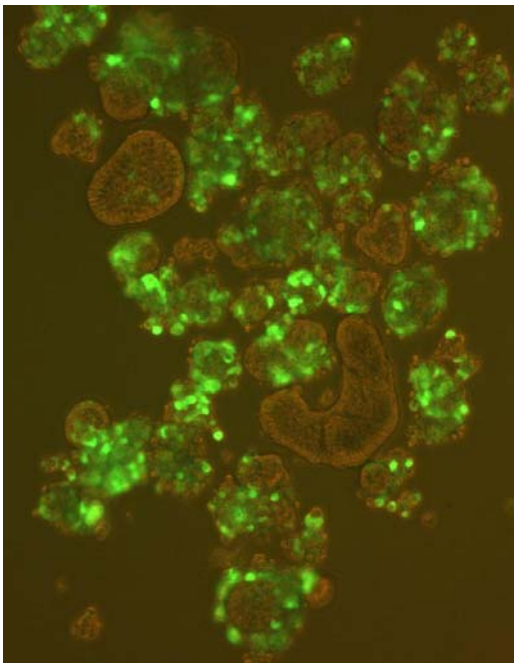

Figure 8. Micrograph of islets transduced with lentiviral vector carrying expression cassette CMV-GFP. Only cells on the surface of islets result positive to GFP staining. Magnification 200X.

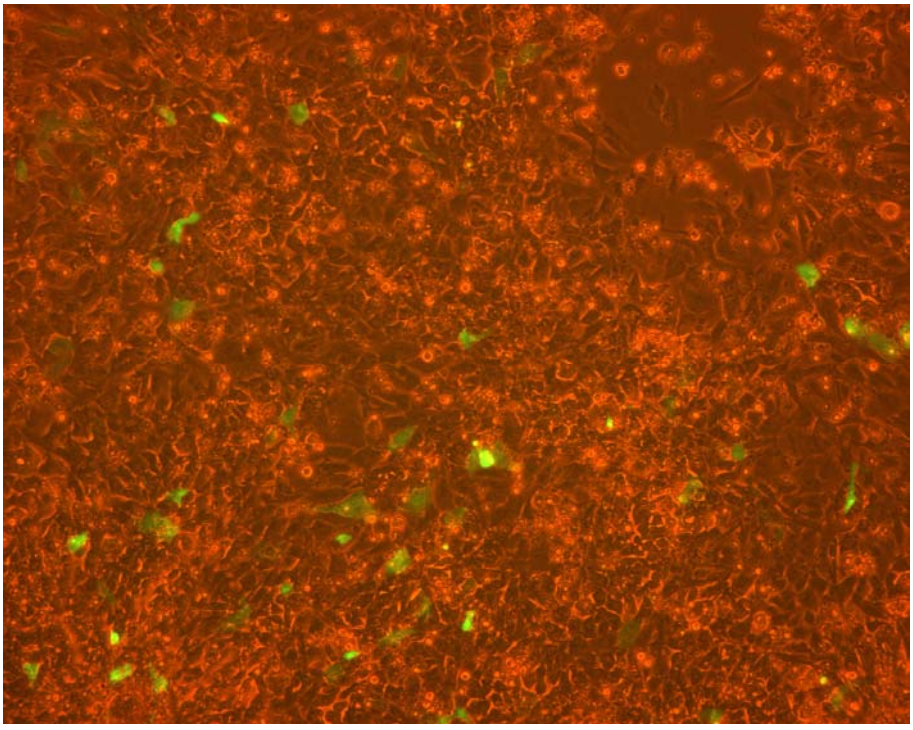

Figure 9. Representative micrografy of islet cells derived from digestion with Accutase. Magnification 400X. Only few cells were transduced by the lentiviral vector.

In addition, an initial post-transplantation inflammatory reaction may damage a considerable mass of tissue (15) and is also favored by the abundance of cells with inflammatory proclivity (i.e. Kuppfer cells) that reside within the liver. It was demonstrated that islet destruction started soon after infusion in portal vein by this thrombotic/inflammatory reaction induced by tissue factors presents on the cells surface. It was recently demonstrated that islet coated with endothelial cells leaded to the inhibition of this reaction with a higher rate of surviving islets to the detrimental effect of inflammation (51, 52, 53). All these consideration leaded to the possibility that islet cells may not be the exclusive target for gene transfer in islet transplantation. It was recently shown that endothelial progenitor cells (EPCs) present in the circulation significantly contribute to transplanted islet revascularization by repopulating transplanted islets (54), and this phenomenon increases transplantation outcome in mice. Indeed, these cells possess angiogenic potential that may be exploited for therapeutic neovascularization (55, 56, 57). Moreover, these cells derived from the mononuclear component of blood and they can be isolated directly from the recipient and expanded *ex vivo.* In this setting we tried to produce chimerical organoids between islets and EPC in order to carry genes of interest and produce a sort of protected niche against thrombotic/inflammatory reaction.

With the aim to evaluate the transduction rate, EPCs were transduced with lentiviral vectors carrying an expression cassette for green fluorescent protein (GFP). After transduction EPCs were analyzed under a fluorescence microscope to assess that transduction succeeded by GFP staining analysis.

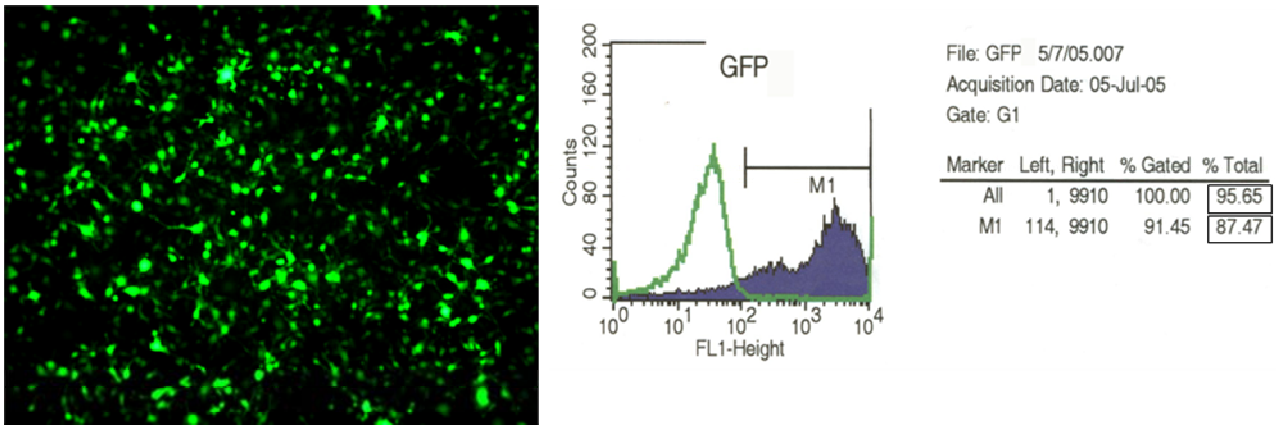

Figure 10. Representative micrograph of EPCs transduced with lentiviral vector. Magnification 400X. Flow cytometry analysis showed that about 90% of cells are positive for GFP.

Obtained cells were named GFP-EPCs and were used for experimental procedures. Before trying co-culture with islets, cytofluorimetric assay was performed with the aim to obtain the percentage of cells transduced by the vector. EPCs transduction resulted in about 80-90% of cells GFP positive (Figure 10).

All these results leaded to the hypothesis that EPCs may be a good substrate for gene therapy approach based on their high susceptibility of transduction with lentiviral vector and the possibility to expand cells *ex vivo*.

Development of a Method to Generate Chimerism Between Pancreatic Islets and EPCs

With the aim to perform chimerism between islets and GFP-EPCs we use microgravity conditions. These conditions are useful to avoid cell adhesion on the surface of the vessel. Indeed, EPCs grew in adhesion condition and are able to adhere on the surface o microplates. In the same way, islets can be cultured in adhesion conditions and, islet cells, can grow out of the whole organoid. To perform chimerism between islets and EPCs we used a rotating system and cells were co-cultured for 24 hours in standard culture conditions (Figure 11).

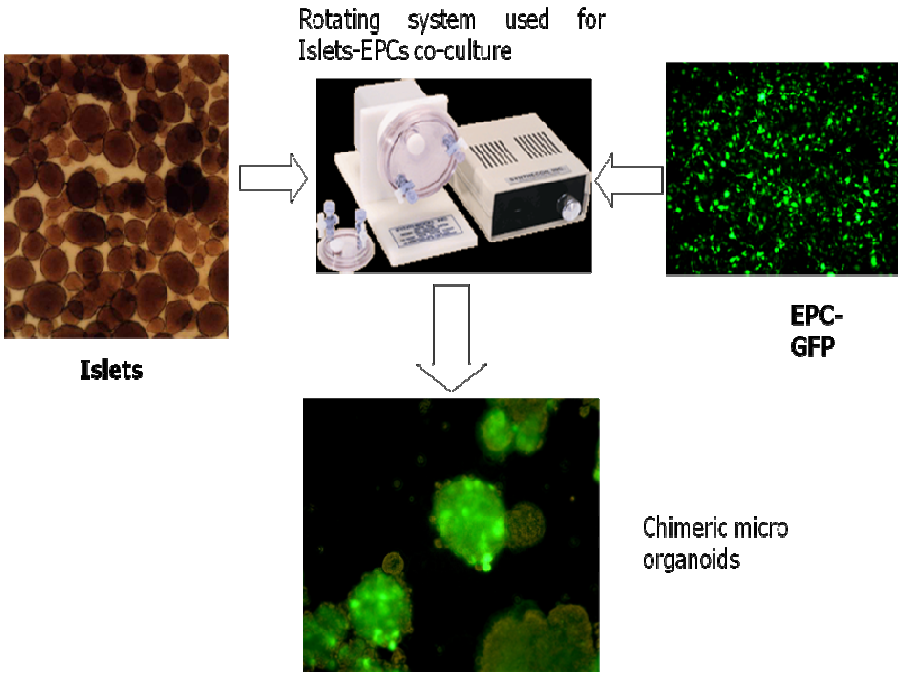

Figure 11. Schematic picture of the system to obtain Chimeric micro-organoids.

After this process, products were analyzed for micro-organoids formation and evaluation of islet surface covering by GFP-EPCs. Under fluorescence microscope, at least 80% of islet surface results covered by EPCs. This procedure was performed with the aim to produce a micro environment to shield beta cells from inflammatory reaction (FIG 12).

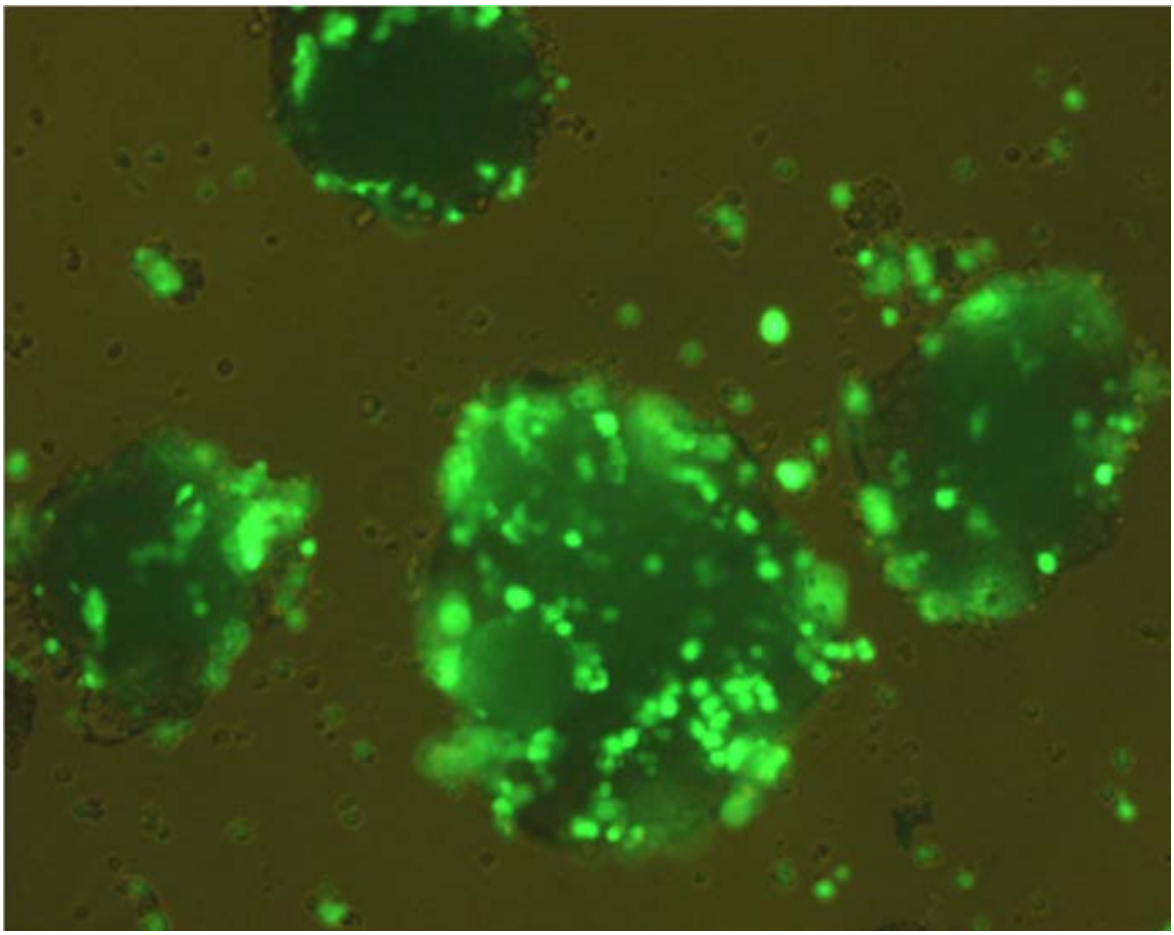

Figure 12. Representative micrograph of chimeric micro organoids islets-EPC GFP. Magnification 400X.

Recent work has showed that a consistent beta cells mass is destroyed by the detrimental effects of a pro-inflammatory and pro-thrombotic environment following islet infusion generated by tissue factors presents on islets after enzymatic digestion (36, 37). It was shown that islet destruction in the bloodstream could be avoided by islets-endothelium chimerism (58). Moreover, EPCs can be derived from the recipient's peripheral blood to avoid autoimmune phenomena and create a protect environment against the immune-system. This approach will allow to design strategies based on transfer of genes of interest into EPCs, due to their ability to be isolated from peripheral blood and to be cultured, expanded and manipulated *ex vivo*.

Effect of Chimerism on Islet Cell Viability

One of the problems derived from islet manipulation *in vitro* is their fragility. Indeed, islets are extremely fragile and all manipulations can affect their structural integrity. These observations underlined that this process can be detrimental for islets.

After chimerization, islets will be analyzed with the aim to evaluate if this process can affect islet viability.

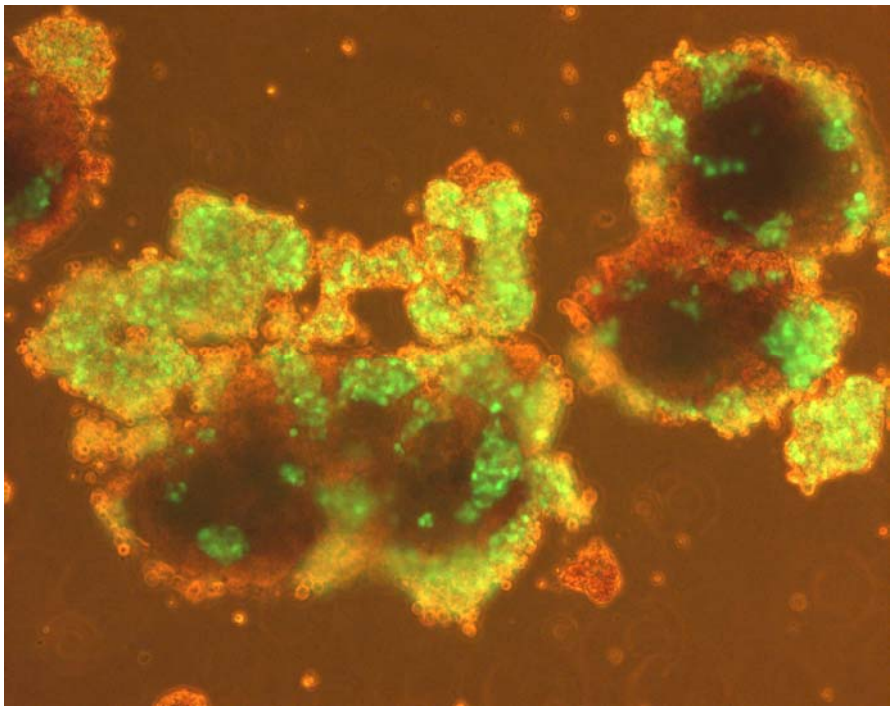

Figure 13. Representative micrograph of micro-organoids stained with ditizone. Magnification 200X.

Then, islets were stained with dithizone, to evaluate degranulation after the process. Indeet, dithizone is able to bind zinc contained the insulin granules. Beta cells under stress condition then results in poor or no staining for dithizone. In this setting, the evaluation of viability by dithizone staining result very good for islets contained in MOIE in comparison with islets alone (Figure 14).

To confirm data obtained by dithizone staining, islet viability was evaluated by propidium iodide (PI) and fluorescein diacetate (FDA) method. Briefly, PI is able to stain death cells with red fluoscence instead of FDA that give out green light.

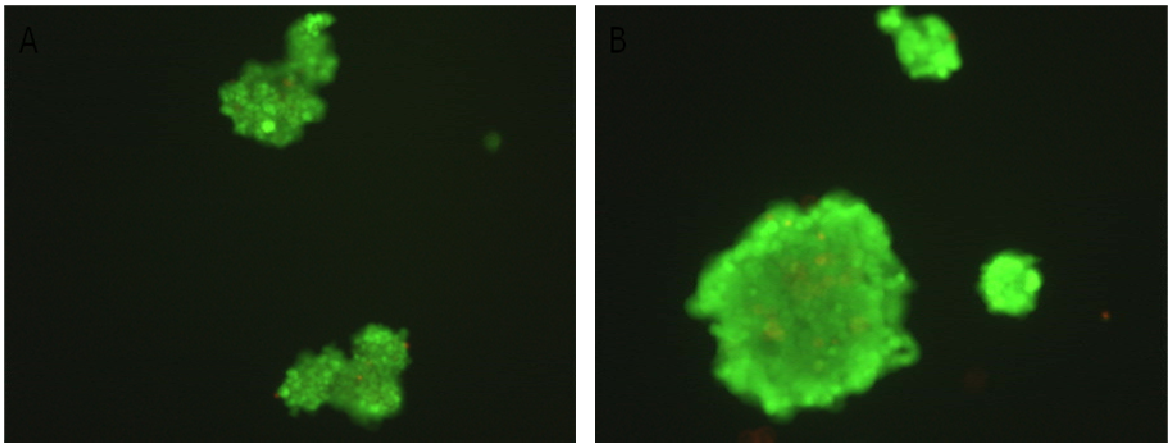

Figure 14. Representative micrograph of islets and MOIE staining with PI (Red) and FDA (Green). MOIEs (right) showed a better viability in comparison with islets alone (left). Magnification 400X.

By fluorescence analysis, resulted a very low rate of death cells in MOIEs after chimerization process to underline that this procedure is effective to make complexes between islets and EPCs and moreover do not affect beta cells viability (Figure 14).

Moreover, by dithizone staining we can observe that there are not morphological compromission of islet integrity.

Maintenance of Pro-Angiogenic Behavior of EPCs After Microchimerism

One of the main problems in islet transplantation is the revascularization of transplanted tissue. Many studies demonstrated that this process is slow and the rate of neo-formed vessel is lower in transplanted than native islets. Poor vascular graft and low angiogenetic processes lead to a big loss of transplanted tissue in the early phase post-transplantation. Moreover, use of immunosuppressant drugs affects revascularization by inhibiting it.

A

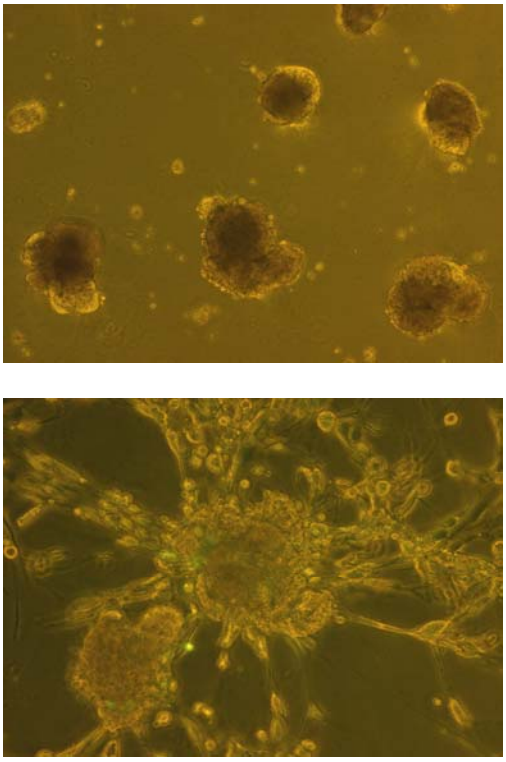

Figure 15. Representative micrograph of islets and MOIES *in vitro* neoangiogenesis. Islets (A) showed no tubular like structures in comparison with MOIEs (B) after 24 hour of culture on matrigel plugs. Magnification 400X.

After chimerization procedure EPCs proangiogenic behavior was evaluated. MOIEs obtained, were seeded on tissue culture plates and cells outgrowth was analyzed for GFP presence under an inverted fluorescence microscope. Fluorescence analysis showed that, after 24 hr EPCs outgrew from MOIEs (Figure 15 and Figure 16). Moreover, MOIEs seeded on tissue culture plates coated with matrigel, an analogous of basement membrane, cells produced tubular vessel like structure.

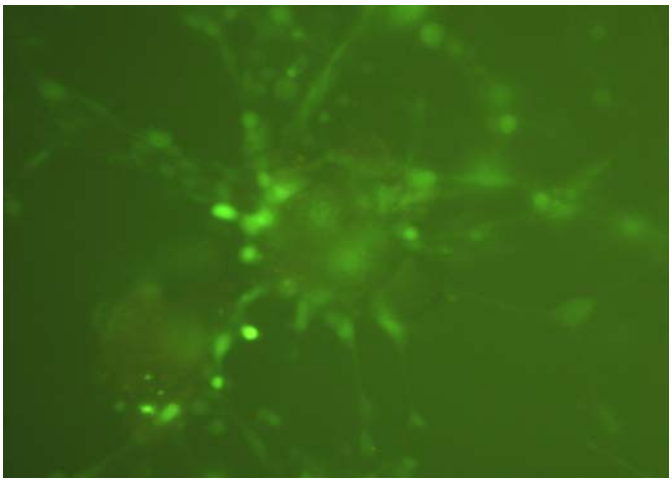

Figure 16. Representative fluorescence micrograph. Tubular like structures in MOIEs are derived from GFP-EPCS. Magnification 400X.

These observations lead to the conclusion that EPC behavior is not affected by chimerization procedure, and that these cells could be a good tool to improve revascularization after transplantation. Moreover, due to EPCs growth rate, these cells can be a good substrate to carry genes of interest in gene therapy.

Discussion

Pancreatic islet transplantation can represent a suitable way for substitutive therapy of beta cell function in patients affected by Type I Diabetes Mellitus. However, many problems have yet to be solved related to organ procurement and conservation, islet isolation and, above all, transplantation and revascularization into liver sinusoids after portal vein infusion. Moreover, recent studies demonstrated that infused tissue is lost soon after portal infusion by activation of inflammatory reaction mediated by tissue factor derived from the *ex vivo* digestion process and exposed on the surface of isolated islets (36, 37, 60). Also, new immune-suppressive therapies with lower toxicity for islets and kidney are necessary. Indeed, a detrimental role of rapamycin in the revascularization processes was recently demonstrated. Rapamycin acts on the endothelial cells present in islets by inhibiting cell proliferation and motility, that are essential in neo-revascularization of tissue after transplantation.

The first limiting phenomenon that occurs in islet transplantation, soon after infusion of tissue in portal vein, is the activation of a thrombotic/inflammatory reaction derived from the contact between the infused tissue and blood. Bennet and colleagues (60) demonstrated *in vitro* that exposure of islets with non heparinized ABO-compatible human blood elicited prompt binding and activation of platelets, binding of leukocytes, and activation of the coagulation and complement systems, resulting in disruption of the integrity of the islets.

All these events are activated by platelets binding on islet surface, with a rapid formation of a capsule around islets. Moreover, this processes and activation of immune system are the basis of loss of tissue in the early periods after islet transplantation with a consistent loss of tissue soon after infusion.

To address the immunological problems and increase the limited supply of human tissue available for grafts, attempts have been made to transfer exogenous DNA into mature beta

cells. Several viral gene delivery systems, such as Moloney-based retroviral vectors, adenoviral vectors, adenoassociated vectors, or lentiviral vectors have been employed for *ex vivo* gene transfer into pancreatic islet cells. Moloney-based retroviral vectors were inefficient in transducing mature insulin-expressing cells due to the lack of proliferation of mature beta cells. Adenoviruses were highly efficient in transducing non-dividing beta cells, however, the expression of the transgene was transient. This could be due either to the absence of genome integration of the virus or to the elimination of transduced cells by the host immune system.

In this study, we performed preliminary experiments combining lentiviral gene transfer technology and *ex vivo* cell manipulation to allow for the generation of engineered microorganoids. The ultimate scenario is the delivery of immunomodulatory and proangiogenetic factors into the tissue to be transplanted, for the purpose of improving engraftment efficiency and neovascularization *in vivo*.

Lentiviral vectors are able to transduce quiescent cells, and transduced genes are permanently integrated into the target cell's genome. Induction of specific tolerance for αGal under nonmyeloablative conditions was achieved by gene transfer into BM cells using a lentiviral vector encoding porcine GalT in a mouse model of heart transplantation. Tolerance induction to tissues expressing αGal plays a key role in successful xenotransplantation, as this carbohydrate is a major target of the immune response to xenografts. Mice reconstituted with GalT accepted αGal heart grafts over 100 days. Transduction with lentiviral vectors results in chimerism at levels sufficient to induce long-term tolerance under nonmyeloablative conditions (61).

Based on these premises, we established optimized protocols to transduce pancreatic islets with lentiviral vectors carrying a cassette of expression CMV-GFP. Islet analysis after transduction showed that a limited number of cells were transduced, mostly located at the islet surface.

Johansson and colleagues demonstrated that chimerical organoids islets-endothelial cells can be produced and they showed that endothelium is able to protect surrounded islets from detrimental effect of IBMIR (58). Recently, it was demonstrated that adding mesenchymal stem cells to this chimerical organoids rate of endothelial cells was increased and endothelium exerted more efficiently its protective and angiogenetic effects (64).

We therefore evaluated the possibility of using a combined approach of cells and gene therapy to protect islets both from IBMIR then from immune system. In our model were used endothelial progenitor cells (EPCs). EPCs can be isolated from peripheral blood of patients and have a strong proliferative and angiogenic potential. Transduction of EPCs with a lentivector carrying CMV-GFP expression cassette demonstrated about 90% of transduced cells, analyzed to fluorescent microscope and flow cytometry. These data confirmed that EPCs can be a good target lentiviral for transduction.

Engineered cells were co-cultured with islets in micro gravity in a rotating system to avoid adhesion to the substrate. After 24 hour of co culture islets demonstrated about 90% of surface was covered from EPCs. Islet integrity in micro organoids were evaluated to analyze effect of the chimerization process. Staining with ditizone showed no morphological damages of islets. Indeed, ditizone stains zinc contained in insulin granules. Islet viability was also not affected from this process. These data showed that production of EPCs-islet micro-organoids can be generated without affecting islet viability. Pro angiogenetic behavior of EPCs in micro organoids were also evaluated. Compared with islets alone, MOIEs showed formation of tubular like structure after 24 hours of culture in 24 wells tissue plates coated with matrigel, showing no modification in angiogenic behavior of EPCs in MOIEs.

These data will lay the foundation of further experiments to explore the clinical applicability of *ex vivo* genetic manipulation to islet transplantation. Although islet transplantation has been proven successful, its clinical impact will remain marginal until new strategies to protect islet survival and function will allow expanding the number of transplants. Generation of local immunoprivileged environments for islet engraftment by gene transfer of immunoregulatory molecules may lead to a reduction in systemic immunosuppression requirement, thus strongly improving the risk/benefit cost of islet transplantation.

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