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Tesi di Dottorato

"Glycaemic control with Mesenchymal Stem Cells and Endothelial Progenitor Cells in an experimental model of pancreatic islet transplantation"

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ABSTRACT

Insulin-Dependent Diabetes Mellitus (IDDM or type 1) is an autoimmune, chronic disease characterised by hyperglycaemia, resulting from an inflammatory infiltration of the islets of Langerhans. The selective destruction of β-cells leads to a lower insulin secretion from the endocrine pancreas. The mainstay treatment for IDDM patients is chronic insulin injection. Although insulin therapy has dramatically reduced mortality from diabetes, patients often incur in complications such as nephropathy, neuropathy, angiopathy and retinopathy. Moreover, patients are risk severe and sometimes fatal hypoglycaemic events.

Pancreas transplantation is currently the only therapeutic approach to restore normoglycaemia and maintain long-term glucose homeostasis; moreover, this procedure improves patients' quality of life.

An alternative to the replacement of the whole pancreas is the transplantation of islet of Langherans cells, isolated from donor pancreata and infused into the recipient's liver via the portal vein.
Compared to solid organ transplantation, the advantages of islet transplantation consist in a relatively simple surgical procedure with low incidence of peri-operative risks. Nevertheless, the particular structure of pancreatic islets resulted in being injured after the isolation procedure.
However, the recurrence of immune response after transplantation and the diabetogenic and growth-stunting side effects of immunosuppressants are major challenges to the application of islet transplantation.

In the last decade many studies have demonstrated the efficacy of cell therapy either with Mesenchymal Stem Cells (MSCs) or Endothelial Progenitor Cells (EPCs) treatment when co-transplanted with pancreatic islets. The first type of cells were reported to modulate the immune response in an allogeneic transplant, preventing the graft-versus-host disease (GVHD) and also improving graft function in the long-term by maintaining glucose homeostasis. The EPCs showed to have strong revascularization properties in several diseases, such as cardiovascular disorders, atherosclerosis and diabetes.

This thesis aims to investigate the role of MSCs and EPCs in prolonging graft survival of pancreatic islet transplantation in a chemically induced rat model of

type 1 diabetes in order to prolong the graft function and reach normoglycaemic levels in the long-term.

We used a rat model to investigate the effect of MSCs and EPCs in combination with islets of Langerhans (700 IE + 500,000 EPCs and 700 IE + 500,000 MSCs) in a syngeneic and an allogeneic diabetic-induced rat model which underwent pancreatic islet transplantation via the portal vein. These types of transplants were compared with islet alone treatment (700 IE), either syngeneic or allogeneic.

We obtained the reversal of the diabetic status in animals up to 6 months after the transplant when they had received islets and EPCs, and up to 75 days post transplant when they had received islets and MSC therapy. The glycaemic values were also confirmed by intraperitoneal glucose tolerance test measures for animals transplanted with IE and EPCs either in syngeneic or allogeneic models.

From data obtained from molecular biology assays on *ex vivo* liver tissues deriving from transplanted animals, we observed that a regulation in the revascularization and angiogenesis genes (VEGF-A, ANG-1, PECAM-1, SDF-1) occurred. Thus, EPCs could act through a regulatory mechanism as shown by their angiogenic gene expression.

These data suggested that both MSCs and EPCs were able to revascularize pancreatic islets and improve the syngeneic graft survival up to a complete healing in our diabetic animal model.

1. INTRODUCTION

1.1 Diabetes Mellitus

Diabetes mellitus is a chronic disease characterised by hyperglycaemia, which is caused by lower insulin secretion from the endocrine pancreas or by insulin resistance of peripheral tissue.

According to data available from WHO, about 346 million people worldwide have diabetes, in 2004 an estimated 3.4 million people died from the consequences of high blood sugar, and more than 80% of diabetes deaths occur in low- and middle-income countries. Furthermore, it is predicted that diabetes deaths will double between 2005 and 2030 (http://www.who.int/diabetes/en/index.html).

Two main types of diabetes mellitus are currently known: type 1 (Insulin-Dependent Diabetes Mellitus, IDDM) and type 2 (Non-Insulin-Dependent Diabetes Mellitus, NIDDM).

The first type is an autoimmune disease that mainly affects young people and is caused by the presence of an inflammatory infiltrate in islets of Langerhans and a selective destruction of pancreatic beta cells which provokes insulin deficiency[1].

NIDDM generally has its onset in persons of over 40 years of age; it is untied up to immune mechanisms and relates to cellular insulin-resistance by peripheral tissues[2]. Furthermore, it is linked to major metabolic dysfunctions such as obesity, altered insulinic secretion and functionality, and also high endogenous glucose levels. Insulin resistance is the basis of the disease, nevertheless high blood glucose levels derive from a combination of reduced insulin secretion. The latter can be due to a failure in β cells functionality, a chronic exposition to fatty acids and hyperglycaemia[3]. Type 2 diabetes is the most common form of diabetes mellitus as approximately 80% of diabetic patients are affected by NIDDM. Due to changes in human behaviour, increasing body mass index, obesity and life style, there is a rising incidence of the disease[4]. Type 2 diabetes develops with a minor tolerance of glucose in the blood after a progressive increase of insulin resistance deriving from tissues. Insulin resistance is balanced at the beginning of the onset by an increase in the functionality of β cells (from an hyperplasia of β cells) and it is subsequently characterized by a loss of the mass in these (the same) cells.

Only 5-10% of diabetic population is affected by type 1 diabetes, although it represents the main social and health problem. As the disease rapidly develops, young people are mostly affected and there are long-term consequences due to serious complications with the cardiovascular, nephritic and nervous system[5].

Nowadays, 5 million people in the world are affected by IDDM and millions of new cases are being registered with severe diabetic complications. In contrast to type 2 diabetes, IDDM is connected to chromosome 6 gene modification in correspondence of the Human leukocyte antigen region (HLA), responsible for gene codification of class II Major Histocompatibility Complex, (MHC-II), which is able to bind and present antigen to T-helper lymphocytes. This triggers the immunological response. In the HLA-D region, either DR3 or DR4 alleles in DR gene class are present in the majority of people affected by IDDM. The only factors that cannot lead to the income of the disease are genetic ones; several environmental factors, difficult to be revealed, play a key role in its appearance. The most probable and suspected factors are viruses, such as enterovirus[6], rotavirus[7] and rubella[8]. In type 1 diabetes, the genetic alteration of MHC-II molecules of the HLA system compromises the identification T cells-mediated; activated clones of T cells identify and destroy β cells with a consequent severe insulin deficiency. These clones are able to escape thymus check during the process of tolerance induction and, once released into the blood flow, can be activated and destroy self antigens.

More than 30 decades ago it was observed that the antibodies directed against the sections of pancreatic islets were present in the serum of patients affected by type 1 diabetes. These antibodies were named ICA, "islet cell antibodies", directed against islet cells. The main identified auto-antigens was the isoform 65 of glutamic acid decarboxylase (GAD65)[9], the transmembrane protein that belongs to the protein tyrosine phosphatase family (IA-2)[10] and the endogenous insulin (IAA)[11]. In the pre-diabetic phase, auto-antibodies directed against islet cells, ICA, are the most useful for predicting the disease. In most cases, in patients who developed diabetes later, ICA levels were present since they were 5 years old[12]. This implies that the autoimmune process remains in a sub-clinical phase for many years and clinical signs are not evident until 80% of β-cells are destroyed. In the early phase of diagnosis, about 90% of patients affected by IDDM have a high auto-antibody level (Type 1A DM); the remaining 10%, who have no level of auto-antibodies in their serum, develop type 1 diabetes in adult age, the *maturity-onset diabetes of the young* (MODY). This can be the

result of a dysfunction in β-cells with a dominant autosomic inheritance (Type 1B DM)[13].

Some patients affected by type 1 diabetes exhibit an insulin resistance typical of type 2 diabetes which can lead to neurological and vascular diseases[14]. There is an important controversial overlap between type 1 and type 2 diabetes: about 10% of patients affected by type 2 diabetes have a high level of auto-antibodies in the blood[15].

Long-term studies have shown that tight control of blood glucose either through conventional intensive insulin treatment, self-monitoring and the patient's food education, significantly prevents the onset of the disease and delays the development of chronic complications associated with it[16]. Nevertheless, in these patients severe hypoglycaemic events can occur which, in some cases, can become fatal[17].

A new and promising approach to improve glucose homeostasis is based on insulin pump and insulin secreting devices, but the development of reliable glucose sensory technology at present remains a limiting factor.

1.2 Pancreas Transplantation

Pancreas transplantation is at present the only therapy able to restore normoglycemia and maintains long-term glucose homeostasis[18]. The first pancreas transplant was performed in 1996 in combination with a kidney transplant in a patient affected by IDDM with terminal renal insufficiency; it proved to re-establish normoglycemic conditions without endogenous insulin injection. Nevertheless, primitive surgical techniques reported a high level of morbidity, graft difficulty and a low survival rate amongst patients[19]. Improvements in surgical techniques, the introduction of immunosuppressive therapies and the monitoring of rejection grade and organ functionality after transplantation reduced patients' morbidity and prolonged graft survival[20].

Three distinctive types of pancreas transplantation have been identified: simultaneous kidney-pancreas transplantation (SPK), pancreas after kidney transplantation (PAK), in patients who have already received a kidney transplant because of severe renal insufficiency, and a pancreas transplantation alone (PTA). Different kinds of surgical approaches can be estimated with their results and risks. About 25,000 patients throughout the world undergo pancreas transplantation, because

it improves quality of life and regresses several diabetic complications[21]. Simultaneous kidney-pancreas transplantation is considered to be standard treatment for patients (selected on main criteria) with type 1 diabetes and end stage renal insufficiency[22]. Numerous studies show the beneficial effects on survival, quality of life and the reversal of further complications related to diabetes in these patients[23].

Simultaneous kidney-pancreas transplantation can indeed solve the metabolic and renal problem together, requiring no further treatment for insulin-dependence and no need for dialysis treatment (also in cases where the transplant is executed before entering into the dialysis). Another advantage is that possible rejection can be diagnosed early related to functional and structural modifications by the kidney. In this case, the right surgery is able to recover the kidney, and, above all, the pancreas.

Though pancreas transplantation can lead to insulin independence (in the first year after transplantation), more than 80% of patients who have received transplants have been exposed to numerous and severe post-operative risks due to the complexity of surgical methodology[24], such as graft vessel thrombosis, intra-abdominal haemorrhages, graft organ pancreatitis, and pancreatic fistulas development etc., requiring subsequent laparotomy and producing further graft loss. As a matter of fact some aspects of pancreas transplantation have to be improved, such as the role of surgical procedures, the selection criteria of the recipients, the correct immunosuppressive therapy and the individual impact of both metabolic and genetic immunological factors on survival and graft functionality.

Post-operative complications limit the wide diffusion of pancreas transplantation, which is a surgical procedure with high morbidity and occasional mortality[25].

Because of surgical-related risks and immunosuppressive treatment, pancreas transplantation is limited to patients affected by type 1 diabetes with severe clinical complications; in such cases the severity of the disease justifies the risks related to the transplant procedure. Finally, pancreas transplantation is not considered a valid alternative for young patients who do not have apparent complications.

1.3 Rejection and immunosoppressive treatment within pancreas transplantation

In order to avoid the functional deficit of a living organ, patients are exposed to organ transplantation.

Transplantation is a procedure involving cells, tissues or organs sampling from an individual (donor) with the graft being re-transferred to a different one (recipient). Based on both donor and recipient identity, the following types of transplantation can be identified:

Autotransplantation: the transfer of graft tissue from one part of the body to another in the same individual;

Isotransplantation: organs or tissues from a donor are transplanted in a genetically identical recipient (i.e.homozygous twin);

Allotransplantation: the most common clinically performed transplant whereby a donor gives an organ to a genetically different recipient;

Xenotransplantation: a tissue or an organ from a donor is transferred to a different species recipient (maximum genetical variability).

The introduction of a foreign organ or foreign tissue into the organism can lead to the development of rejection, due to the recipient's immunological system identifying the graft donor tissue as foreign cells which either give a humoral or cellular response, with consequential graft loss. The intensity and time required to verify a rejection grade depends on the donor and recipient genetic compatibility. If there is perfect compatibility (auto- or iso-transplantation), no rejection grade will develop. Furthermore it is compulsory to prevent rejection infiltration by selecting the best donor possible and the right immunosuppressive treatment.

Several rejection grades have been classified according to the histopathological characteristics and the time gap between the operation and the onset of rejection.

Hyperacute rejection focuses on haemorrhage and vessels thrombosis: it begins in the early minutes after transplantation, particularly after donor vessel anastomosis with recipient vessels. This causes the host's preformed circulating antibodies to bind to the antigens expressed on endothelial cells of the graft.

This link activates the complement system with further alterations on graft vessel walls, thus favouring the development of vessels thrombosis. Endothelial cells have been induced to secrete high molecular weight types of vonWillebrand factor,

which are able to mediate adhesion and platelet aggregation. All of these phenomena contribute to thrombosis and vessel closure and graft tissue also has an irreversible ischemic impairment. Hyperacute rejection is mainly due to G immunoglobulin, IgG, which has turned into proteic alloantigens due to the development of foreign MHC molecules after a blood transfusion, prior to the graft procedure or repeated pregnancies. If their levels are low, the phenomenon develops slowly, over several days, before establishing itself.

Acute rejection occurs one week after transplantation mediated by T lymphocytes, macrophages and antibodies inducing parenchymal or vascular impairment.

The delayed onset of this type of rejection is due to the time required for T effector lymphocytes to differentiate themselves and for the production of antibodies to mediate the reaction[26, 27]. They can induce the direct lysis of grafted cells or cytokines production by recruiting and activating inflammatory cells responsible for tissue necrosis[28]. Acute rejection appears in vascularized grafts, just as in kidney grafts, mediated by CD4+ and CD8+ lymphocytes often detected by acute inflammation through the microvasculature: the first targets of this process are endothelial cells[29]. The graft infiltrate present in acute cellular rejection is mainly made up of graft alloantigens specific CD8+ T cells[30]. CD4+ T lymphocytes can exert their role through cytokines production and the induction of a delayed type hypersensitivity (DTH) reaction; these cells alone can induce an acute rejection[31].

Over a long period of time a *chronic rejection* is characterized by graft tissue fibrosis with normal structural loss[32]. Nowadays the "graft arteriosclerosis" or "accelerated arteriosclerosis" is recognized as the main cause of graft failure and appears as an arterial vessel occlusion due to smooth muscle cell proliferation of the intima vessel. This condition is an expression of a delayed hypersensibility reaction in the organ parenchyma where lymphocytes, activated by alloantigens expressed on endothelial cells, induce macrophages to secrete growth factors to smooth muscle cells. Therefore, macrophages activation and subsequent mesenchymal stem cell growth factor production, just like platelet-derived growth factor (PDGF) which stimulates proliferation of fibroblasts and synthesis of collagen, are typical fibrosis-based rejection grade factors. Without the right immunosuppressive treatment, the transplanted organ gives rise to increasing impairment mediated by the recipient's immunological system.

In the last forty decades, the development of immunosuppressive drugs have changed solid organ transplantation (the kidney, heart, lung, liver and pancreas) into a

clinical routine procedure. Increased short-term graft survival is due to better rejection prevention and its more efficacious treatment in cases where the process appears.

Immunosuppressive treatment can induce its effects by acting through the activation of T cells, cytokines production or clonal espansion. Immunosuppression standard protocols consist of early therapies and maintenance to prevent acute rejection and predict short cycles of more aggressive therapies for the treatment of further acute rejection events.

1.3.1 *Early immunosuppression*

Very strict immunosuppressive protocols are applied in the immediate post-operative days when the grade of rejection is at its maximum. Most patients' initial therapy consists of high drug doses used in maintenance therapy, even though in some cases potent antibodies anti-T cells are administered. The latter protocol is mainly used in subjects with higher risk of acute rejection (children, sensitive patients who have had previous transplants and transfusion or multiple pregnancies).

Standard induction therapy consists of a 7-14 days cycle with p*olyclonal anti-lymphocytes antibodies or anti-CD3 antibodies.* These drugs successfully decrease the incidence of acute rejection and its extent, though their long-term use is not advised since they can increase severe infections and malignancies.

In early therapy, the efficacy of several clinical trials has shown that some human monoclonal antibodies (basiliximab, daclizumab) are active towards interleukin-2 receptor (IL-2) on T cell activated surfaces, whose inhibition causes a specific immunosuppressive effect.

1.3.2 *Maintenance therapy*

The immunosuppressive state of maintenance in grafted patients can be achieved by the right combination of immunosuppressive drugs. This protocol reduces side effects and blocks several molecular mechanisms involved in T cells activation. The standard combination is generally made up of *corticosteroids, calcineurin inhibitors* and *antiproliferative agents.*

Transplant characteristics such as donor/recipient HLA compatibility rate, the organ to be transplanted and the patient's state of health, all influence the choice of

and doses of immunosuppressive drugs. Actual immunosuppressive protocols, used in pancreas transplantation require a combined use of drugs: tacrolimus and mycophenolate mofetil in 80% of cases or cyclosporine and mycophenolate mofetil in 20% of cases. Cyclosporine and tacrolimus belong to calcineurin inhibitors; they both bind to cytoplasmatic receptors (cyclophyllin and FKBP-12) and resulted as calcineurin-inactivating complexes, a T cells receptor signalling pathway enzyme, preventing the IL-2 gene transcription and relative T lymphocytes-dependent activation. The introduction of Cyclosporine A in graft treatment has significantly increased graft survival even though the drug has severe side effects such as acute and chronic nephrotoxicity, hypertension, hepatotoxicity, neurotoxicity and hyperlypidemia etc.[33].
In order to minimize cyclosporine toxic effects and at the same time maintain an adequate immunosuppressive effect, its dose has to be monitored and personalized for each patient.

Tacrolimus is a highly nephrotoxic, neurotoxic and diabetogenic drug, but in response to cyclosporine it reduces hypertension, hyperlipidemia, hirsutism and gingival hypertrophy cases[34].

Mycophenolate mofetil is a micophenolate semisinthetic acid derivative, which acts through the de novo selective synthesis block of purins and several lymphocytes responses; among the various collateral effects, severe gastrointestinal outcomes and an increasing infectious risk by cytomegalovirus were present.

The combination of tacrolimus and mycophenolate mofetil has largely substituted that of cyclosporine and mycophenolate mofetil due to evidence of a rejection grade reduction and minor side effects.

The use of sirolimus in different concentrations, in association with other immunosuppressants has led to great advantages in grafted patients.
Sirolimus (Rapamycin) is an immunosuppressive drug which is able to inihibit T cells response towards cytokines stimuli, by blocking IL-2 receptor intracellular signalling and inhibiting T lymphocytes development. Sirolimus binds to the same intracellular protein over which the tacrolimus acts (FKBP-12) and is often used together with tacrolimus or cyclosporine. Its use can reduce an associated drug quality with a consequent decrease in side effects.

Among the various drug combinations, tacrolimus/sirolimus is the most frequent, followed by sirolimus-cyclosporin, tacrolimus-sirolimus-mycophenolat

mofetil and mycophenolate mofetil-sirolimus; sirolimus is used alone in only very few cases[35].

In some transplant centres, corticosteroids continue to be used in immunosuppressive treatments but in most cases it has been a real challenge to turn towards drug free protocols in order to minimize glucose intolerance, dyslipidemia and osteoporosis.

Calcineurin inhibitors can also show these side effects, including the β-cell damage within islets because of apoptosis[36]. Recent times have seen an increase of patients treated with antibodies, mostly those receiving a non-combined pancreas transplant or simultaneous kidney-pancreas transplant[37]. In 90% of cases the treatment entails a combination of several antibodies but occasionally just one alone is used[38].

The most involved anti-lymphocytes agent is the monoclonal antibody OKT3, together with polyclonal treatments, such as anti-thymocyte globulin (ATG). T anti-lymphocytes antibodies treatment (monoclonal or polyclonal) can begin immediately after the post-operative period or during the pre-operative protocol in order to create an immunoprotective condition within the organism.

1.4 Pancreatic islet transplantation

The islets of Langerhans are a promising alternative to whole organ transplantation; they are collected from a donor's pancreas and injected through the portal vein into a recipient's liver (figure 1).
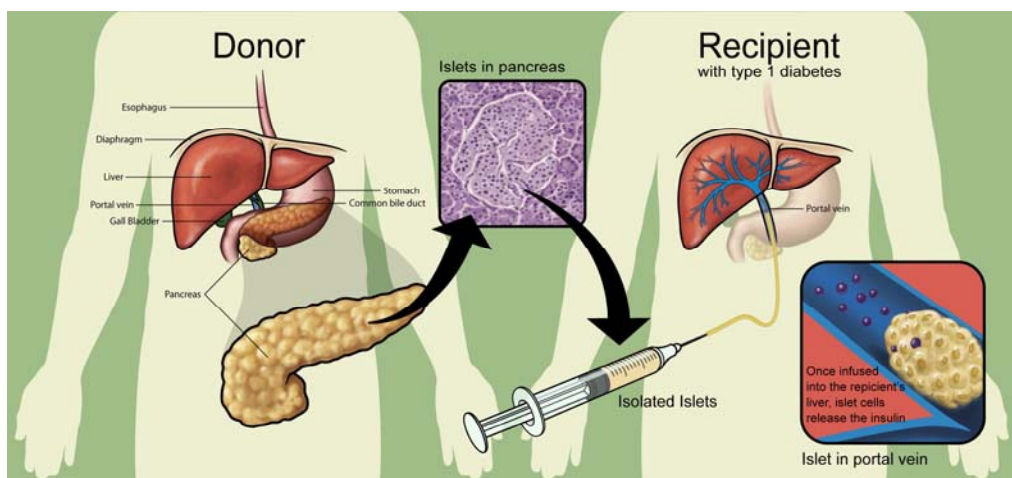

Figure 1. **Islet transplantation**. Islets were purified and infused via the portal vein of diabetic recipients, once they were extracted and isolated from the pancreas.

In 1893 the English surgeon W.Williams attempted to transplant the extract of sheep pancreas into a 15-year-old boy with a severe form of diabetes, marking the first pioneeristic xenograft transplant[39].

More than 50 years later, in 1967, Paul Lacy suggested that a better solution to exogenous insulin treatment was the transplant of pancreatic islets[40]. Later, in 1972, Lacy successfully reversed diabetes for the first time in a murine model of chemically induced diabetes through a pancreatic islet infusion[41].

It was only in 1986 that an automated method for islet isolation with a sufficient number of islets to be transplanted was introduced into the clinical setting, and in 1989 the Paul Lacy group exploited this method to reverse diabetes and obtain insulin independence after human islet transplantation[42]. Unfortunately the graft failed a few days after surgery, perhaps due to an inadequate immunosuppressive regimen.

Until 1990, none of the above-mentioned transplants were successful until the group from Pittsburg, USA, performed the first series of human islet allograft, prolonging insulin independence through steroid-free immunosuppressants treatment (FK506). The patients remained insulin independent up to 5 years after the transplant. The long-term survival of intrahepatic human islets was confirmed by liver biopsies, revealing that allogeneic human islets were successfully engrafted in the hepatic microenvironment[43].

In 1996 in Giessen, Germany, there was an improvement in peri-transplant management as well as the immunosuppression results in 100% initial islet-graft function and 40% insulin independence after one year.

In 1999 a very successful protocol was achieved in clinical trials by the Edmonton group which combined islet transplantation with the use of Rapamicyn by implementing a steroid-free protocol. The result was a series of transplants with insulin independence in 7/7 human islet-cell transplant recipients within the first year[44]. All patients received pancreatic islets from 2 donors (only in one case 4 donors were necessary). The Edmonton protocol used a combination of immunosuppressive drugs, including Daclizumab (Zenapax), Sirolimus (Rapamune) and Tacrolimus (Prograf). Daclizumab was given intravenously right after the transplant and then discontinued, while Sirolimus and Tacrolimus, the two main drugs that maintain the immune system preserved from the destruction of the transplanted islets, must be administered throughout the patients' life[44].

The difference between the Edmonton protocol and previous ones was a convenient number of high purity level islets, but, most of all, a less diabetogenic and steroid-free use of immunosuppressive drugs.

Islet Transplant Registry reported more than 450 allogeneic pancreatic islet transplantations between 1974 and 1999 in patients affected by type 1 diabetes, but less than 10% reached insulin independence within one year, though 28% of patients secreted high levels of C peptide. Several factors contributed to the failure, such as a poor transplanted islet mass, inadequate anti acute rejection treatment, diabetes autoimmune recurrence and extremely toxic immunosuppressive drugs such as cyclosporine and glucorticoids. The clinical introduction in the Edmonton protocol led to progress in pancreatic islets transplantation, with the general acceptance of islet transplantation as a feasible clinic therapy, especially for the treatment of patients affected by type 1 diabetes with developed frequent hypoglycaemic episodes.

A further study in the University of Alberta on a 66-year-old patient showed that 82% of patients who received a transplant remained insulin independent up to one year after transplantation; this percentage decreases as the years go by: up to 70% in the second year and 50% in the third year. In spite of this, many patients continued to produce sufficient amounts of insulin in order to reduce hypoglycaemic risky episodes; therefore, islet transplantation mostly became efficacious in glucose level control.
Similar results were produced by other studies involving islets which were kept in culture before transplantation up to three days (2001, Miami, USA).

The National Institute of Health, Immune Tolerance Network (ITN) set up a multicentre trial of islet transplantation. The Collaborative Islet Transplant Registry (CITR) was established in 2001 by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

The Registry was created with the aim of collecting, analyzing and comprehensively communicating data from all islet/beta cell transplantation centres in the US, Canada and some European and Australian centres to guide transplant centres in developing and refining islet/beta cell transplant protocols.

A close network of collaboration between initiatives and programs of the National Institutes of Health, the Juvenile Diabetes Research Foundation International (JDRFI) and other health centres, such as the Health Care Finance Administration-HCFA, the Health Resources and Services Administration (HRSA), and the Food and

Drug Administration (FDA), ensure that the outcome measures used by CITR are appropriate, standardized, and relevant.

The last annual scientific report, specifically 2010 CITR (7th) Annual Report, presents data from the past ten years, namely the large majority of the islet transplant programs active in 1999-2009 (www.citregistry.org). The following data refer to the last decade and shows that 571 allogeneic islet transplant recipients (481 islet transplant alone, 90 islet after or simultaneous kidney) received 1,072 infusions from 1,187 donors. The mean age of recipients who received an islet allograft transplant rose from 42 to 49 years and the mean duration of diabetes grew from 26 to 32 years over the decade. The donors, on the other hand, rose from 42 to 44.8 years of age; about 58% of them were male, deceased due to cerebrovascular accidents/strokes.

The pancreas were processed better and faster; in addition the islet product showed that the mean total IE rose from 417 to 463 per infusion and the β-cells growth amount from 217 to 335. Nevertheless, the immunosuppressive regimen revealed a change only using IL2R antagonists to a replacement or supplementation including T-cell depletion with or without TNF antagonist in over 80% of the transplants in the last three years.

The maintenance of the immunosuppressors was achieved by a calcineurin-inhibitor and a combination of inosine monophosphate dehydrogenase-inhibitor (IMPDH). The overall results demonstrate that there is 65% of insulin independence in the first year post-infusion, increasing up to 75% by the second year. Moreover the long-term graft function significantly improved, i.e. in 2004-2007 the insulin independence lasted for a longer time than in 1999-2003. By observing the global data of CITR, it can be concluded that over the last decade the allograft recipients exhibited benefit with relatively low risks, (low levels of infusion-related complications, few events of immunosuppressors-related cancer and death), and islet transplantation was engrafted in recipients over 35 years of age with good glycaemic control. Finally, the use of vaso suppressors and insulin in the donors, as well as the use of T-cell depletion and calcineurin-inhibitors for immunosuppressive maintenance in the pre-operative period, create the best conditions for obtaining improved outcomes (http://www.citregistry.org/).

Islet transplantation offers various advantages compared to the whole organ transplant, such as the fact that it is a relatively simple surgical procedure (mini-invasive

surgery) with consequent low post-operative risks and low morbidity as well as culturing or cryopreserving islets.

The protocols used to handle pancreatic islets for reducing their immunogenicity revealed that a short time of culture was adequate. Furthermore, this allows the recipient's conditioning to be optimized, with a consequent easier tolerance induction, and less risk of rejection. In addition, the requirement for immunosuppressive drugs treatment was low[45].

Islet transplantation is indeed considered a promising strategy for the treatment of diabetes mainly in young patients and children with no evident insurgence of severe complications (i.e. when possibility of whole organ transplant has been discarded)[46].

There are some key points to consider in order to perform a successful human pancreatic islet transplant:

- *correct islet handling in pre-transplant phase*: the cold preservation of the pancreas before islet isolation has to be less than 8 hours. The same procedure has to be executed in as little time as possible in order to obtain maximum yield from a single pancreas and preserve the vitality of the mix to be transplanted[47].

- *correct number of grafted pancreatic islets*: to assess an intervention with a good probability of success, at least 12,000 IE/Kg should be transplanted into a recipient (IE is Islet Equivalent, islet volume converted into the number of islet with a diameter of 150 μ).

On average, a human pancreas contains 300,000 to 1.5 million pancreatic islets and it has been evaluated that only 60% is necessary to maintain a normal glucidic metabolism[48].

Nevertheless, at least 2 donors are required in order for pancreatic islets to obtain an insulin independence, but occasionally the same result has been obtained by collecting islets from a single donor[49].

- *ischemic and nonfunctional processes which can hit transplanted islets*: islets have a characteristic microvasculature in a normal pancreas: arterioles are dispersed in capillaries in the centre of the islet, emerge through the islet peripheral area and then drain into the portal system (insulo-acinar portal system)[50]. This structure is destroyed after the isolation of the islets and their transplantation. During the isolation and culture of the islets, the endothelium loses its differentiation and is able to degenerate.

A rapid revascularization is required to let the islets survive and function after transplantation[51]. Therefore, the survival of grafted islets most of all depends on endothelial cells and capillaries originating in the implanted organ which creates the new vascular system of the islets. It also has to be considered that the early ischemic period significantly damages the islet centre, which contains the great part of the insulin secreting β-cells[52].

It is known that blood vessels inside the transplant begin to be visualized 3-5 days after transplantation, and 7-14 days after transplantation a normal blood flow is re-established.

- *adverse effects of actual immunosuppressive protocols*: immunosuppressive drugs are essential to prevent the processes of rejection linked to transplantation, even though immunosuppressive protocols based on the use of corticosteroids, cyclosporine and tacrolimus (FK 506) are far from being effective in islet transplantation, because the same drugs can induce diabetes[33].

The potential inefficacy of human islet transplantation could be linked to an inadequate number of grafted IE and to technical factors linked to isolation that reduces the vitality and functionality of the islets[47].

Moreover, the presence of non-specific inflammation and the onset of rejection processes[53] mediated by the immune system can lead to the destruction of grafted islets.

Despite increasing interest in this technique, its clinical application remains limited mainly because of low yield in islet achievement and side effects associated with the immunosuppressive drugs used. Grafted islets appear to have a greater aptitude towards acute rejection to verify autoimmune relapse compared to the whole pancreatic organ transplantation[54]. In the immediate post-operative period, a loss in grafted islets occurs[55]; in animal models grafted islets are about 50% less than transplanted ones.

Moreover, it has recently been observed that patients treated with Edmonton protocol occasionally reached graft tolerance due to the homeostatic expansion of autoreactive memory cells which are able to induce rejection[56]. Though tolerance induction can be induced by using mycophenolate mofetil, the risk of normal growth blockage and child development[57] prevent its use, except for cases in which children risk losing their lives because of organ insufficiency or other severe complications due to diabetes[58]. As a consequence, several aspects of islet transplantation have to be defined in order to introduce this clinical therapy as a routine cure which is valid for the

treatment of type 1 diabetes. Graft survival needs to be improved and side effects linked to the use of immunosuppressive drugs avoided[59], by introducing new therapeutic strategies.

Several approaches have been proposed in order to induce tolerance instead of administrate immunosuppressive therapy. These alternatives consist in the infusion of stem cells[60] or bone-marrow cells[61] or mesenchymal stem cells[62] in the pre-transplant phase.

1.5 Islets of Langerhans
1.5.1 Architecture of Pancreatic Islets

The islets of Langerhans are the regions of the pancreas or micro-organs that contain at least four types of endocrine cells: α-cells that produce glucagon, β-cells that produce insulin, δ-cells that produce somatostatin, PP-cells that produce pancreatic polypeptide, and ε-cells that produce ghrelin.

In rodent models the distribution of these cells in the islets is not randomized, but β-cells are located in the inner core of the islets, while the non-β cells form the mantle region. This spatial position within the islets contains several properties so that when insulin secretion is decreased, the organization of islet cells is found to be changed; for example β-cells are bound with non-β-cells[63]. Since the non-β-cells which are in contact with β-cells show no increase in insulin levels in vitro studies, it is important to observe the that the mantle position of non-β-cells is pivotal for insulin regulation by homologous contacts between β-cells[64].

Scientific literature provides evidence that there is no clear agreement on the architecture of human pancreatic islets and their distribution, even though it is accepted that the endocrine components between humans and rodents are not that different and that β-cells are less numerous in humans than in rodents[65].

In 1976 Orci and Unger located α- and δ-cells of the human islets in the mantle and grouped against capillary walls within the core of β-cells[63]. Later Grube proposed that β-cells are located in the islet core and α-cells are arranged at the periphery and along intraislet capillaries[66].

During the past three decades it has been a great challenge to identify the distribution of pancreatic islets, but Bosco and his colleagues have recently proposed a

model showing human islet cells distribution in a trilaminar plate (a sandwich structure), where α-cells are in the external layers and β-cells in the internal ones. He also reported no vascular channel in the β-cells in the centre of the structure, but a vascular network was observed at the α-cells level[67], (figure 2).

A   **B**
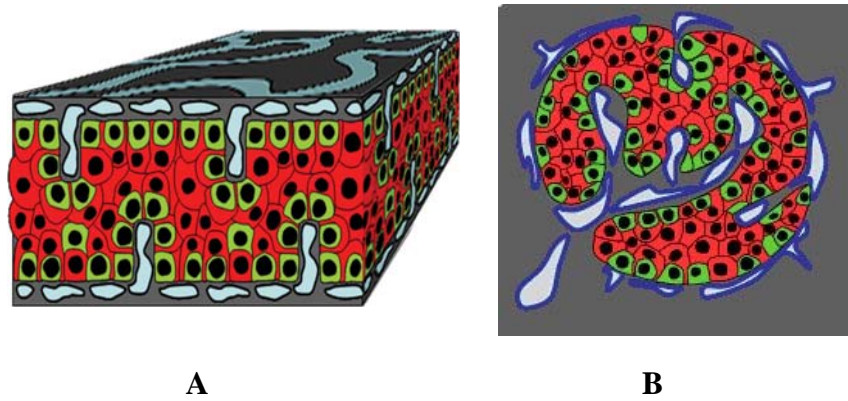
Figure 2 **Endocrine cell and vessel organization in human islets**. **A**: α-Cells (green) reside at the periphery of the plate in close contact with vessels (blue). β-Cells (red) reside in the central part of the plate and the developed cytoplasmic extension runs between α-cells reaching the vessels surface. **B**: The plate with adjacent vessels is folded so that it forms an islet. (Bosco D., Diabetes, 2010)

1.5.2 *Physiological site for pancreatic islet transplantation*

Recent work by Merani and his colleagues extensively discussed the main role of the site of infusion by means of an animal model and human clinical practice[68]. Since the liver has been widely studied and has the advantage of a double arterial and venous vascular supply, many of the islets are lost during or immediately after portal vein transplantation. Moreover, the oxygen tension of the parenchyma is lower than that of the pancreas and the liver as a site of implantation is associated with procedure-related complications, including haemorrhage and thrombosis. Patients remain insulin independent immediately after the surgical approach, but the effect is not long lasting[69].

Many efforts were made to achieve the best sites for pancreatic islet implantation in order to optimize the engraftment and function of the islets, reduce the number of transplanted islets and decrease immunogenicity.

Islet milieu requires a direct access to oxygen, glucose and other metabolites and need to be free from toxic metabolites and oxygen free radicals. The slow revascularization of the transplanted pancreatic islet is another key issue and is required to have a good vascular supply and high oxygen levels[70]. The insulin hormone, which is

released by β-cells, should be delivered through the portal vein, as the liver and muscle are primary sites of insulin action, therefore it could be beneficial to transplant the islets in a site that is drained into the liver[71].

In addition it is conceivable that the implantation site should be immunoprivileged, thus the pancreatic islets should be infused in a minimal inflammatory reaction site to increase long-term survival and the loss of β-cells should be minimal. Moreover, the implantation site should have easy access to reduce further complications, and the number of islets should be minimal in order to have the lowest transplant volume for multiple donors[72].

Merani also reported that the advantages of portal vein infusion of islets of Langerhans were the simple way of injecting them by cannulation of a mesenteric venous tributary during laparotomy or percutaneously using fluoroscopic and ultrasonographic guidance, and also the low requested number of islets[73]. At the same time, the intraportal site reflects that a reduced function of islets in a model without allorejection and autoimmune diabetes is not suitable for long-term function[74]. What is more, the instant blood-mediated inflammatory reaction (IBMIR) remains crucial in order for the β-cells to function after transplantation into the vascular site.

The kidney subcapsular site is most commonly used in rodent models and has good results in that diabetes is reversed within a few days. The metabolic engraftment and function of human fetal islet-like clusters transplanted into immunodeficient nude mice under the kidney capsule is better than the lung, liver and spleen from both a morphological and functional perspective[75]. The disadvantage of the kidney capsule is the scanty blood supply that leads to a low-oxygenated microenvironment for the islets.

Literature provides evidence that only 250 syngeneic subcapsular islets are required for the subcapsular site to reverse streptozotocin-induced diabetes in mice a considerable amount less compared to the 600–800 required for the intraportal site[76]. Besides the best preserved structure of rodents compared to human islets, it is not easy to lift or infuse islets under the rodent kidney capsule. Finally, the surgical site is very invasive and co-morbidities in the animal recipient population, including diabetic nephropathy, may reduce the hospitality of the kidney.

The spleen, which is a metabolically suitable site for islet transplantation, is an alternative site but it is not as advantageous as the liver. Indeed, there are several

risks of haemorrhage and the improved access of lymphocytes to transplanted tissue in the spleen makes this site unsuitable for islet transplantation[77].

Merani investigated other main sites for pancreatic islet transplantation. The pancreas is in theory a good site for supporting the function of the islets in the long-term because of the high partial pressure of oxygen in the tissue. On the other hand, the relatively invasive nature of the isolation procedure and known evidence regarding the autoimmune recurrence of type 1 diabetes make the pancreas a clinically irrelevant site for transplantation[68].

Moreover, several papers showed that the intraperitoneal and omental pouch sites are attractive as they provide a large implantation volume and the concurrent use of transplant devices or capsules[78]. As well as its simple surgical approach, the omentum offers other advantages[79], but the large number of islets required for both intraperitoneal and omentum sites and reduced data on long-term survival make these two sites less attractive than others.

Islet transplantation has also been performed into bone marrow of non-diabetic rats and in the os femoris in diabetic ones: results reported that there is a persistence of insulin up to 21-30 days post-transplant[80]. On the other hand, the intramuscular site is very attractive due to its simple accessibility and monitoring, even though this site is associated with a higher frequency of leucocytic infiltration compared to others[81]. Another site for islet transplantation was the fat pads in the mouse model that showed a simple way of infusion, which was similar to the intramuscular one because of the low required islet number[82].

Finally, some immunoprivileged sites, such as the brain, thymus and testis[83], were reported because they have shown to confer protection to an allotransplant and these sites are more suitable for transplants of cells. Indeed, they can protect the organism from allorejection or xenorejection without further immunosuppressive therapy.

In conclusion, further research in humans is required before the adoption of any clinical routine, so the careful evaluation of five criteria is suggested (the cellular physiology of the site, the endocrine function for good glycaemic detection, the immunological role of the site, surgical and technical complications due to the chosen site and supporting evidence from studies on large animals) before choosing a clinical application for the alternative site for islet transplantation, even though it is actually indicated that the portal vein remains the main method of infusion[68].

1.5.3 *Murine models of islet transplantation*

A strategy to reduce the graft islet mass for transplantation to a recipient has been investigated in a rat model of islets transplantation. Hara et al has demonstrated that animals transplanted with 1500 syngeneic or allogeneic islets in the portal vein or under the kidney capsule restored to normoglycemia one day after transplantation. The authors reported no significant difference in loss of graft function between syngeneic and allogeneic islet transplantations during the three days after transplantation, neither did they observe any significant difference in deterioration of graft function between portal vein or kidney capsule sites of transplantation[84].

Cornolti has recently investigated whether syngeneic islet transplantation can obtain adequate islet function during glucose stimulation by employing a continuous glucose monitoring system in the diabetic rat model. Islet encapsulation through immunoisolation systems requires no further immunosuppressive treatment, thus leading to a new clinical approach. Cornolti's group has developed two types of islet transplantation: islets immunoisolated in microcapsules inserted in the peritoneal cavity and free islets inserted under the kidney capsule. They observed an homogeneous state of normoglycemia after the two protocols, but the microcapsulated one obtained a better control of glucose sensing and insulin release.

Even though encapsulated islets are separate from the microcirculation with this method, islets can react to high glucose levels in the blood and efficiently release insulin. On the other hand, islets transplanted beneath the kidney capsule showed less viability and function, concluding that despite being nearer to the microcirculation, the host cell reaction was involved[85].

1.5.4 *Clinical outcomes of islet transplantation*

In order to perform human islet transplantation in patients affected by type 1 diabetes, the required clinical indications for hypothetical subjects should be the onset of frequent and severe hypoglycemic events. Moreover, other possible required indications should be severe clinical problems associated with the use of exogenous insulin therapy and an unsuccessful insulin-based therapy to prevent acute complications.

As reviewed by Fiorina et al., the patients that could undergo islet after kidney transplantation were subjects with an end-stage renal disease who also received a kidney transplantation alone or who rejected the pancreas after simultaneous kidney–pancreas transplantation; otherwise, for islet transplant alone, numerous patients should have to be selected[86].

Data collected by the CITR revealed that 80% of graft function was successful at 1000 days of follow-up for islet after kidney transplantation, while for islet transplantation alone almost 60% at 1000 days were considered successful[86]. Several articles reported insulin independence and normalizing glucose homeostasis after an islet transplantation, but only few patients maintained insulin independence in the long term with sustained C-peptide secretion[87].

Since insulin is secreted by intrahepatic tissue in grafted patients and cleaved by the liver, Luzi et al. reported that not only a successful islet graft returned to basal hepatic glucose and improved the action of insulin[88], but it also achieved a loss of insulin independence, in most cases, by means of long-term partial islet function[89].

The positive effect of islet transplantation was also seen in the patients' survival rate; data regarding the morbidity and the mortality of patients affected by type 1 diabetes were evaluated after the transplantation protocol.

In 2005 Fiorina performed two types of transplants in diabetic patients in an attempt to determine islet transplant improvement with regard to cardiovascular function: a kidney-islet transplant and a kidney alone transplant. The first successful group of patients reached long-term C-peptide secretion, whereas the second group showed a loss of C-peptide secretion 6 months after transplantation. The authors concluded that type 1 diabetic patients with end stage renal disease who received kidney-islet transplantation showed an improvement in aspects of cardiac function for a 3year follow-up in comparison with the group who had received a kidney transplant alone[90].

According to available data from clinical trial, it is evident that stringent glucose control can reduce the risk of microvascular complications[91], even though no controlled studies have clearly answered the question of whether islet transplantation can halt the progression of long-term diabetic complications. Apart from the difficulties of performing large clinical trials and also other factors, such as the differences in islet isolation procedures, the absence of standardized protocols and the persistence of many regional-based immunosuppressive approaches, the restoration of islet function could in

fact be protective against long-term diabetic complications, as confirmed by a few uncontrolled preliminary studies from Milan, Miami and other groups. Nevertheless, clinical complications largely remain a strong unresolved issue, since the above mentioned cardiovascular function should be kept under strict observation after islet transplantation[90].

Patients with type 1 diabetes are also at high risk for macro/microangiopathy. Nephropathy is also considered to be one of the most serious complications of this disease, even though a study on the combined effect of islet transplantation and immunosuppression on kidney function has shown promising results, which are to be confirmed, in patients transplanted with islets alone[92]. Finally, diabetic retinopathy and complications of the central nervous system have been recently investigated by using islet transplantation as an improved type of treatment.

1.5.5 *What happens to islet structure after an islet transplantation*

The vasculature of islets cultured *in vitro* and their subsequent transplantation can dedifferentiate or degenerate from its original architecture. In addition, the immediate days after islet transplantation are critical since this process is characterized by substantial islet cell dysfunction and β-cells death – a more pronounced characteristic in diabetic recipient[55]. The blood flow of the transplanted islets seems to be lower in diabetic recipients and could be caused by an altered regulation of the blood flow rather than a defect in the revascularization process[93].

Furthermore, islet hypoxia is generally thought to be the major reason for the vulnerability of islets in the first few days of engraftment. In 1998 Carlsson demonstrated that oxygen tension immediately after islet transplantation was 50% of that in native islets and slightly lower than that in the adjacent renal cortex. One month after implantation, the exact time when revascularization is likely to be complete, oxygen tension in transplanted islets was lower than after one day. Although the newly born microvessels within the graft are responsible for oxygen transportation, they have a reduced ability to transport oxygen to the islet graft compared to the highly specialized vascular network of the endogenous islets. This means that transplanted islets are exposed to an environment containing a much lower concentration of oxygen compared to the native pancreas[94].

Another major issue concerns islet vascularization after their implantation. Islets have less blood flow than surrounding pancreatic exocrine tissue, greater vessel density with greater volume, and intra-islet capillaries are lined by fenestrated endothelial cells[95]. Indeed, the revascularization of transplanted islets begins within 2–4 days and is completed within 14 days after transplant[96].

In various implantation sites it has been observed that grafted islets have reported a decreased vascular density compared to endogenous ones one month after transplantation. In islets implanted into the spleen, as opposed to islets implanted into the liver or kidney, a more pronounced decrease in vascular density is well established, suggesting that the revascularization of transplanted islets is impaired, at least quantitatively, regardless of the implantation site. On the other hand, a large number of blood vessels was found in the connective tissue surrounding the renal subcapsular and intrasplenic islets, so that the predominant location of capillaries in the connective tissue stroma is the immediate vicinity of the endocrine tissue[97]. This structure was later confirmed by a pancreatic islet graft under the renal capsule rat model where a lymphatic network was shown from one week up to 9-12 months after transplantation, with a major number of capillaries within the connective tissue stroma between the transplanted islets[98].

The survival of islets in the graft depends on the diffusion of nutrients and oxygen[99] from the surrounding tissue until the revascularization process of the grafts is complete. A part of the revascularization process is likely to depend on the hypoxic activation of genes encoding for angiogenic factors, which lead to new blood vessel formation and improved oxygen delivery[100].

An array of peptides including acidic fibroblast growth factor (aFGF), basic acidic fibroblast growth factor (bFGF), tumor growth factor- (TGF) α, TGF-β, PDGF, hepatocyte growth factor (HGF), tumor necrosis factor- (TNF) α, vascular endothelial growth factor (VEGF), angiopoietin (ANG) and interleukin- (IL) 8 were supposed to be the regulators of angiogenesis[101]. Among these angiogenic factors, the most potent hypoxia-inducible gene that regulates blood vessel growth has been demonstrated to be VEGF, a selective mitogen for endothelial cells *in vitro* able to promote also vascular permeability *in vivo*. VEGF is also expressed by pancreatic islets[102]. A study on VEGF gene expression carried out by Vasir et al. has shown a different pattern on normoglycaemic and diabetic animals after an islet transplantation in the first few days after transplantation. They found a slight increase in the VEGF receptor expression at

days 3 and 5 in the normoglycemic recipients and, at the same time, a decrease in VEGF expression; the beginning of angiogenesis in the grafts could be responsible for this trend. On the contrary, VEGF receptors in the diabetic group at days 5, 7, and 14 showed a marked expression, perhaps in accordance with the concept that vascularization of transplanted islets is delayed by the presence of hyperglycemia, which derives from an increase in local oxygen consumption, as it is known that glucose oxidation in β-cells increases as glucose levels rise[103].

Vasir and his colleagues also found that both VEGF isoforms in normal and diabetic recipients at days 1 and 3 after transplantation increased their expressions, suggesting that revascularization had already begun. A parallel increase in the expression of VE-cadherin was also found during the process of revascularization that could in part have been mediated by VEGF. Finally they reported that the diabetic milieu could have impaired the early vascularization of transplanted islets, thus providing an explanation for the less favourable outcomes[104].

More recently Golocheikine et al. have studied a model of subcutaneous islet transplantation in matrigel basement membrane matrix to assess the role of indirect proangiogenic growth factors in islet revascularization. The group has studied whether VEGF, a direct agonist capable of activating endothelial cell proliferation and migration, and HFG, a growth factor leading to a blood vessel formation *in vivo* besides inducing VEGF, could enhance graft survival and function *in vivo*[105]. A further confirmation of intercellular adhesion molecules (ICAM) and vascular cell adhesion molecule (VCAM) involvement has also suggested that a cross-talk mechanism between VEGF and HGF could improve the blood vessel formation toward a better vascularization in the graft[105].

In order to point out the importance of revascularization after islet transplantation, in 2006 Brissova showed that there is a reciprocal communication endocrine cell–endothelial cell, (figure 3), since early differentiating endocrine cells produced angiogenic factors including VEGF-A and ANG-1. Moreover, it was observed that insulin deficiency in mice with reduced VEGF-A expression was not a result of β-cell dysfunction but an abnormality in the islet vasculature. This was in line with the fact that β-cells and islet microvasculature participate cooperatively in the maintenance of glucose homeostasis. VEGF-A was hence considered to be a key regulator of islet vascularization so that insulin levels in the systemic circulation could be influenced by modulating VEGF-A expression; this suggested that a new mechanism was able to

connect the impairment in islet vascularization with the defect in the release of insulin associated with diabetes[106].
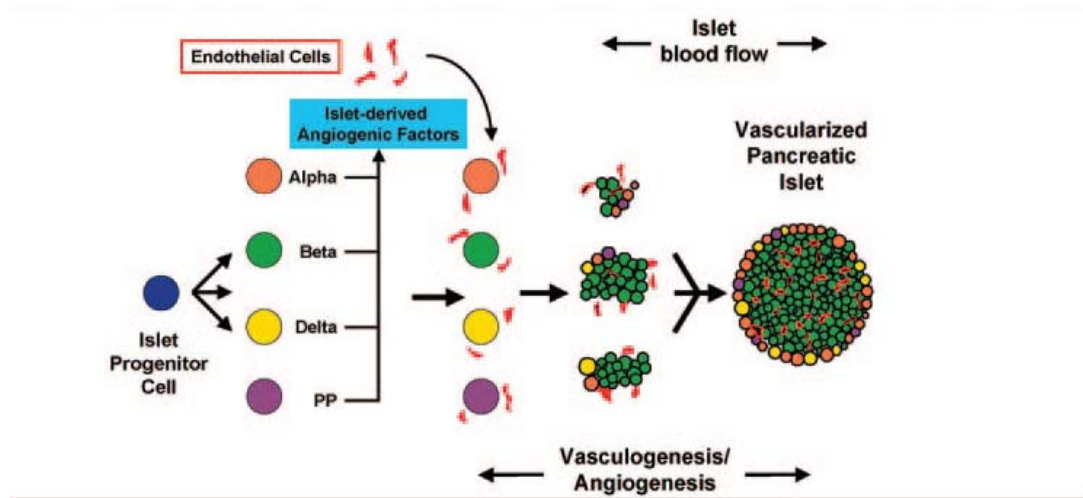

Figure 3. **Model of pancreatic islet vascularization**. After an initial production of angiogenic factors released by early developing islet cells, a recruitment of endothelial cells and their association with developing islet cell clusters occurred. Next, blood flow to small endocrine cell clusters established itself and a concurrent organized structure of islet cell formation occurred. (Brissova M., Diabetes, 2006).

1.5.6 *Angiogenic features of intra-islet cells*

β-cells in mice are in direct contact with basement membranes of endothelial cells, whereas the intra-islet microvasculature of human islets is surrounded by a double basement membrane[107]. An extensive capillary branching in the islet core is arranged by one to three arterioles entering an islet.

In this structure, the secreted hormones are forced to exit from the islet through small venules formed from these capillaries. The main characteristic of intra-islet vasculature is the lining of fenestrated endothelial cells[108]. These fenestrae are thought to be induced by factors such as VEGF-A and angiopoietin-1 both secreted by the islet cells[109]. It is reported that islet endothelium induce insulin gene expression, stimulating β-cell proliferation and affecting adult β-cell function by secreting several known/unknown paracrine factors[110].

Moreover, the islet microvasculature is involved in the regulation of leukocyte recruitment into the islets in type 1 diabetes during the autoimmune destruction of β-cells. During the early phase of type-1 diabetes, islet endothelial cells particularly express surface leucocyte-homing receptors. These receptors can help the

entry of leucocytes into the islets with the consequent destruction of β-cells[111]. It was recently confirmed that the direction of blood flow within mice islets is from the inner-outer area as well as from the top to the bottom[112]. This trend implicates that blood can flow from β-cells to non-β-cells thus involving the influence of insulin on the function of non-β-cells. However, in top to bottom blood flow, the cell types do not influence each other in any way.

As assessed by Linn in 2003, endothelial cells contained within the islets required growth factors and were stimulated by FGF-2, VEGF and TNF-α to allow their migration. FGF-2 was indeed found to be an important factor for endothelial proliferation of isolated islets, while VEGF promoted a cord formation. TNF-α was instead able to enhance the effect of VEGF on EC proliferation. The authors demonstrated the significance of donor endothelial cells either *in vitro* as well as *in vivo* in the revascularization process. To this aim they observed that the pancreatic islet microvasculature was identified as a target site for the graft rejection process. In addition, this structure was involved in the revascularization process since the donor endothelial cells remained in the graft up to 21 days after transplantation at its periphery and surrounded by renal tissue[113].

In order to determine whether intraislet endothelial cells participate in the revascularization and access the structure and composition of blood vessels in the revascularized grafts, Brissova et al used a murine model in which endothelial cells were tagged with lacZ, which was labelled for the mouse endothelial marker PECAM-1 (expressed on the surface of both donor and recipient endothelial cells). Brissova found the existence of two types of blood vessels in the revascularized mouse islet graft: 1) capillaries formed by either donor or recipient endothelial cells directly connected to each other and 2) chimeric blood vessels formed from a mixture of donor and recipient endothelial cells. The chimeric structure was not evident in the human graft. The involvement of both donor and recipient intraislet endothelial cells in the revascularization of transplanted islets was finally confirmed, thus demonstrating that those cells could integrate into the functional vasculature of revascularized islet grafts[114].

These data were later confirmed by Nyqvist who stated that intraislet endothelial cells were able to participate in the formation of functional blood vessels within the islet graft vasculature subsequent to transplantation. He observed that the transplantation of freshly isolated islets with a relevant number of endothelial cells, in

contrast to cultured islets, markedly improved their vascularization, thus a preservation of intraislet endothelial cell mass was able to improve the long-term graft function[115]. Nyqvist recently found that donor islet endothelial cells contributed to the revascularization process of fresh islets and they were also observed in the long-term, but the endothelial cells deriving from donors were not as sufficiently effective as the cultured islets in the reversal of diabetes[116].

1.6 Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) were described for the first time by Asahara et al. as human adult cells involved in postembryonic neovasculogenesis[117]. Neovasculogenesis, the *de novo* formation of blood vessels from avascular tissue, differs from neoangiogenesis, the formation of new blood vessels that sprout and grow from pre-existing vessels. Up to Asahara's findings it was considered that neovasculogenesis only occurred during embryogenesis, within the extra-embryonic yolk sac mesoderm, where primitive haemangioblasts arranged to form islands and tubular structures with further differentiation into blood and vessels[118].

The discovery of EPCs led to the new concept that vasculogenesis and angiogenesis could develop at the same time in postnatal life because these cells could differentiate into vascular endothelium, when required, through a mechanism that recapitulates embryonic vasculogenesis. Fadini et al. also reported that EPCs originate in the bone marrow where they are localized in either a quiescent state or a more undifferentiated one.

EPCs can be mobilized from the bone marrow into the peripheral circulation in response to many stimuli, including growth factors and cytokines, released by ischaemic or damaged tissues, as well as during physiologic processes[118]. When EPCs reside in the blood circulation, they can home specifically towards sites of ischaemia or tissue damage, because of the interaction between soluble chemokines and their receptors expressed on EPCs surface[119-124]. Once the EPCs have adhered to activated endothelium, they either serve as patches to repair any endothelial denudation or migrate into the surrounding tissue following chemokine gradients. When EPCs are exposed to appropriate environmental stimuli, including growth factors and nutrients, tissue EPCs proliferate and organize themselves into three-dimensional tubular

structures that eventually connect with the pre-existing circulation system and mature to become new blood vessels. EPCs consequently show two main activities in the vascular system: the healing of endothelial damage and the formation of new blood vessels in ischaemic tissues.

Studies with induced experimental mechanical endothelial damage have shown that EPCs are able to repair the vessel wall, re-establishing anatomical and functional endothelial integrity[125]. The functions of EPCs also include paracrine activity by the secretion of growth factors and cytokines[126] and possible trans-differentiation into cardiomyocytes[127].

As a result, EPCs can be considered as an integrated component of the cardiovascular system prone to pathological alterations and pharmacological modulation. The inability of EPCs to maintain a normal endothelial homoeostasis and to promote the development of new vessels appears to be the reason for accelerated vascular disease and ageing, with the potential blockage of compensatory angiogenesis, thus favouring the development and progression of ischaemic syndromes. Moreover, EPC alterations have been shown in subjects with no theorical classic cardiovascular risk factors, such as diabetes, hypertension, dyslipidemia, cigarette smoking, obesity and a family history of cardiovascular disease[128].

In addition to being altered in the presence of risk factors, EPCs are further impaired in patients with early or advanced atherosclerosis. By using different methods, it has also been demonstrated that levels of EPCs are directly linked with brachial artery flow-mediated dilation, a measure of peripheral endothelial function, regardless of classic risk factors.

A relevant decrease in the level of EPCs represents an advanced atherosclerosis of coronary or peripheral arteries[129]. This evidence indicates that a drop in EPCs is in accordance with the natural history of atherosclerosis[130]. At present there is a general agreement concerning EPC levels which provide a measure of the endogenous vascular repair capacity related to atherosclerotic burden and influence of cardiovascular risk[118].

1.6.1 *Origin of Endothelial Progenitor Cells*

Besides the much research interest in the field of endothelial cells, EPCs can be isolated by multiple precursors and there has been a great variability in their functional evaluation, regarding future characteristics of the cells[131].

Literature has reported several methods for isolating EPCs, and the main sources presented are peripheral blood, bone-marrow and umbilical cord blood.

Ingram et al. found a method based on the clonogenic and proliferative potential of EPCs isolated from umbilical cord and adult peripheral blood which was strictly related to the hematopoietic cell system, because of its similar clonogenic and growth characteristics. In human cord blood the authors identified a unique population of high proliferative potential–endothelial colony-forming cells (HPP-ECFCs) that could give rise to a substantial number of cells with final secondary and tertiary colonies[132].

Kirton instead showed that the isolation of mononuclear cells from peripheral blood or bone marrow (seeded on a fibronectin-coated plate in a medium supplemented with endothelial growth factors) resulted in an adherent spindle-shaped cell expressing endothelial cell (EC) markers and typical endocytosis of acetylated low density lipoprotein (LDL). This type of EC could not form EC tube-like structures in an *in vitro* angiogenesis assay model and, most of all, these cells showed leucocytic (CD45) and monocytic/macrophages (CD14) markers[131]. This method which was used to isolate mononuclear cells from peripheral blood resulted in a high contamination of platelets, also due to their expression of CD31 and vWF[133]. The further elimination of platelets microparticles led to a depletion in the angiogenic properties of the EPC medium, thus reporting an *in vitro* characteristic due to the presence of platelets after the mononuclear isolation[134].

Moreover, a similar method to Asahara's first one consisted in isolating CD34+ and VEGFR2+ mononuclear cells from peripheral blood and in cell labelling with antigen specific antibodies by employing fluorescence activated cell sorting to select EPCs[135]. After the characterization of putative EPCs by CD34+ marker, Peichev et al. introduced a further expression of CD133 in combination with CD34 and VEGFR2, showing that the expression of both markers established a phenotipically and functionally distinct population of circulating endothelial cells with a role in the neoangiogenesis. Under specific stimuli such as incubation with growth factors VEGF,

FGF-2 and IGF, the circulating endothelial cells differentiated into mature adherent endothelial cells[135]. Peichev's findings were later reversed by studies which highlighted that those types of cells were not EPCs but distinct primitive haematopoietic progenitors also expressing CD45 marker with no ability to form vessels[136].

A method developed by Hill showed the colony forming cell assay to isolate EPCs from peripheral blood; this method was further divided into two other assays. One derived from a phenotypical expression of EC markers as well as from haematopoietic cell-specific surface antigens (CD14, CD45, CD115) known as Colony Forming Unit cells. These cells showed phagocytic abilities, low proliferative activity and were unable to form vessels *in vivo*. Central clustered cells possess myeloid progenitor potential[137].

The other method, involving cells named Endothelial Colony Forming Cells which showed robust proliferative potential, highlighted the formed capillary-like structures *in vitro[132]* and vessels *in vivo* with no expression of CD14, CD45 and CD115 or showed phagocytic abilities. However, previous transplant studies identified a type of cell with a similar phenotype residing within the bone marrow[138].

'EPCs' appeared to represent two distinct populations with overlapping antigen expression (e.g. CD34/VEGFR2).

Early-EPCs	Late-EPCs
Morphology: Form colonies with sprouting cells/ Spindle shaped cells (circulating angiogenic cells)	Morphology: Form flat cobblestone monolayer
Low proliferative capacity (last up to 4 weeks)	High proliferative capacity
Marker expression: CD34+/-, VEGFR2+, CD133+, CD31+, CD14+, CD45+,CD115+, up-take of Ac-LDL, vWF+, CD144-	Marker expression: CD34+, VEGFR2+, CD31+, up-take of Ac-LDL, vWF+, CD144+, CD105+, CD146+, CD14-, CD45-, CD115-, CD133-
Can phagocytose bacteria	Can not phagocytose bacteria
Lack vessel formation ability in vivo	Can contribute to vessel formation in vitro and vivo
Secrete angiogenic factors	

Figure 4. **Early- and late-EPCs**. Different morphology, proliferative ability, markers expression and in vivo properties characterize two different types of EPCs (Kirton J.P., Microvascular Research, 2010).

Two different EPC subpopulations, early and late EPC, were described with distinct cell growth patterns and the ability to secrete angiogenic factors[139], (figure 4).

Hematopoietic-derived spindle shaped cells, as described in the first and second method (also referred to as circulating angiogenic cells), and CFU colonies were generally classified as early-EPCs; they developed after 4-7 days with a peak growth in culture at 2–3 weeks and died after approximately 4 weeks *in vitro*. Moreover, they secreted an array of angiogenic, antiangiogenic and neuroregulatory cytokines[139].

The Endothelial Colony Forming Cells, the cobblestone shaped cells known as late-EPCs, grew after 3-4 weeks with characteristics of mature endothelial cells; they represented the limited subset of cells that do not express hematopoietic antigens (CD45−)[140] and were the only cell type described that exhibited vessel forming ability, hence they were considered to be 'true EPCs'[131].

In consideration of the literature data it can be argued that there is no general agreement on the best antigenic combination to define a population of purified EPCs. Therefore, it has been suggested to consider the original antigenic phenotype of EPCs as those cells positive to CD34 and VEGFR2 markers; this could be the best compromise in terms of specificity and sensitivity in the clinical setting, even though it is not completely distinguishable between EPCs, mature endothelial cells and haematopoietic progenitor cells[118].

1.6.2 *Homing of Endothelial Progenitor Cells*

On account of the vessel forming abilities of EPCs, it is useful to induce the late-EPC within the blood system to promote homing to sites of vascular injury and ischemia or for *ex vivo* expansion prior to implantation. The mechanisms of EPC homing are still unclear, even though tissue resident EPCs have been identified[141].

The initial steps in mobilization involve the activation of Matrix MetalloProteinase-9, which catalyses the conversion of membrane bound Kit ligand to a soluble Kit ligand. The subsequent cKit-positive progenitor cells can then move from the osteoblastic to the vascular zone of the bone marrow microenvironment[131]. Several studies confirm that this process is enhanced by high levels of SDF-1 and VEGF and appears to be nitric oxide dependent[142].

A relevant number of studies have shown that VEGF, SDF-1, in addition to G-CSF, GM-CSF, oestrogen, erythropoietin, statins and physical exercise, enhance the mobilization of total/early-EPCs within circulation.

In comparison to early-EPCs, the number of late-EPCs within the circulation was increased by G-CSF, a CXCR4 (SDF-1 receptor) antagonist and statin treatment[132]. Moreover, the proliferation, migration and ability of tube forming via up-regulation of CXCR4 and SDF-1 were shown to be improved by an activation of PAR-1 (main thrombin receptor expressed by ECs) *in vitro*[143].

Similarly, the proliferation of late-EPCs and their ability to form vessels decreases following exposure to oxidative stress/ROS generation, hyperglycaemic conditions, hypoxia or pro-inflammatory stimulation (TNF-α)[144]. However, inhibition of p38 mitogen-activated protein kinase pathway blocked TNF-α induced senescence.

Using a mouse model of hind limb ischemia, Chavakis et al. demonstrated that β2-integrins are involved in the homing of hematopoietic progenitor cells to sites of ischemia and that the pre-activation of β2-integrins is fundamental for the neovascularization process[120]. Integrins are crucial transmembrane molecules that mediate cell adhesion, migration and the homing of progenitor cells to sites of ischemia. Furthermore, Chavakis reported that there is much evidence to support the following mechanism of homing. As well as hematopoietic stem/progenitor cells, EPCs express β2-integrins. These molecules can be activated by Mn^{2+} and can mediate the adhesion of EPCs to mature endothelial cells, recombinant human ICAM-1 and the fibrinogen; they also mediate the chemokine-induced transendothelial migration of EPCs. Moreover, β2-integrins are involved *in vivo* in the homing of progenitor cells to the sites of ischemia, and the vascular integration of the integrins significantly contributed to the neovascularization capacity of infused bone marrow progenitor cells in the mouse model of hind limb ischemia. The preactivation of the β2-integrins on EPCs by activating antibodies was shown to significantly increase the *in vivo* neovascularization ability of EPCs, indicating a new therapeutic tool to promote the homing of EPCs. β2-integrins were found not only expressed on differentiated leukocytes but also on hematopoietic stem/progenitor cells[145]. β2-integrins were also shown to mediate adhesion and transmigration of hematopoietic stem/progenitor cells[146].

The role of β2-integrins was also to mediate the adhesive interactions of EPCs to mature endothelial cells and to extracellular matrix proteins; integrins are also crucial for the chemokine-induced transendothelial migration of EPCs *in vitro*, while *in vivo* they were reported to improve the neovascularization ability of infused bone marrow progenitor cells[120].

As also reported by Yoon et al., the bone marrow-derived EPCs expressed β2-integrins, the ligand of ICAM-1, suggesting that ICAM-1/β2-integrin binding plays a role in the homing of EPCs to ischemic muscle tissue. The importance of ICAM-1 was observed, as confirmed by using a blocking antibody for ICAM-1, which markedly decreased both the adhesion of EPCs to endothelial cells in vitro and the homing of EPCs to ischemic limb in vivo. Yoon revealed that in response to ischemia, ICAM-1 was selectively overexpressed in the ischemic muscle compared with the nonischemic muscle, suggesting that ICAM-1 could be the key molecule to induce preferential recruitment of EPCs to ischemic sites[147].

Carmona later investigated the role of activation of Epac1 (a nucleotide exchange protein for Rap1, which is directly activated by cAMP) on integrin-dependent homing functions of progenitor cells *in vitro* and *in vivo* as a mechanism directing the homing of EPCs. Epac1 is expressed in human EPCs and its stimulation with a specific pharmacologic activator is able to increase the activity of integrin and integrin-dependent homing functions, such as adhesion and migration. Moreover, short-term preincubation of human EPCs with the pharmacologic Epac activator increased *in vivo* homing to sites of ischemia and neovascularization-promoting capacity of EPC suggesting that activation of Epac1 is a useful strategy for increasing the therapeutical potential of human EPCs[148].

Another mechanism involved in the homing of EPCs was further assessed by Lev and colleagues who found that activated platelets, interacting with EPCs in vitro under static and flow conditions, played an important role in the recruitment of EPCs to sites of injury[149]. As previously reported[150], activated platelets may facilitate the homing of EPCs by recruiting and "directing" them to sites of vascular injury.

Hematopoietic progenitor cell rolling in murine bone marrow micro-vessels is mediated by P- (and E-) selectin and could be inhibited by antibodies to these selectins[151]. The involvement of P-selectin in the platelet-EPC interaction also accounts for the need for platelet activation to facilitate this interaction, since platelet P-selectin is expressed mainly when platelets are activated[152].

1.6.3 *EPCs and diabetes: impaired function of EPCs and mechanisms involved*

Fadini and his colleagues reported that diabetes mellitus is associated with a twofold to fourfold increase in the incidence of cardiovascular disease[118]. The high risk of vascular disease in diabetes is generally attributed to the negative effects of glucotoxicity and lipotoxicity on the vessel wall in general, and in particular to endothelial cells. Endothelial dysfunction and damage is an early and widespread complication in patients with diabetes and it is also the first stage in the pathogenesis of atherosclerosis[153]. This could represent, at least in part, the accelerated atherosclerotic process that characterizes diabetes mellitus.

It has recently been discovered that the endothelial repair process plays an important role in determining overall vascular health. An impaired endothelium is repaired by being partially covered by resident endothelial cells together with the contribution of circulating EPCs.

The reduction of the proliferation rate in culture of EPCs and dysfunction has been shown in type 1 and type 2 diabetes; Tepper found that early EPCs from patients with type 2 diabetes exhibit impaired proliferation, adhesion and incorporation into vascular structures[154].

Loomans et al. obtained similar results in patients with type 1 diabetes, by showing a reduced number of early cultured EPCs compared with control subjects. Patients' EPCs were functionally impaired in an *in vitro* angiogenesis assay and conditioned media from patients' EPCs showed a reduced capacity to support endothelial tube formation[155]. They both showed that the rate of proliferation of EPCs from plated PBMCs in diabetic patients was inversely correlated with levels of glycated haemoglobin, suggesting a possible relation between glucose control and EPC function. Reduced adhesion of EPCs to HUVECs demonstrated altered cell-to-cell interactions which could indicate that EPCs are recruited less avidly *in vivo* at sites of ischemia, and also that reendothelization by means of bone-marrow derived cells is less likely to take place in the presence of EPC dysfunction.

Furthermore, compared to matched controls, type 2 diabetes is associated with a reduction in circulating CD34+KDR+ EPCs by using a flow cytometry that is not subjected to bias inherent in cell culture protocols. Besides CD34+KDR+ cells, generic CD34+ cells were also severely decreased in patients with diabetes as compared to controls[156]. It has been found that a smaller but significant reduction of circulating

CD34+ cells is detectable in patients with prediabetic conditions, such as impaired fasting glucose and impaired glucose tolerance, suggesting that this parameter is an early indicator in the natural course of the disease.

Despite data on *in vitro* cultured EPCs, the literature does not provide clear evidence that apoptosis of EPCs is increased *in vivo* in patients with diabetes. On the contrary, the evidence from *in vivo* animal experiments has shown that diabetes leads to an inability to mobilize EPCs from the bone marrow to the peripheral circulation in response to ischaemia. In rats with experimental diabetes, Fadini et al. showed that ischaemia reperfusion injury did not trigger any increase in Sca-1+c-kit+ cells (rat homologues of EPCs) because of the inability to upregulate the hypoxia-sensing system HIF-1α and the defective release of mobilizing cytokines, such as VEGF and SDF-1α. As a consequence, post ischaemic angiogenesis was significantly compromised in diabetic animals compared to non-diabetic ones[157].

Diabetes represents a clinical condition connected to an extensive progenitor cell reduction[158]. Hypothetical mechanisms that could account for circulating EPC reduction in diabetes include: (i) decreased survival; (ii) altered mobilization from the bone marrow; (iii) deranged differentiation and (iv) extravascular homing or waste.

Regarding the mechanisms involved in the impaired function of EPCs within diabetes, besides several factors that have been involved in EPC mobilization, proliferation, migration, their differentiation and homing to sites of vascular injury, it was found that the expression of angiogenic factors, such as VEGF and hypoxia-inducible factor-1 (HIF-1) is reduced in the hearts of diabetic patients during acute coronary syndromes[159]. The expression of HIF-1 gene decreases in hyperglycaemic conditions and is associated with myocardial infarct size in rats[160].

SDF-1, also known as CXCL12, and its receptor CXCR4 played a critical role in regulating hematopoietic cell trafficking. In non-obese diabetic mice, the onset of diabetes is significantly delayed by reducing the level of SDF-1, either by antibody-mediated neutralization or G-CSF-induced suppression of SDF-1 transcription[161]. In a diabetic environment, EPCs could decrease because of increased apoptosis, as confirmed by many studies.

In rats with streptozotocin-induced diabetes, reduced circulating EPC levels were associated with uncoupled eNOS in bone marrow[162]. Dernbach showed that EPCs are more protected against oxidative stress compared to mature ECs, and therefore it

seems unlikely that the decrease and dysfunction of EPCs are mediated by an increase in oxidative stress[163].

Furthermore, a dysfunction of EPCs in type 2 diabetes patients was associated with oxidative stress due to a significant production of reactive oxygen species (ROS). A prolonged exposure of EPCs to high glucose concentrations in vitro increased superoxide anion production, and reduced NO bioavailability[164]. A generation of superoxide anions appeared by using several processes that included glucose auto-oxidation, increased protein kinase C (PKC) and NAD(P)H oxidase activity[165].

1.6.4 *Mobilization of EPCs into the circulation*

As previously reported, EPCs can be released into the circulation in response to injury (e.g. ischaemia) or mobilizing or angiogenic factors, though without the presence of specific biomarkers, the identity of the mobilized cells and the site of mobilization are still unclear.

Several studies have been carried out with regard to burn injuries and it is widely accepted that VEGF is one of the most largely studied angiogenic factors. Gill et al. demonstrated that VEGF levels were high in the plasma of patients 6–12 h after burn injuries or coronary artery bypass grafting, and this further correlated with the rapid mobilization of proangiogenic cells into the bloodstream, with a return to basal levels by 48–72 h post-injury[166].

Levels of factors such as VEGF and SDF-1 in adult human peripheral blood were linked to the severity or total surface area of the burn injury; these factors reached their maximum within 24 h of the thermal injury and declined over several days to weeks[167].

ECSM2, known as endothelial cell-specific chemotaxis receptor (ECSCR), is an endothelial-restricted cell surface receptor that appears to enhance the sensitivity of VEGF in angioblasts during vasculogenesis. This receptor was found to be increased in the plasma of burn patients[168]. The increased levels of ECSM2 continued to remain high even after VEGF was shown to decrease[169].

These data suggested that after a burn injury and increased levels of circulating VEGF and SDF-1, both proangiogenic and endothelial cells were rapidly mobilized into the peripheral blood. Pro-inflammatory factors, such as eotaxin and

granulocyte colony-stimulating factor (G-CSF), have also been shown to be elevated after burn injury[170].

Several studies were recently reported concerning mobilizing factors and heart disease. Hill and his colleagues indeed reported that patients at risk of developing cardiovascular diseases have a reduced number of proangiogenic cells in their circulation[137], while others found conflicting data on the number of such cells in patients with coronary artery disease[171]. As for severe burn injuries, acute myocardial infarction (AMI) can produce a rapid mobilization of proangiogenic cells into the circulation; this effect is related to increased levels of circulating angiogenic factors.

In a study aimed at examining the role of EPCs and their precursors CD34+ mononuclear cells in the mobilization in patients with AMI, enhanced VEGF levels in the circulation of patients 3–28 days post-AMI was observed. These data was related to high levels of cells, identified by phenotypically proangiogenic cells[172].

Another study showed relevantly increased numbers of circulating cells with a proangiogenic phenotype in the early stage after AMI. These cells reached maximum growth within 7 days post-AMI and then declined to basal levels after 60 days. The increased levels of 'proangiogenic progenitor cell' strongly correlated with increased VEGF levels in the patients' plasma[173]. In the circulation of patients affected by AMI increases in VEGF, G-CSF and CXCL12 levels[171] have been reported, even though it has been shown that VEGF presents two patterns of release during AMI: the first in the acute phase (24–48 h) and the second in the subacute phase (7 days)[174].

Leone later suggested that an increase in CD34+ cell mobilization and the conflicting coronary artery disease data on cell mobilization can be correlated with the myocardial ischaemic burden in these patients[171]. At the same time, it has been demonstrated that for those patients affected by AMI, the proangiogenic cells were mobilized due to acute cardiovascular injury[173].

1.6.5 *EPCs in the clinical practice*

A relevant number of early studies in animal models showed that the bone marrow or blood-derived EPCs could improve vascular repair. Research using animals was carried out on several vascular pathologies such as hindlimb ischemia, acute myocardial infarction, and cardiovascular diseases linked to diabetes. As a result, in the

last ten years emerging studies on the proangiogenic role of EPCs in wound repair and tissue regeneration were translated into early clinical trials.

Several efforts focused on treating cardiovascular disease by means of mononuclear cells or CD34+/CD133+ cells selected from mobilized peripheral blood, bone marrow or cultured MSCs[170].

Other studies were developed on animal models of hindlimb ischemia as reported by a substantial amount of data in literature and briefly exposed as follows.

Kalka[175] performed a transplantation with human endothelial progenitor cells (hEPCs) in an athymic nude mice model of hind limb ischemia. hEPCs were isolated from human peripheral blood and after *ex vivo* expansion were systemically injected to assess the enhancement of neovascularization in ischemic tissues. It was observed that not only did the heterologous cell transplantation improve neovascularization and blood flow recovery, but the improved perfusion was sufficient to increase the success of limb salvage (reduction of the rate of limb necrosis and autoamputation).

Chavakis[120] subsequently used murine Sca-1+/Lin- hematopoietic progenitor cells from β2-integrin–deficient mice in an attempt to demonstrate the role involved by integrins in the adhesive interaction of progenitor cells to mature endothelial cells. The group studied a mouse model of hind limb ischemia by intravenous infusion of bone marrow hematopoietic progenitor cells: the result was an incorporation of the transplanted cells in newly formed vessels and an improvement of neovascularization. With the aim of confirming *in vitro* data showing that E-selectin was expressed on activated endothelial cells, Nishiwaki performed a transplantation of EPCs in a murine ischemic hind limb model. The group used HUVECs transduced with a recombinant adenovirus of E-selectin (AdRSVE-sel) or that of β-galactosidase (AdRSVLacZ) and their effect was examined in the ischemic model. A significant improvement of tissue recovery with an enhancement in neovascularization was observed in mice overexpressing E-selectin following EPC transplantation.

In the same model of hindlimb murine ischemia, it was thought that a local injection of sE-selectin to an ischemic limb could enhance the recruitment of transplanted GFP-positive EPCs from the systemic circulation to an ischemic limb in WT mice. The incorporation of transplanted EPCs into the microvasculature of ischemic limbs was further quantified. The histological examination showed that sE-selectin pretreatment increased EPC homing in the ischemic muscle just seven days

after the systemic injection, thus the number of EPCs able to home to ischemic limbs was markedly increased by sE-selectin[176].

Besides limb ischemia, progenitor cells were also employed as cell therapy in some pathologies in animal models as well as in human trials.
Functional alterations similar to those observed in diabetic patients were found in EPCs isolated from aged patients with coronary artery disease or ischemic cardiomyopathy[177].

The number of EPCs has been greatly associated with a risk of cardiovascular disease, as suggested by the result that EPCs circulating expression level predicted the occurrence of cardiovascular events and vascular tissue injury[178].

In a swine chronic myocardial ischemia model a catheter-based transplantation of a freshly isolated, autologous EPC-enriched fraction was performed. In order to verify the therapeutic usefulness of these cells, they were transplanted into the myocardium in a nude rat model of myocardial ischemia. As a result, the locally transplanted CD34+ cells were integrated into the foci of myocardial neovascularization and differentiated into mature ECs, consequently they enhanced vascularity in the ischemic myocardium thus preserving left ventricular systolic function. The reendothelialization of the denuded blood vessels, neovascularization and paracrine effect were considered to be the mechanisms involved in EPC-mediated myocardial protection and repair after acute myocardium ischemia.

In a pilot study of cell therapy for ischaemic cardiomyopathy, progenitor cells cultured from patients' peripheral blood were compared with freshly isolated bone marrow mononuclear cells. The aim was to assess if an intracoronary infusion of progenitor cells into the infarct-related artery at least 3 months after myocardial infarction could improve global and regional left ventricular function. The results demonstrated that infusion of progenitor cells derived from bone marrow into the infarct-related artery was associated with moderate but significant improvements in both global and regional left ventricular contractile function, showing that only bone marrow and not peripheral blood progenitor cells were able to improve left ventricular ejection fraction[179].

Due to promising results from clinical trials of cell transplantation involving endothelial progenitor cells (EPCs) in hindlimb and cardiac ischemia models, other settings of occlusive thromboatherosclerotic diseases, such as strokes, were considered where the acceleration of angiogenesis might be expected to enhance the outcome. It was demonstrated that in immunocompromised mice subjected to stroke, 48 hours

earlier a systemic administration of human CD34$^+$ cells led to an acceleration in the neovascularization at the border of the ischemic zone followed by endogenous neurogenesis[180].

Recently *ex vivo* expanded EPCs were injected via the jugular vein after 1 hour of transient middle cerebral artery occlusion (tMCAO) in a mouse model in order to explore the role of EPCs following ischemic brain injury. After 3 days of MCAO, EPC transplantation prominently decreased the ischemic infarct volume compared to the control. EPC therapy reduced mouse cortex atrophy 4 weeks after tMCAO, and was accompanied by improved neurobehavioral outcomes; moreover, the injection of EPCs was able to strongly increase angiogenesis in the periinfarction area. Following a long-term stroke, the systemic delivery of EPCs was shown to be protective against cerebral ischemic injury thus promoting neurovascular repair[181].

The possibility of cellular therapy with EPCs as a treatment of diabetic macular ischemia and the vasodegenerative phase of diabetic retinopathy was discussed by Li Calzi[182]. Diabetic retinopathy is thought to largely be a result of diabetes-induced retinal microvascular dysfunction and is characterized by capillary leakage (loss of the functional integrity of the blood retinal barrier) or capillary closure resulting in ischemia (the formation of acellular capillaries, with loss of blood supply to the neural retina). The same group observed that relevant numbers of bone-marrow derived cells contributed to both repair and improve pathological neovascularization in the eye.

It was investigated whether EPCs isolated from healthy patients were better able to repopulate degenerate (acellular) retinal capillaries in chronic (diabetes) and acute (ischemia/reperfusion [I/R] injury and neonatal oxygen-induced retinopathy [OIR]) animal models of ocular vascular damage. The results were that healthy human CD34+ cells could repair injured retina and there was a defective repair of vasculature in patients with diabetes[183].

In order to observe the role of hematopoietic stem cells in revascularizing ischemic retinas a novel model of retinal neovascularization in adult mice was developed. Progenitor cells were durably engrafted and afterwards retinal ischemia was induced to promote neovascularization[184].

1.7 Mesenchymal Stem Cells

Stem cells possess two properties:

- *self-renewal*: the ability to go through numerous cycles of cell division while still maintaining their undifferentiated state;

- *potency*: the ability to differentiate into specialized cell types under appropriate stimuli, (figure 5). During the regeneration process a stem cell, just like at the beginning of the process, and the second is a progenitor cell that gives rise to a specialized cell under the right stimuli. The stem cells are divided into three classes:

- *totipotent*;

- *pluripotent*;

- *multipotent*;

- *oligopotent*;

- *unipotent*.

The zygote, the only *totipotent* cell (the first few divisions of the fertilized egg), has the potential to create all cells and tissues that generate the embryo and support its development in the uterus. This stem cell can differentiate into embryonic and extraembryonic (umbilical cord and placenta) cell types and create a complete, viable organism[185].

Pluripotent stem cells come from totipotent cells and can differentiate into nearly all cells, mainly the three embryonic sheets: mesoderm, endoderm and ectoderm. These sheets create all body cells, thus pluripotent cells are able to give rise to all cell types. Embryonic stem cell lines (ES cell lines) come from the inner cell mass of the blastocyst, one of the early stages of embryonic development, prior to the embryonic implantation in the wall of the uterus. ES cells are pluripotent because during their development they give rise to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm[186].

Multipotent stem cells can differentiate into a number of cells, but only those of a closely related family of cells.

Oligopotent stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells.

Finally *unipotent* cells can produce only one cell type (their own) but have the property of self-renewal and differentiate into all cell types of the tissue from which

they are created, and they are also distinguishable from non-stem cells (adult stem cells, undifferentiated, not specialized).
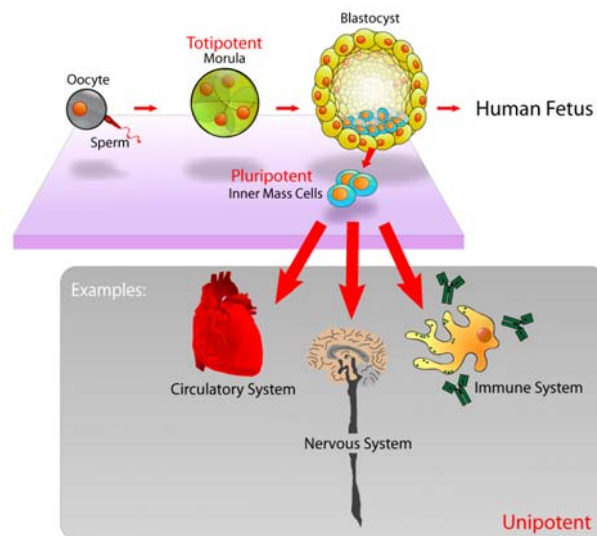

Figure 5. **Potency of cells**. The pluripotent stem cells, deriving from the inner mass of the blastocyst, give the three embryonic sheets that further develop their stemness into any tissue in the body except a placenta. The morula is the only cell able to develop in all tissues and a placenta (totipotency).

Adult stem cells, typically present in healthy tissues, give only one cell line in normal conditions and this mechanism guarantees the renewal of the tissue. Nevertheless, if the tissue is impaired, it is necessary to recreate more cell types, so pluripotent stem cells are activated[187].

Most adult stem cells are generally referred to by their tissue origin (bone-marrow, cornea, retina, dental pulp, fat, liver, skin, endothelium, etc)[188]. Among the various stem cells, a relevant role is given to mesenchymal ones, which are able to create all the cells of the mesodermic line.

In 1924 Alexander A. Maximow, Russian morphologist, used extensive histological findings to identify a singular type of precursor cell within mesenchyme that developed into different types of blood cells[189]. Later, scientists Ernest A. McCulloch and James E. Till first revealed the clonal nature of marrow cells in the 1960s[190].

An *ex vivo* assay for examining the clonogenic potential of multipotent marrow cells was reported only in the 1970s by Friedenstein and colleagues: stromal cells, which referred to an adherent cell population and had an elongated shape were similar to fibroblasts called colony-forming unit-fibroblasts (CFU-F). These cells were

able to differentiate into osteocytes, thus they were also identified as osteogenic precursors[191]. Further studies showed their differentiative potential which has also been used in the mesodermic line, with relation to condrocytes, tenocytes and myoblasts[192].

Based on these several differentiation abilities, in the 1980s Caplan introduced the expression Mesenchymal Stem Cells, MSCs[193]. Though MSCs are subjected to adult stem cells criteria, such as self-renewal and differentiative potential, the problem of whether the term "stem cell" is appropriate when referring to a single MSC cell remains unsolved. It was proposed recently to use a new expression "multipotent mesenchymal stromal cells", maintaining the acronym MSCs, to describe fibroblast-like plastic-adherent cells[194].

Later Bonnet and colleagues were able to isolate a single-cell-derived population capable of differentiating abundantly into different mesenchymal cell types *in vivo* from the murine mesenchymal compartment; they identified a subpopulation based on the expression of stage-specific embryonic antigen-1 (SSEA-1)[195].

MSCs were initially isolated by bone marrow[196] and were subsequently also cultured from adipose tissue, placenta, amniotic fluid and fetal tissue such as lung and blood, as well as from Umbilical Cord Blood (UCB). In each tissue originating from the mesenchyme, MSCs can maintain a physiological cell turn-over even if present only in a minimal amount[197].

There is still controversy today surrounding the phenotypic definition of MSCs. In fact there is no marker or combination of markers that could define MSCs in a unique way, so a different series of factors that need to be verified are considered[194]. Expanded MSCs express the following markers: CD105 (SH2 or endoglin), CD73 (SH3 or SH4), CD90, CD166, CD44 and CD29[196]. Hematopoietic or endothelial markers are not expressed by MSCs: CD11b, CD14, CD31 and CD45[196].

At present, the functional standard used to define MSCs is based on their differentiative ability in several mesenchymal lines, such as osteocytes, adipocytes and condrocytes. Pluripotent cells were recently shown to differentiate not only in the mesoderm line, but also in the endoderm and the neuroectoderm line, as neurons, hepatocytes, and endothelial cells. These cells were identified in bone marrow and were named as multipotent adult progenitor cells (MAPC)[198], hBMSCs ("human BM-derived multipotent stem cells")[199], MIAMIs (marrow isolated adult multilineage inducible cells")[200] and VSELs (very small embryonic-like stem cells)[201].

Similar pluripotent cells were also found in adipose tissue[202] and more recently in amniotic fluid. Particular growth conditions are required to culture these primordial cells, such as fibronectin-coated culture dishes, a culture medium rich in growth factors and a long culture time with low cell density. Compared to these, a higher density culture can stimulate the differentiation towards mesenchymal progenitors with reduced differentiative potential.

It has not been possible until now to isolate such cells from fresh tissues, blood or bone marrow; at present it is not still clear whether these are primary cells with a physiological role or whether they are the result of a prolonged culture in specific conditions.

Due to the lack of specific markers, the isolation of MSCs is based on their plastic adherence, obtained from a heterogeneous population of adherent cells. MSCs are structural components of the bone marrow stroma, which support the hematopoiesis by producing extracellular matrix components, like cytokines and growth factors. The main physiological role of MSCs is their ability to repair cells, which are ready to mobilize and differentiate in response to signals of pathological or traumatic conditions[203].
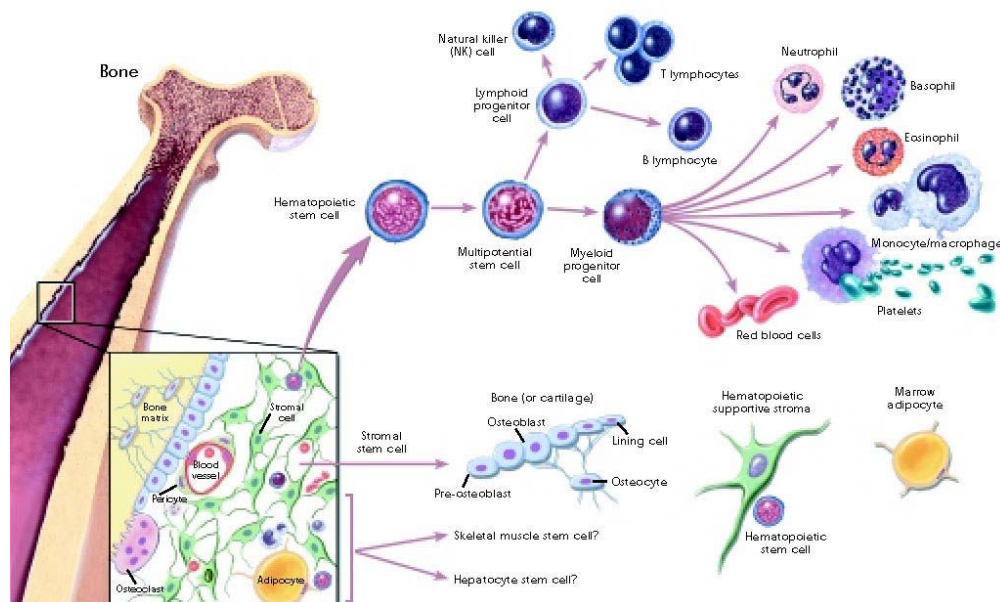

Figure 6. **Mesenchymal Stem Cells in the bone-marrow niche**

Even though bone marrow is the main source, the extraction site of MSCs influences the behaviour of isolated cells; those extracted from alveolar bones show less condrogenic and adipogenic potential than those isolated from iliac bones.

MSCs are a small cell component, but generally using 10-20 ml of bone marrow aspirate it can be isolated and expanded to form hundreds of colony-forming cells. The isolation begins through centrifugation with a different density gradient to separate the bone marrow mononucleated fraction as well as to exploit the adherence ability of MSCs to culture over plastic dishes[204]. The colony-forming unit test (CFU) is an *in vitro* method used to evaluate proliferative capacity and count colonies derived from single cell expansion. These populations are expanded for 40-50 cycles until they reach the "Hayflick limit"; they do not lose their multipotential characteristic although their proliferative characteristic is prone to decline.

Immunological studies evaluated MSCs as surface markers involved in T lymphocytes interaction such as the adhesion molecules VCAM-1, ICAM-1[196]; they also express the HLA[205], the MHC-I and also negligible levels of MHC-II and Fas ligand, but they do not express B7-1, B7-2 and CD40. Besides the MSC characteristic found in the bone marrow, these markers indicate their role in the immunoregulation processes.

MSCs were found to alter cytokines secretion by means of dendritic cells (DCs), T naïve and effector cells (Th1, Th2) and natural killer cells (NK), inducing an anti-inflammatory and more tolerant phenotype[206], (figure 7).
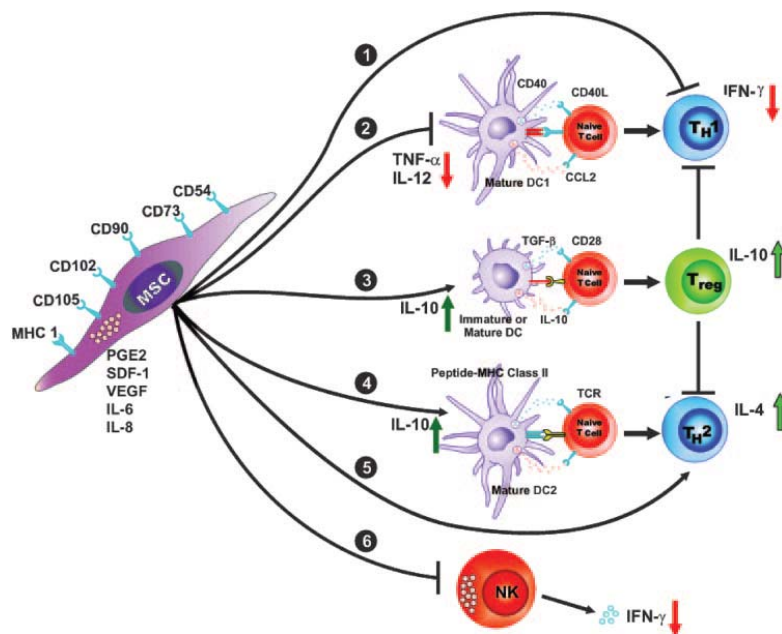

Figure 7. **Proposed mechanisms of action of MSCs.** The immunomodulatory effect of MSCs due to interactions with both the innate (DC, pathways 2-4; NK cell, pathway 6) and adaptive immunity systems (T cell, pathways 1 and 5). The DC maturation state could be influenced by MSC inhibition of TNF-α

secretion and promotion of IL-10 secretion with a resultant switch of the immune response toward in an anti-inflammatory/tolerant phenotype. MSCs could also inhibit IFN-γ secretion from Th1 and NK cells and increase IL-4 secretion from Th2 cells, thus reporting a Th1 -> Th2 shift (Aggarwal S., Blood, 2005).

Interaction between allogeneic MSCs and immune system cells can play a basic role *in vivo* in the induction of tolerance; indeed, MSCs mediate the reduction of the incidence and the severity of GVHD[62], as well as prolong graft survival in skin transplantation, reduce inflammation and suppress lymphocytic responses in allogeneic transplants[207].

In allogeneic settings these properties of MSCs were exploited as a valid alternative to immunosuppressive treatment due to the avoidance of side effects linked to the actual use of immunosuppressive pharmacological protocols[208].

At present, one of the unsolved questions on the use of mesenchymal stem cells in grafts is their dispersion in the entire body of the recipient. Several studies showed the ability of autologous and allogeneic MSCs to migrate and establish themselves in different tissues and organs of the recipient: gastrointestinal tract tissues, kidneys, skin, thymus, liver, spleen, bone marrow and mesenchymal tissues, even though data report that the preferred destination of MSCs after the transplant is the lung[209].

Mesenchymal stem cells are probably trapped into the lungs because of the major difference between the cell diameter and the inner diameter of the capillary lung lumen (> 15μm vs. 10 μm, respectively)[209].

Allers showed that the lung of a murine recipient is the long term destination site of human MSC. A similar distribution of MSCs was observed both for cells injected into the venous circle or the arterious one[210]; the distribution is time dependent[211] and, in a non-conditioned recipient, the level of grafted cells after a transplant is relatively low[210].

1.7.1 *Mesenchymal Stem Cells and islets*

In 2008 Jacobson studied the role of MSCs in the rejection of allogeneic pancreatic islet transplantation in a BALB/c mouse strain model in order to evaluate the immunosuppressive properties of MSCs. Since the MSCs showed the capacity to suppress T cell proliferation *in vitro*, Jacobson demonstrated that mice transplanted under the left kidney capsule with allogeneic islets and cultured BM-derived stromal

cells had a minor grade of rejection compared to mice receiving islets alone, suggesting also a prolongation in the survival of transplanted islets[212].

Later Figliuzzi et al., who used the same site of injection, reported that co-transplantation of MSCs with pancreatic islets improved islet graft function with a promotion in graft vascularization. They showed that co-transplantation of 2,000 pancreatic islets and 1×10^6 bone marrow–derived MSCs, under the kidney capsule of syngeneic diabetic-induced rats, were effective in that the number of capillaries increased when compared to animals receiving a greater number of pancreatic islets alone. MSCs were also able to reduce the number of transplanted islets by 30%.[213]

Moreover, Xu explored the ability of MSCs on blood glucose levels when administered by intraperitoneal injection in diabetic-induced rats if MSCs were cultured with rat pancreatic extract (RPE). MSCs treated with RPE were found to secrete several growth factors such as IGF-1, VEGF, bFGF which were considered as trophic factors when compared to the cytokines produced by normal RPE or either MSCs alone treatment. Secreted factors by conditioned RPE-treated MSC media showed to be preventive against apoptosis in pancreatic beta cells as supported by histological evaluation and morphometric evidence; what is more, a significant decrease in blood glucose levels was found[214].

Similarly, MSCs enhanced the survival of insulin-secreting β-cells when MSCs were injected together with islets into the omental pouch. By transplanting a mean value of both syngeneic 700 IE and 3×10^6 MSCs in a diabetic STZ-induced rat model there was an enhancement in long-term islet graft survival. The same protocol using allogeneic cells did not exhibit a similar long-term efficacy, thus demonstrating that the long-term survival was dependent on the presence of autologous MSCs[215].

Park et al. hypothesized that MSC-secreted molecules would induce a trophic effect in pancreatic islet culture conditions. They showed an improvement in islet survival and function after islet transplantation and human MSC-conditioned medium in a mouse streptozotocin diabetes-induced model[216]. To this aim, since successful islet transplantation is dependent not only on the number or mass of the islets, but also on the stable engraftment and prevention of immune-mediated rejection of transplanted islets, Park was intended to demonstrate the involvement of trophic agents in improving islet survival. In this context MSCs are known to repair and regenerate tissue through the secretion of active or trophic factors[217].

Therefore the role of appropriate trophic molecules that could effectively inhibit islet quality degradation during islet culture was investigated as well as whether MSCs might secrete active agents able to maintain islet viability and function. By using islet-MSC co-culture and islet culture using MSC-conditioned medium, *in vitro* data reported an improvement in insulin secretory function and viability for islets. Differences in VEGF and angiogenesis-related signalling as well as β-cell function enhancement supported the hypothesis that increased blood vessel formation of MSC-CM–cultured islet grafts is caused by a trophic factor related to MSCs, which induces signals involved in β-cell survival and intra-islet endothelial revascularization. Islet transplantation performed under the kidney capsule together with human umbilical cord blood-derived MSCs reported an increase in the intensity of insulin staining and significantly enhanced blood vessel formation in islets cultured with MSC-conditioned medium, as well as a facilitated regulation of blood glucose at normoglycaemic levels and body weight gain.

Finally, trophic agents secreted by MSCs were responsible for the prevention of islet cell death due to the induction of anti-apoptotic and survival signalling, the improvement of islet function, and the stimulation of migration and revascularization of intra-islet endothelial cells. Islets co-cultured with MSCs revealed enhanced islet quality, thus demonstrating the *in vivo* glycemic control. It can be said that MSC-originated factors have a positive effect on isolated islets[216].

Analogous results about the protective role exerted by MSCs in islet transplantation was further assessed by Ito et al. who performed an islet transplantation with 500 IE combined with 10^7 MSCs in the portal vein of diabetic-induced animals. The *in vivo* data reported that the co-transplantation of MSCs promoted capillary formation in and around the islets, showing an increased vWF-positive cell per β-cell ratio. The increased capillaries per β-cell ratio *in vivo* supported the MSC-mediated EC proliferation; some detected MSCs were found to express VEGF, which contributed to new capillary formation. From histological sections it was reported that the islets transplanted with MSCs maintained a normal, undisturbed structure, whereas islet-alone transplants appeared fragmented. MSCs prevented cell loss during the early post-transplantation period and the loss of islet cells may also be caused by proinflammatory cells and cytokines. Finally, they argued that the co-transplantation of islets and MSCs significantly reduces the number of islets required for diabetes reversal by promoting graft revascularization and improving islet cell survival and graft function[218].

In order to evaluate the effect of MSCs with islets and determine their role on the remodelling process, the graft function in a mouse model of minimal islet mass was recently studied. In the syngeneic mouse model of diabetes the effect of co-transplanting kidney-derived MSCs on the morphological and vascular engrafting, as well as the function of the transplanted islets, was assessed.

1.7.2 *Mesenchymal Stem Cells and Endothelial Progenitor Cells*

Another intriguing tissue-engineering system was the co-culture between MSCs and endothelial cells in several scaffold systems.

Since MSCs and pericytes have a similar vessel-support role due to their migration towards vessels in response to factors secreted by EC, MSCs can be considered as interacting with vascular signals *in vivo* because of their location in the perivascular niche. In 2009 Lozito studied the interaction between MSCs and micro-EC found in the microvasculature as capillaries, and macroEC found in the larger blood vessels[219]. These ECs are considered to be in cell-surface markers, and cellular and matrix interactions. He focused on the effects of extra cellular matrix produced by ECs (EC-matrix) on MSC differentiation. As ECs re-establish the differentiation signals which enable MSCs to be involved in the perivascular niche, a possible role for MSCs was also investigated in order to understand an involvement in the vascular milieu better. By analyzing the co-culture system EC-MSC, macro-EC were reported to be involved in smooth muscle cells interaction. Similarly, micro-EC deriving from capillaries without interacting with smooth muscle cells can only support EC-differentiation. In this context, MSCs cultured on macro-EC matrix were able to develop both ECs and smooth muscle cells differentiation, while their culture on micro-EC matrix only revealed an EC-differentiation. This study also highlighted an increase in MSC expression of vascular cells markers with the development of functional vascular cell phenotypes, as MSCs cultured on EC-matrix showed subcellular cytoskeletal protein distributions similar to those of vascular smooth muscle cells and improved incorporation into EC tubes on Matrigel.

Finally, EC-matrix supports MSCs differentiation toward EC and smooth muscle cells lineages[219].

Aguirre et al. later studied the physical and biochemical interactions between BM-EPCs and MSCs in an *in vitro* co-culture system to explain their roles in

vascularization, through BM-EPCs obtained from hematopoietic stem cells expressing Flk-1+ CD34+[220]. If BM-EPCs were mature enough, the co-culture caused angiogenic differentiation and tubulogenesis[221]. The co-cultures of BM-EPCs and MSCs studied by Aguirre were examined for 14 days; an early high cell motility was observed with the subsequent reorganization and formation of multicellular, elongated structures and the stabilization of cell–cell contacts.

The BM-EPCs/MSCs co-culture demonstrated the importance for direct cell contact, as seen by the early formation of stable cell unions, since many cells presented dual fluorescence in the first few hours in culture and the number of cells increased over time. The functional ability of co-cultured cells to form pre-vascular structures was evaluated by co-culturing BM-EPCs/MSCs in Matrigel-coated plates. With no VEGF supply, the co-cultured system developed into tube-like structures, even in serum-starved conditions, highlighting the importance of paracrine signalling, which can be attributed to cytokine production.

Aguirre and colleagues also showed that MSCs participated in tube-like formation analogously to BM-EPCs, thus providing evidence that MSC could differentiate to endothelial-like cells or vessel supporting cells. Another important result was the reduced cell proliferation in the co-culture system in line with the differentiation and formation of tight links observed both in Matrigel and normal conditions.

The cross-talk between BM-EPCs and MSCs was also investigated with the aid of angiogenic markers to determine the temporal profile in a 14-day period. The first three days marked an up-regulation of cell–cell and cell–matrix adhesion proteins, growth factors and signalling cytokines (Cdh-5, PECAM-1, Angpt-1, Flt-1, VEGFR-1 and Matrix metalloproteases), while growth factor members of the TGF-b family, VEGF-B, VEGF-C and the transcription factor inhibitors of differentiation Id1 and Id3, were down-regulated. In contrast, VEGF-A was slightly down-regulated at all points of time. The TGF-b family down-regulation was considered to be the cause of the reduced cell proliferation in the co-culture system[222].

A similar system of co-culture was the recently found peripheral blood-derived Outgrowth Endothelial Cells (OEC)/bone-marrow-derived MSCs in EGM-2, a special endothelial medium. The OEC supported the neovascularization process by also forming perfused vascular structures after co-implantation with other cell types. The co-culture system in EGM-2 medium arranged more microvessel-like structures compared

to co-cultures in the medium for osteogenic differentiation of MSCs due to an increased release of angiogenic factors by the mesenchymal cells in the co-culture and an increase of markers and growth factors associated with vessel stabilization by pericytes. Although MSC are considered to be a natural source of angiogenic growth factors, the cell culture medium also appears to have a significant impact on the angiogenic activation of endothelial cells in the co-culture. The increased arrangement of vascular structures in EGM-2 is related to higher levels of the proangiogenic factors VEGF and PDGF in the supernatant of co-cultures as well as in monocultures of MSCs when cultivated in EGM-2[223].

As mentioned above, pericytes play a crucial role in the process of vessel stabilization and are essential for building long-lasting vessels after the implantation of prevascularized constructs. They are resident in the outer wall of microvessels and have stabilizing functions relevant to angiogenesis[224].

Some studies report that pericytes may derive from MSC residing along abluminal surfaces of the microvasculature where they interact directly with vascular endothelial cells[225]. Furthermore, pericytes express PDGF-b receptors while MSC and endothelial cells release PDGF-BB, thus providing a paracrine interaction between pericytes, MSC, and endothelial cells[226].

The increased expression observed of sm-actin and desmin in co-cultures of MSC and OEC in response to EGM-2 has to be related to increased PDGF levels in co-cultures and monocultures of MSC when cultured in EGM-2, indicating an effect of the medium on factors responsible for vessel stabilization[223].

1.7.3 *Mesenchymal Stem Cells – Endothelial Progenitor Cells – Pancreatic Islets*

In order to enhance the islet angiogenesis better, MSCs were added to the culture medium of EC with isolated islets. The ability of ECs (with or without MSCs) to adhere to and grow into human islets was analyzed and when MSCs were present this ability was enhanced by covering the islet surface. In a system consisting of MSCs cultured with islets, the EC sprout formation was mostly stimulated in the islets where intra-islet capillary-like structures were formed.

The stimulatory effects of MSCs on angiogenesis in three-dimensional fibrin gels were reported to induce vessel formation caused by the probable degradation of the fibrin matrix by proteases produced by the MSCs, and sprout formation was improved

in matrices in which the matrix density was too high for the ECs to invade when they were cultured alone[227].

Since MSCs were able to drive the EC migration into dense micro-organs such as islets, the MSCs provide growth factors and extracellular matrix able to support the stabilization and maturation of the EC sprouts[228].

In fibrin gels the MSCs were evidenced by the increased sprout formation by EC-MSC islets, where a tight link between EC sprouts and MSCs occurred with a high degree of frequency. Compared to untreated islets, in the formation of EC-MSC islets and their culture (6 days) no additional loss of islets was observed. To promote EC adhesion and proliferation on the islet surface, the microvascular EC survival was applied; furthermore, the culture conditions did not promote the pro-inflammatory and pro-coagulant capacity of ECs (same expression of TF, IL-6, IL-8 or MCP-1 between the EC-MSC and control islets). The authors finally suggested that the use of composite EC-MSC islets could have beneficial effects on revascularization and immune regulation, constituting a technique for improving islet engraftment[229].

1.7.4 *Mesenchymal Stem Cells and revascularization*

As reported by Wu BM-MSCs were able to promote angiogenesis in a model of BM-MSC-treated wounds in diabetic mice[230]; this supported previous findings concerning newly formed granulation tissue and the survival of keratinocytes in the formation of new blood vessels during the process of neovascularization[231].

In this model, BM-MSCs were not found in the vascular structures but in close proximity, so the paracrine effect of BM-MSCs in angiogenesis was then examined; BM-MSC conditioned medium promoted endothelial tube formation and expressed high levels of VEGF-α and ANG-1 but not ANG-2, highlighting the neovessel structure role[230]. The results of Wu demonstrated that BM-MSCs engrafted in the wound release proangiogenic factors, with a consequent role for MSC mediated enhanced angiogenesis[230].

A system consisting of direct interactions between BM-MSCs and vascular endothelial cells with no further addition of dermal fibroblasts was shown to be involved in the release of angiogenic factors to regulate vessel formation *in vitro*. In an attempt to use a suitable *in vitro* model to provide both essential matrix molecules and proangiogenic factors, Sorrell et al. investigated whether a Matrigel modified model

could provide enough quantities of growth factors to sustain vascular cords through the inclusion of BM-MSCs instead of growth factors. The influence of BM-MSC was found to release both angiogenic and stabilization factors and adult human BM-MSCs acted as facilitator cells with respect to the angiogenic process[232].

Bianco finally found that mesenchymal stem cells were able to contribute to the formation of blood vessels given an adequate supply of differentiated endothelial cells. He defined this characteristic as angiopioiesis, to be distinguished from angiogenesis and vasculogenesis. The angiopoietic property of ''MSCs'' is their ability to supply ''mural cell''/pericyte characteristics where angiopoietin-1 (a gene mediating pericyte characteristic functions), and other genes are expressed in response to factors mediating endothelial–mural cell interactions. Though the issue needs to be clarified, one important question is whether MCAM/CD146 is involved in mural cells in microvascular districts of multiple tissues, since the known function of MCAM is to mediate interactions of MSC with other cells. Furthermore, BM-MSCs were reported to be involved in the formation of sinusoids, as opposed to capillaries, so it can be argued that a sinusoidal-type microvascular network was considered as a key developmental event in hematopoiesis[233].

2. AIM OF THE THESIS

The overall aim of this thesis is to investigate the role of MSCs and EPCs in decreasing acute rejection of pancreatic islet transplantation in a chemically induced rat model of type 1 diabetes. Previous studies based on the use of pancreatic islets alone and in combination with cell therapy confirmed that pancreatic islets alone are insufficient in re-establishing normoglycaemic levels due to their poor microvascularization in the resulting grafts. We therefore decided to perform pancreatic islet transplantation together with MSCs and EPCs, both of which were administered with pancreatic islets via the portal vein in syngeneic and allogeneic models in order to re-establish the normal architecture and function of pancreatic islets and reach normoglycaemic levels in the long-term. An assessment of the re-establishment of the revascularization and neoangiogenesis of the islets according to our protocols was then carried out using *ex viv*o molecular biology assays (RT-PCR).

3. MATERIALS AND METHODS

3.1 Animals

Inbred male Lewis (L) rats as recipients and Wistar Furth (WF), Lewis and Lewis *LEW-Tg F455/Rrrc* (Harlan) donor rats weighing approximately 250-300 g, were purchased from Charles River Laboratories, Calco, Italy. The animals were fed on standard rodent chow (Rieper, Italy) and water *ad libitum;* they were kept under a 12 h light/dark cycle. All the experimental procedures were carried out with the approval of the Ethical Committee for Animal Experimentation of the University of Pisa and the EEC/609/86. All efforts were made to reduce the number of animals used.

3.2 Pancreatic Islets

3.2.1 *Pancreas isolation, digestion and islet culture*

Donor animals were anaesthetized with an intraperitoneal injection containing a single dose of Pentothal sodium (75 mg/Kg, Gellini International) and underwent a median laparotomy. The pancreas was highlighted and the bile duct was clamped at the duodenum point of release[234], (figure 8 A).

The bile duct was incanulated (figure 8 B) in proximity to the hepatic triad (portal vein, hepatic artery, bile duct) and the animal was sacrificed. The pancreas, distended by bile duct injection through a cannula with a diameter of 23 gauche, was perfused with 15 ml of Collagenase P, (Roche Diagnostics, Italy), 1 mg/ml maintained at T= + 4°C to avoid the activation of the enzyme. Collagenase P was able to disrupt the exocrine parenchyma, leaving the endocrine part intact, with a subsequent separation of the pancreatic islets.

After the enzyme perfusion the pancreas resulted as being a well-defined organ so it was easily excised and minced. The pancreas was collected in a 50 ml tube and kept in ice until digestion started. The organ was incubated at T = + 37°C for 15 min while being continuously shaken, so the Collagenase P was activated by heat, and at the end of the incubation time, the pancreas was homogenized using a Pasteur's pipette.

The organ was washed with Hank's solution (HBSS), centrifuged (300 g, 7 min, T = + 4°C) and the solution above was discarded; the procedure was repeated three times (with these washing cycles, traces of fat and blood were removed with a parallel activation of Collagenase P). Islets were then separated from exocrine tissue by centrifugation on discontinuous gradient: the homogeneous tissue was resuspended in a tube containing 10 ml of Histopaque® 1119 (density: 1,119 g/l, Sigma, Italy) and then 10 ml of Histopaque® 1077 (density 1,077 g/l, Sigma-Aldrich, Italy) and 10 ml of HBSS were stratified one after the other on the top of the sample. The tube was centrifuged for 20 min. at 546 g at T = + 4°C. The islets forming 2 rings were removed from the interface of the three layers, washed two-three times in HBSS, and finally resuspended in 10 mL of RPMI (Eurobio, Italy) supplemented with 10% Fetal Calf Serum (Eurobio, Italy), 1% L-glutamine (Biowest), 10 mM glucose (Baker Analized reagents, Netherlands), 50 U/ml penicillin (Sigma-Aldrich, Italy), 50 μg/ml streptomycin (Sigma-Aldrich, Italy), 0,2 μg/ml amphotericin B and 1% HEPES buffer (Sigma-Aldrich, Italy) on a free floating culture flask.

Islets were handpicked under an inverted microscope, (figure 8 C), under sterile conditions and purity was assessed by Dithizone staining (Sigma-Aldrich, Italy). Pancreatic islets were cultured in RPMI medium (Eurobio, Italy) and incubated at T = + 37 °C (95% air and 5% CO_2), for a maximum time of two days before transplantation. The total islet mass, for each graft, expressed as the 150μm diameter islet equivalent number (IE), was calculated based on volumetric assumptions.
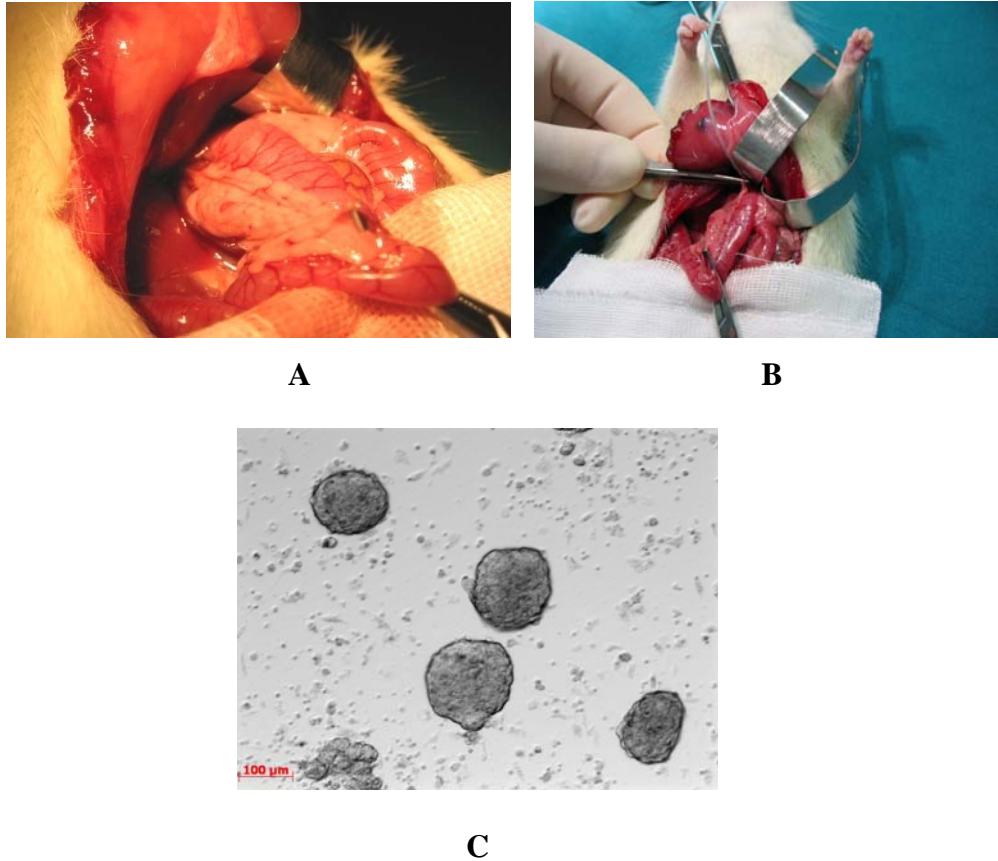
Figure 8. **Pancreas isolation and islets culture.** Clamping of bile duct at the duodenum point of release (**A**); incanulation of bile duct in proximity of the hepatic triad (**B**); pancreatic islets at optical microscope, magnification 10x, referred to a 100 μm scale bar (**C**).

3.2.2 *Pancreatic islet viability assay*

The number of viable islets was determined by a 0.4 % Trypan Blue staining assay (Sigma-Aldrich, Italy). The blue pigment did not enter the membrane of viable cells so they appeared as shiny, circular and bright, with a dark side, (figure 9). The dye entered the non-intact membrane dead cells, so they became blue. After a cycle of centrifugation at 180 g for 5 min and a resuspension of the pellet in 1 ml of PBS-1X, the number of viable cells was determined by evaluating a 400 μl sample with 10 μl of Trypan Blue. Viable islets were evaluated under the optical microscope and only bright ones were counted.

If less than 30% of the surface of the islet contained dead cells, the islet was considered viable because the visibly coloured surface means that there is a higher number of dead cells in the inner part of the islet. The cell count was completed within 3 min to avoid the dye also entering the viable cells.
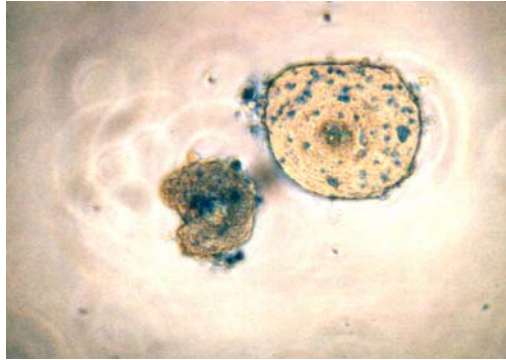
Figure 9. **Islet viability by means of Trypan Blue**.

3.2.3 *Islet quality assessment by Dithizone*

Dithizone (DTZ) (Sigma-Aldrich, Italy) was used to assess the functionality of pancreatic islet. DTZ binds zinc ions, which are necessary for insulin assembly in a resultant exameric structure, and are present in the islets' ß cells, and therefore stains the islets red, (figure 10). Exocrine tissue, which the preparations also contain, does not bind DTZ and is therefore not stained[235]. Islets containing insulin-secreting ß cells were transplanted into animals with type 1 diabetes. Briefly, DTZ solution (0.1 mg/ml) in RPMI was filtered by a 0.2 µm filter and used to assess red stained viable islets.
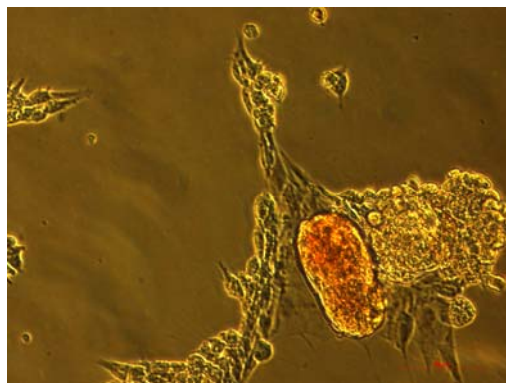

Figure 10. **Islet staining with Dithizone.**

3.3 Mesenchymal Stem Cells

3.3.1 *MSCs isolation, expansion and characterization*

Rat bone marrow cells were collected from tibiae and femurs of Wistar Furth, Lewis and *LEW-Tg* (*F455/Rrrc*, University of Missoury, USA) animals according to Dobson's procedure[236]. Briefly, once the femurs and tibiae were extracted, their proximal ends were removed, and the bones were placed in microcentrifuge tubes supported by plastic inserts cut from 1 ml hypodermic needle casings and briefly centrifuged at 700 g for 2 min. The marrow pellet was resuspended in 10 ml of Hank's balanced salts solution (HBSS, w/o calcium and magnesium; Euroclone, Milan, Italy) + 1% fetal bovine serum (FBS, HyClone, South Logan, Utah, USA) and washed at 300g for 7 min. After the cells were passed through a 22-G needle, they were re-suspended in culture medium (DMEM-Low Glucose, with L-glutamine, 25 mM HEPES and pyruvate, GIBCO™–Invitrogen, Milan, Italy, supplemented with 10% FBS), counted using a hemocytometer and seeded in a 24 x 10^6/75 cm^2 flask.

Cells were incubated at T = + 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Half of the complete medium was changed after 1 week and thereafter the whole medium every 3–4 days. When approximately 80% of the flask surface was covered, the adherent cells were incubated with 0.05% trypsin–0.02% EDTA (Eurobio, Courtaboeuf, Cedex B, France) for 5–10 min at T = + 37°C, harvested, washed with HBSS and 10% FBS, and resuspended in complete medium (primary culture, P0). Cells were then re-seeded at 10^4 cells/cm^2 in 100-mm Ø dishes (P1): expansion of the cells was obtained with successive cycles of trypsinization and reseeding[237]. The number of colony forming units-fibroblasts (CFU-F) was used as a surrogate marker for MSC progenitors frequency: two 100-mm Ø dishes were seeded with 1 x 10^6 total nucleated cells. After incubation for 14 days at 37°C in a 5% CO_2 humidified atmosphere, the dishes were rinsed with HBSS, fixed with methanol and stained with Giemsa: visible colonies formed by 50 or more cells were counted and reported as the number of CFU-F/10^6 seeded Total Nucleated Cells (TNC)[238].

3.3.2 *Immunophenotyping*

At the fourth or fifth passage, the morphologically homogeneous population of MSCs was analysed for the expression of particular cell surface molecules using flow

cytometric procedures: MSCs recovered from flasks by trypsin–EDTA treatment and washed in HBSS and 10% FBS were resuspended in a flow cytometry buffer consisting of CellWASH (0.1% sodium azide in PBS; Becton Dickinson, San Jose, CA, USA) with 2% FBS. Aliquots (1.5×10^5 cells/100 µl) were incubated with the following conjugated monoclonal antibodies: CD45-CyChrome™, CD11b-FITC (in order to quantify hemopoietic-monocytic contamination), CD90-PE, CD106-PE, CD73-PE, CD54-FITC, CD44-FITC (BD Pharmingen, San Diego, CA, USA). Non-specific fluorescence and morphologic parameters of the cells were determined by incubation of the same cell aliquot with isotype-matched mouse monoclonal antibodies (Becton Dickinson, San Diego, CA, USA). All incubations were performed for 20 min, and after incubation the cells were washed and resuspended in 100 µl of CellWASH; 7-AAD (7-amino-actinomycin D) was added in order to exclude dead cells from the analysis. Flow cytometric acquisition was performed by collecting 10^4 events on a FACScalibur (Becton Dickinson, San Jose, CA, USA) instrument and the data were analysed on DOT-PLOT bi-parametric diagrams using CELL QUESTpro software (Becton Dickinson, San Jose, CA, USA) on a Macintosh PC.

3.3.3 *Differentiation of MSCs*

The ability of MSCs to differentiate along osteogenic and adipogenic lineages was assayed, as described previously by Sbano et al[355]. Osteogenic and adipogenic differentiation were evaluated by cytochemical analysis. Petri dishes were stained to assess extracellular matrix mineral bound by Alizarin Red-S. Adipogenic differentiation was evaluated by staining lipid–rich vescicles with Oil Red O.

3.3.4 *Osteogenic differentiation*

The primary (P0) MSC culture was stimulated with osteogenic differentiating factors: 10^5 cells/plate were seeded in a 6 well-plate and cultured with the culture medium supplemented with 100 µg/ml Ascorbic Acid (Sigma-Aldrich, Italy), 10 mM β-Glycerophosphate (Sigma-Aldrich, Italy) and 10nM Dexamethasone (Sigma-Aldrich, Italy). The medium was replaced every 3 days and the deposition of mineral nodules was revealed after 20 days with Alizarin Red-S. The cells were washed with HBSS solution at room temperature and incubated at 4°C with 70% cold Ethanol

(T = - 20 °C) for 1 hour. They were then washed twice with 1 ml of PBS 1X and incubated with Alizarin S, 40 mM, pH 4.2, (Sigma-Aldrich, Italy) at room temperature for 10 min while being continuously shaken. The cells were washed rapidly 5 times with HBSS to minimize the non-specific binding.

3.3.5 *Adipogeneic differentiation*

The primary (P0) MSC culture was stimulated with adipogenic differentiating factors: 10^5 cells/plate were seeded in a 6 well-plate and cultured in adipogenic medium (DMEM-LG with 10 % FBS, 0.5 mM of isobutyl methylxanthine, 10 μM of dexamethasone, 10 μg/ml of insulin, and 70 μM of indomethacin; Sigma). The medium was replaced every 3 days and the accumulation of lipid-rich vacuoles was revealed after 21 days with Oil Red-O staining. The cells were washed with HBSS solution at room temperature and incubated at room temperature with 10% formalin for 5 min; the formalin was then removed and the same volume of fresh formalin was added. The cells were incubated for 1 hour at room temperature. The formalin was then removed and the cells were washed with 60% isopropanol and left to dry. Subsequently, 2.1 mg/ml Oil-Red O solution (Sigma-Aldrich, Italy) was added and incubated for 10 min at room temperature to minimize the non-specific binding. The cells were washed four times with deionized H_2O.

3.4 Endothelial Progenitor Cells
3.4.1 *EPCs isolation and expansion*

Bone marrow was harvested from both femurs and tibias of Wistar Furth and Lewis *LEW-Tg* (EPCs GFP+*) F455/Rrrc* (Rrrc, University of Missoury, USA) male rats, according to Dobson's procedure[239]. Mononuclear cells (MNC) were obtained by density gradient centrifugation. The cells, 25×10^6 MNC/well, were then seeded on 6 well plates coated with 1% Gelatin (Sigma-Aldrich, Italy), and cultured in EGM2 (Lonza, Italy) supplemented with 10% FBS. They were then incubated at 37°C in a fully humidified atmosphere containing 95% air and 5% CO_2. EPC colonies appeared in cell cultures after 1 week and were identified as circumvented monolayers of cobblestone-like cells.

3.4.2 *Flow cytometric assay*

A flow cytometric analysis was performed on cells at the passage P2 using the following surface antibodies: CD45 (in order to quantify hemopoietic–monocytic contamination), CD44, CD90, CD31, Endothelium (AbD Serotec, BD Pharmingen). Non-specific fluorescence and morphologic parameters of the cells were determined by isotype-matched mouse monoclonal antibodies and 7-AAD. The cells were measured in a FACSCalibur (Becton Dickinson) flow cytometer.

3.4.3 *Dil-AC-LDL uptake*

To identify ex vivo expanded EPC the cells were imaged for their incorporation of acetylated low-density lipoprotein (aLDL) labelled with fluorescent Dil dye (Dil-Ac-LDL, Invitrogen). The cells were incubated with 10 μg/ml Dil-Ac-LDL for 4 hours at 37°C on a glass slide. After fixation with 10% formalin for 20 min at 4°C, they were washed and visualized by fluorescence microscopy.

3.5 *In vitro* experiments
3.5.1 *In vitro angiogenesis assay*

BD Matrigel™ Basement Membrane Matrix was thawed on ice overnight, and a volume of 50 µL, added to EGM-2 (Lonza, Italy) supplemented with 1% FCS was spread evenly over each well of a 24-well plate. The plates were incubated for 30 min at 37°C to allow Matrigel to gel. EPCs GFP+ were seeded (3.0×10^4 cells/cm^2) in complete absence of light and cultured in 1 ml of EGM-2 medium (Lonza, Italy) supplemented with 1% FCS. After 2 hours of incubation at 37°C, the cultures were photographed (5 fields for each well: the four quadrants and the center) at a magnification of 10 x, and were checked approximately every 2 hours to observe the tube formation. Phase contrast (bright field) and fluorescence images were recorded by carrying out a double channel experiment with a Carl Zeiss Microscope with a fluorescence camera, (AxioVision 4.8.2 software).

3.5.2 *Monitoring cell growth rate*

The proliferation rate of MSCs and EPCs was assessed by separate assays. The cells, MSCs or EPCs, were seeded as previously mentioned and, after the attachment of the cells, they were removed by trypsinization at 24, 48 and 72 hours to determine their growth rate. The cells were counted in a Thoma's camera under an optical microscope and fluorescence microscope.

Each doubling time (T_d) was calculated, by assuming a constant growth rate, with the following formula:

$$T_d = (t_1 - t_0)*\ln 2/\ln(q_1/q_0)$$

where q_0 is the cell number at time t_0 and q_1 is the cell number at time t_1.

3.6 *In vivo* experiments

Recipient animals received a single high dose (65mg/kg intraperitoneal) of Streptozotocin (STZ) (Sigma-Aldrich, Italy). Streptozotocin is an antibiotic extracted from *Streptomyces achromogenes* and 10 hours after the injection it causes pancreatic beta cells necrosis, with high levels of insulin and hypoglycaemia[240].

Blood glucose concentration was determined by using a commercially available glucose meter on days 3 and 5 after STZ injection (Stat Strip XPress™, Nova Biomedical UK). Animals with fasting blood glucose higher than 16.7 mmol/l (> 300 mg/dl), on at least two consecutive measures, were considered diabetic, while rats whose blood glucose remained lower than 16.7 mmol/l after 1 week were withdrawn from the study.

Donor animals were anaesthetized with an intraperitoneal single dose of Penthotal Sodium, 65 mg/kg, (Gellini international), while diabetic recipients were anaesthetized with an intraperitoneal dose 0.3 ml of Zoletil 100 (tiletamin 90 mg/Kg and zolazepam 10 mg/Kg, Virbac s.r.l., Italy, intraperitoneal) and the portal vein was exposed through a midline incision under the spine. All animals were transplanted either with IE or MSCs or EPCs via the portal vein of the liver.

3.6.1 *Experimental Transplant groups*

Control group
- 700 IE L-L (n=6)
- 700 IE WF-L (n=5)
- 500,000 EPCs WF-L (n=4)

MSCs group
- 700 IE L-L + 500,000 MSCs (n=6)

EPCs group
- 700 IE L-L + 500,000 EPCs (n=11)
- 700 IE WF-L + 500,000 EPCs (n=5)

3.6.2 *Pancreatic islet, MSCs and EPCs transplantation*

Pancreatic islet transplantation

Pancreatic islets were collected from the cultured flasks and transferred to a sterile tube. After being centrifuged at 180 g for 5 min, the pellet was suspended in 0.5 ml of PBS-1X, counted and suspended again in 0.5 ml of PBS-1X for the injection.

Mesenchymal Stem Cells transplantation

MSCs medium was removed from the flasks and treated with a cycle of Trypsin 1X with 7 min of incubation at T = + 37°C, 5 % CO_2, to let the enzyme activate. The cells were then blocked by DMEM; the resultant solutions were collected and centrifuged at 180 g for 5 min. The pellet was suspended in 0.2 ml of PBS-1X solution, of which an aliquot of 30 µl was taken and added to 30 µl of Trypan Blue and counted in Thoma's camera to assess viable cells.

Endothelial Progenitor Cells transplantation

EPCs medium was removed from the flasks and treated with a cycle of Trypsin 1X with 5 min of incubation at T = + 37°C, 5 % CO_2, to let the enzyme activate. The cells were then blocked by HBSS supplemented with 10% FCS; the resultant solution was collected and centrifuged at 180 g for 5 min. The pellet was suspended in 0.2 ml of PBS-1X solution, of which an aliquot of 30 µl was taken and added to 30 µl of Trypan Blue and counted in Thoma's camera to assess viable cells.

Once the cells MSCs or EPCs were counted and placed, either alone or in combination with pancreatic islets, in 0.5 ml of PBS-1X solution. When the cells were

injected with pancreatic islets, they were mixed together in the solution and incubated for one hour maximum at T = + 37°C, 5 % CO_2.

After a median laparotomy and a midline incision, the portal vein was exposed, (figure 11 A) and cells were injected using a syringe (the portal vein was the site of injection where the cells were allowed to enter the hepatic parenchyma), (figure 11 B).
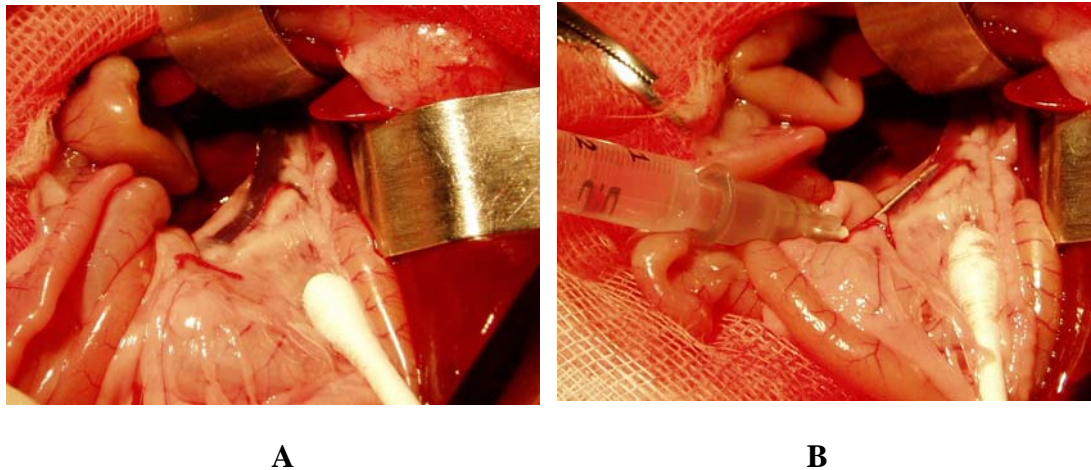

A   **B**

Figure 11. **Portal vein islet transplantation.** The portal vein was exposed through a midline incision under the spine (**A**). Islets were suspended in 0,5 mL of PBS solution and injected through a 26G needle into the portal vein (**B**).

After having padded the site of injection and washed the animal entrails with physiological solution, the abdomen wall was sutured at two different edges. The inner edge, made up of muscles and peritoneum, was sutured with a 4-0 gutstring (Ethicon, Johnson & Johnson); the upper edge was also sutured with a non-reabsorbable gutstring (Ethicon, Johnson & Johnson). The animals were kept in separate cages and checked daily for glucose monitoring in accordance with the Ethical Committee guidelines of the University of Pisa.

3.7 Assessment of Graft Function

Fasting blood glucose levels were used to assess islet graft function. The measures were performed at defined time points (-5, -3, 0, 2, 3, 4, 5, 7, 9, 12, 15, 18, 21, 24, 27, 30, 37, 44, 51, 58, 72, 86, 100, 120, 140, 160, 180 days from transplantation). Graft failure was defined as a reversal of hyperglycemia (> 300 mg/dL) by two consecutive measurements by using a glucometer.

3.8 Intraperitoneal glucose tolerance test (IPGTT)

After 6 h of fasting, the animals of all the groups were injected intraperitoneally with 2 g/Kg body weight of Glucose solution (Baker Analized, Reagent). Their blood glucose levels were measured at 0, 15, 30, 60, 90 and 120 minutes after the injection using a glucometer. The values were compared to diabetic and healthy control animals.

3.9 *Ex vivo* experiments

A maximum amount of 30 mg of fresh liver tissue was collected in a microcentrifuge tube and stored at T = + 4 °C immediately after excision of the organs and up to 2 hours before starting with the purification of total RNA. If it was not possible to process the tissue within 2 hours, the samples were stored at T = - 80 ° C.

3.9.1 *Total RNA extraction and characterization*

Once the samples of the liver tissues were weighed as above, disrupted and homogenized by a syringe in 600 μl of the lysis RLT buffer supplied with β-Mercaptoethanol (a highly denaturing guanidine-thiocyanate–containing buffer, able to immediately inactivates RNases to ensure purification of intact RNA) and incubated for 15 min at room temperature, total RNA was extracted using the RNeasy® Mini kit following manufacturer's 'Purification of total RNA from animal tissues' protocol (see the RNeasy Mini Handbook, Qiagen, Hilden, Germany, http://www.qiagen.com).

Briefly, the lysate was centrifuged for 3 min at full speed; the supernatant was carefully removed by pipetting and transferred to a new microcentrifuge tube. Moreover, one volume of 50% ethanol was added to the cleared lysate and mixed immediately by pipetting, with no further centrifugation (ethanol was added to provide appropriate binding conditions).

Afterwards up to 700 μl of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuge for 15 s at 12000 g. The flow-

through was discarded and exceeding volume was re-transferred in the same RNeasy spin column and centrifuged again at the same conditions (the step in the RNeasy Mini spin column allows the total RNA to bind to the membrane, while contaminants were efficiently washed away). An amount of 350 µl of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at 12000 g to wash the spin column membrane. In order to degrade genomic DNA from RNA preparation prior to RT-PCR, the column was added with 46 µl of the following mix:

- 5 µl Incubation Buffer 10x (Roche Diagnostics GmbH, Germany, www.roche.com)
- 1 µl Recombinant DNase I RNase-free 10,000 units (Roche Diagnostics GmbH, Germany, www.roche.com)
- 40 µl Rnase-free water.

The spin column was incubated with the mix for 30 min at room temperature and at the end of the incubation time, buffer RW1 process was repeated with the same conditions as before DNase treatment. After centrifugation, the RNeasy spin column was carefully removed from the collection tube so that the column did not contact the flow-through. It was then added 500 µl of buffer RPE supplied with ethanol to the RNeasy spin column and centrifuged for 15 s at 12000 g to wash the spin column membrane. The flow-through was discarded and this step was repeated with a final centrifugation for 2 minutes at 12000 g (this long centrifugation dries the spin column membrane, ensuring that no ethanol was carried over during RNA elution, because residual ethanol might interfere with downstream reactions).

The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min to eliminate any possible carryover of Buffer RPE, or if residual flow-through remained on the outside of the RNeasy spin column after previous step.

The RNeasy spin column was placed in a new 1.5 ml collection tube, added with 40 µl RNase-free water directly to the spin column membrane and centrifuged for 1 min at 12000 g to elute the RNA.

The amount of extracted RNA was quantified by measuring the absorbance at $\lambda = 260$ nm with a Biophotometer Plus Eppendorf spectrophotometer diluted as follows: 5 µl RNA + 95 µl RNase free water. The final concentration of the RNA was expressed in ng/µl. The purity of RNA was checked by measuring the ratio of the absorbance at 260 and 280 nm where a ratio ranging from 1.5-2.0 was

considered to be pure. The absence of degradation of the RNA was confirmed by RNA electrophoresis on a 1.5% agarose gel containing ethidium bromide.

3.9.2 *Reverse transcription*

To synthesize first strand cDNA, RNA was reserve transcribed with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Life sciences): the kit provides a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates.

Briefly, in a sterile, nuclease-free tube on ice, a variable amount of total RNA, between 0,1 ng and 5 μg, was added; in the tube was placed a fixed amount of oligo(dT)$_{18}$ primer (1 μl), (the oligo(dT) primer selectively anneals to the 3'-end of poly(A) RNA, synthesizing cDNA only from poly(A) tailed mRNA). In the same tube a variable content of nuclease-free water was added up to a final volume of 12 μl. After a gentle mix by pipetting, the sample was briefly centrifuged and incubated at 65°C for 5 min and maintained on ice.

At the end of the incubation period, the mix was added with the following:

- 4 μl of 5X reaction buffer

- 1 μl of RiboLock™ RNase Inhibitor (20 u/μl) (protect effectively RNA from degradation at temperatures up to 55°C)

- 2 μl of 10 mM dNTP Mix

- 1 μl of RevertAid™ M-MuLV Reverse Transcriptase (200 u/μl) (maintains activity at 42-50°C)

up to a total volume of 20 μl.

After a gently mixing and centrifugation, the sample was incubated for 60 min at 42°C. The reaction stops after heating at 70°C for 5 min. The reverse transcription reaction product could be directly used in PCR applications or aliquoted in equal volumes and stored at T = - 80 °C for several months.

cDNA was quantified by measuring the absorbance at $\lambda = 260$ nm with a Biophotometer Plus Eppendorf spectrophotometer with the following dilution: 1 μl cDNA + 99 μl water. The final concentration of the cDNA was expressed in ng/ml.

3.9.3 *Real-time quantitative RT-PCR*

Real-time quantitative RT-PCR (qRT-PCR) was performed with SYBR Green Master Mix technique (Qiagen), according to the manufacturer's instructions.

A total volume of 5 µl of RNA was used in the assay for each sample and all the genes were tested with the same panel of cDNA samples. All standards and samples were run in triplicate on 96-well reaction plates with the iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad).

Reactions were prepared in a total volume of 25 µl containing 5 µl volume cDNA, 1 µl of each primer (forward and reverse 10 µM), 12.5 µl of SYBR Green master mix, 5.5 µl of sterile water. No template controls were run for each master mix and primer pair.

After having performed preliminary gradient real-time RT-PCR assays, the optimal annealing temperature for all the primer pairs resulted to be T = + 60 °C. At this temperature cycle threshold (Ct) value was generated as well as a sharp melting peak, with no amplification of non-specific products or primer-dimer artefacts. The conditions of reverse transcription and primer concentrations were set according to the recommendations in the manufacturer's instructions. PCR efficiencies were calculated with a relative standard curve using serial 1:5 dilutions of template cDNA in triplicate. Gene expression variation was calculated for individual reference genes based on Ct values, correlation coefficient (R^2), real-time PCR efficiencies (E) and slope values, generated of each standard curve. The curves obtained for each cell line showed a linear relationship between RNA concentration and the Ct value of PCR real time for all genes. Real time efficiency E was calculated from the given slopes in the iQ Real Time PCR System software according to the equation: E=10-(1/b)-1, where b is the regression coefficient. Efficiency of primers pairs ranged from 90% to 120% and correlation coefficient ranged from 0.993% to 1.0%.

The real time amplifications included 3 minutes at T = 96 °C (AmpliTaq Gold activation), followed by 40 cycles at T = 96 °C for 30 seconds and at T = 60 °C for 1 minute. This cycle was followed by a melting curve analysis, ranging from 55°C to 95°C, with increasing steps of temperature of 1°C every 30 seconds.

Baseline and threshold values were automatically determined for all plates using the Bio-Rad iQ5 Software 2.0. The threshold value has been subsequently manually set if necessary.

When comparing gene expression in different samples, it is essential to consider experimental variations such as amount of starting material, inhibitory factors of the tissue, RNA extraction and integrity, reverse transcription efficiencies, loading error. To account for these, accuracy of RT-PCR relies on normalization to an internal control, often referred to a reference gene (RG). Before any gene is chosen as a reference, an exhaustive search is necessary to ensure that no significant regulation occurs.

The expression of the primers VEGF-A, ANG-1, VCAM-1, SDF-1, PECAM-1 for RT-PCR was designed using Beacon Designer software. The primer for the reference genes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also designed using the same software. This procedure has been standardized taking into account the particular structural characteristics that the primers could be due to the chemistry of the SYBR-Green. The aim was to get similar temperatures to perform more analysis at the same time. Special attention was given to primer length, annealing temperature, base composition and 3'-end stability. Exon and intron boundaries were determined by aligning primers with corresponding rat genomic sequences downloaded from GenBank, http://www.ncbi.nlm.nih.gov/genbank/, and ensuring the specificity of the sequence by BLAST.

Sequences of the primers:

SDF-1	TGCATCAGTGACGGTAAGCCA (F)
	GATCCAAGAGTACCTGGACAA (R)
PECAM-1	TCAGCTGCCAGTCAGTAA ATGG (F)
	TCTGGAAGTTGCTCTTTGCTCTT (R)
VEGF-A	GAGGAAAGGGAAAGGGTCAAAA (F)
	AATCCTGGAGCGTTCACTGTG (R)
ANG-1	GTGGCTGGAAAAACTTGAGA (F)
	ACATCCCGTCTTGAAATCCA (R)

3.10 Statistical analysis and image performing

Data were analyzed using GraphPad Prism version 5 software. All numerical data were expressed as the mean ± SE. Data were tested for statistical significance with the paired Student's *t* test, the unpaired Student's *t* test or by analysis of variance (one-way ANOVA), as appropriate. When significant differences were observed, the Tukey test as a *post hoc* comparison test (one-way ANOVA) was done. A value of $p < 0.05$ was considered significant.

Analysis of MSC and EPC samples was realized by flow cytometry with a FACScan (Becton Dickinson, argon laser source, with wave length of emission at 488 nm and power of emission 15 mW) on a PC by CELL QUEST software (Becton Dickinson).

4. RESULTS

4.1 *In vitro* experiments

4.1.1 *Expansion and characterization of rat MSCs*

Rat BM-derived MSCs were successfully culture-expanded. Cells were particularly heterogeneous until the fourth–fifth passage in culture and also comprised numerous lipid vacuoles. Haematopoietic cells were lost during the medium changes as shown by Flow cytometric analysis. Primary culture cells were trypsinised and plated, reaching a cellular expansion up to a mean 10^9 factor in 3 months (Figure 12 A). The time of proliferation of MSCs was represented in fig. 1A, showing a mean value of doubling time of 25 hours.
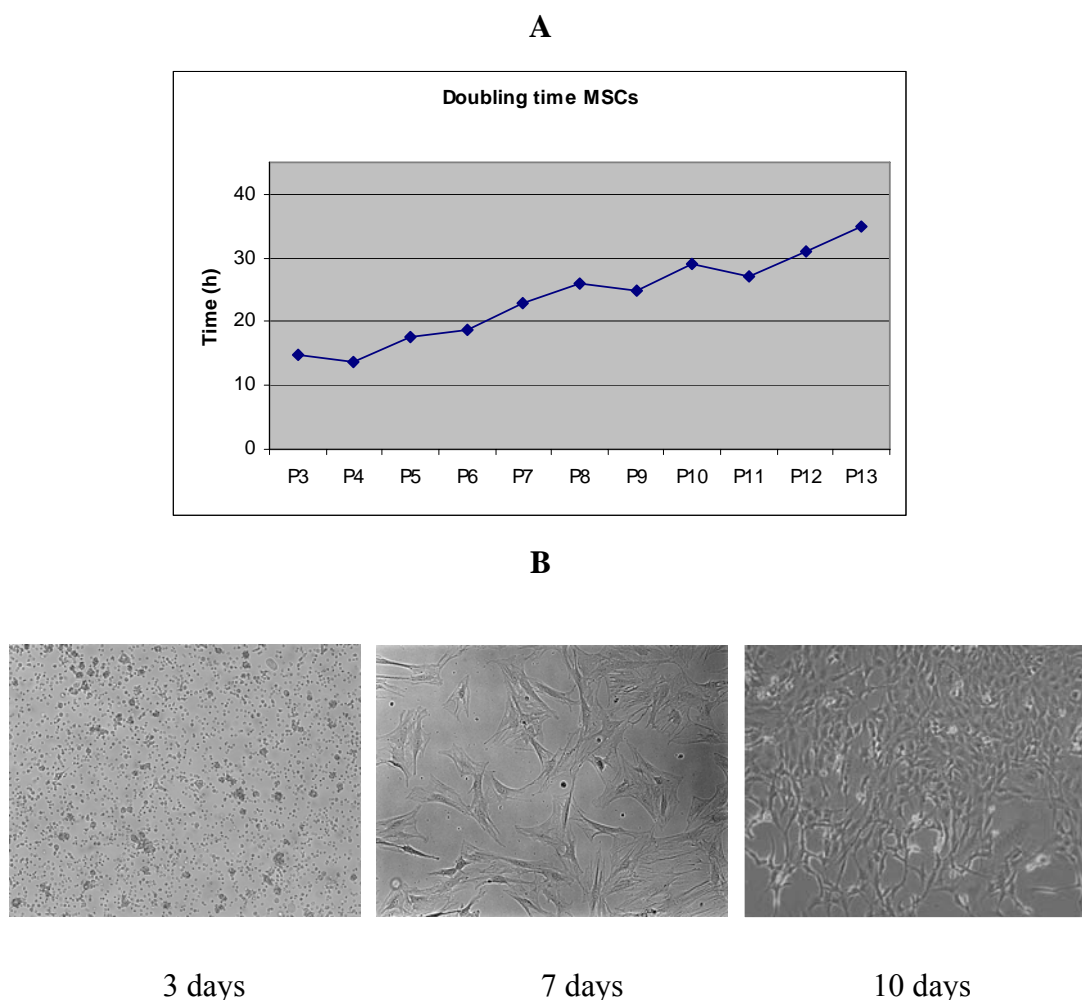
A

B

| 3 days | 7 days | 10 days |

Figure 12. **Characteristics of MSCs**. Doubling time of MSCs represented as the growth curve (**A**); typical morphology in time lapse in the first 10 days from the seeding (**B**).

After the fifth passage, the cells grew exponentially, requiring weekly passages (Figure 12 B). The CFU-F assay was used as a surrogate assay for MSCs. In the BM total nucleated cell population, the estimated CFU mean count resulted as $56/10^6$ TNCs.

Isolated cells from bone marrow of Lewis rats showed, by carrying out a careful study with FACS analysis, a homologous dimensional distribution in the range 300-800 μm, (figure 13 A). Cells resulted negative for isotype control (figure 13 C) because they did not bind non-characteristic and non-autofluorescent antibodies.
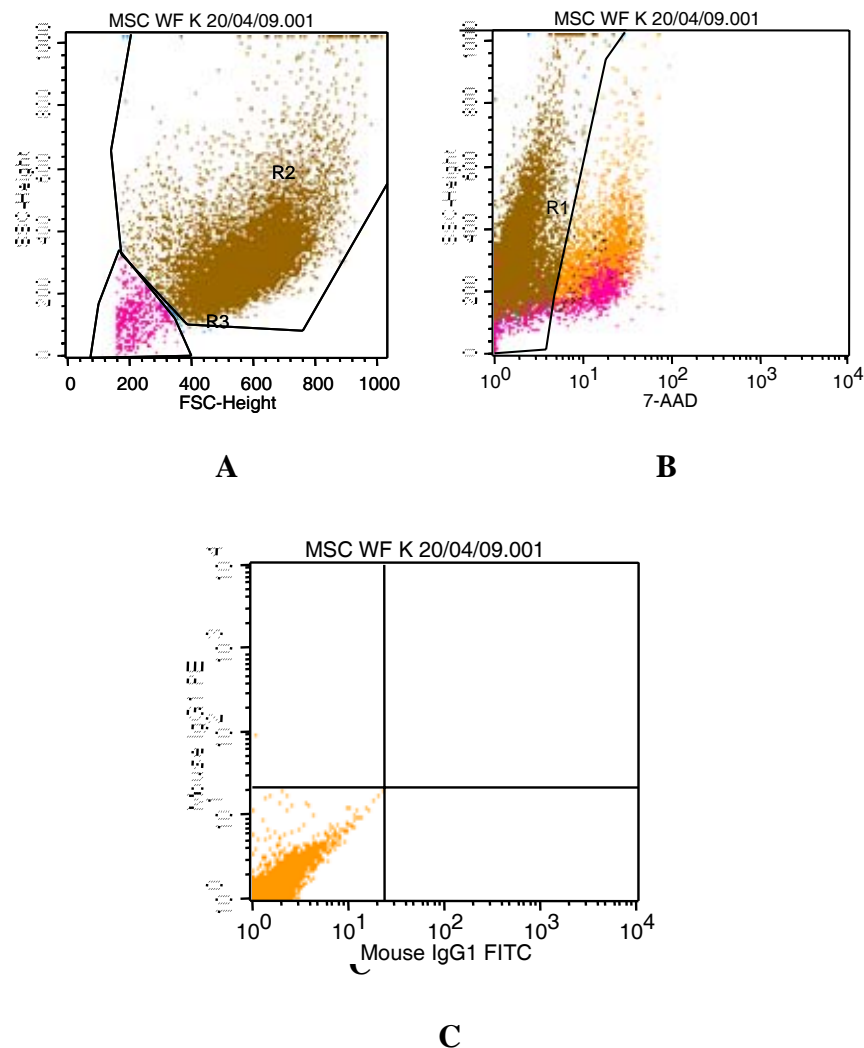

Figure 13. **Distribution of MSCs.** Distribution according to the measure of the population of isolated cells (**A**), viability analysis with 7-AAD (**B**); isotype control with mouse IgG1 (**C**).

FACS analysis was also used to assess the purity of our MSCs and the existence of a homogeneous population of adherent cells (after 4–5 passages). After the

exclusion of dead cells, (R1 on 7-AAD negative elements; cells positive to 7-AAD were excluded because they could give a cross-reaction with non-specific antigens, figure 13 B) the cell population resulted as being uniform positive for CD90, CD44, CD54, CD73, and CD106. There was no significant contamination of haematopoietic cells, as Flow cytometry assay was negative for markers of haematopoietic lineage, including CD11b and CD45, (figure 14).
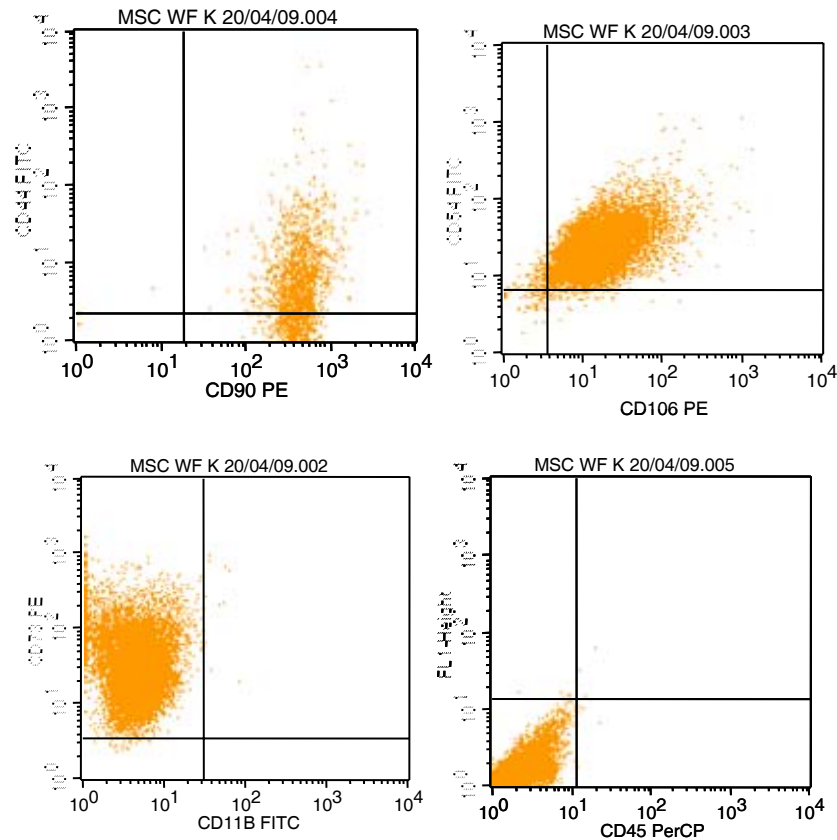

Figure 14. **Flow cytometric analysis of MSCs.** CD11b and CD45 antibodies showed no contamination of haematopoietic cells but positivity for classical mesenchymal markers CD44, CD54, CD73, CD90, CD106.

MSCs treated with osteogenic medium formed small deposits of hydroxyapatite stained intensely red with Alizarin S, (figure 15 A). MSCs treated with adipogenic medium were successfully differentiated towards adipogenic lineages: lipid vacuoles started to accumulate in the cytoplasm of the cells after just 2–3 days of stimulation and were stained orange–red after 21 days (figure 15 B).
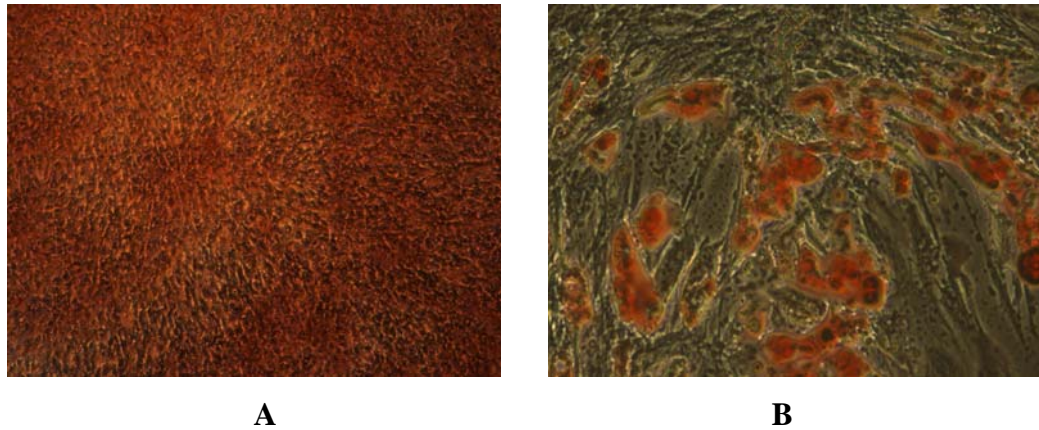
<div align="center">**A**   **B**</div>

Figure 15. **Differentiation of MSCs.** Osteocytes, with visible deposits of hydroxyapatite intensely stained red by means of Alizarin S, (**A**), and orange–red stained lipid vacuoles of the cytoplasm of MSCs treated with adipogenic medium with Oil-Red O, (**B**).

4.1.2 *Expansion and characterisation of rat EPCs*

We cultured-expanded rat BM-induced EPCs from Wistar Furth and Lewis EGFP rats.

Confluent EPCs (after 14 days) showed spindle-shaped cells; primary culture cells (14 days) were trypsinized and plated, reaching a mean cellular expansion of up to 10^6 at P1 (range 10^5–10^7). EPCs used to perform our transplants were EPC WF POOL AG clones.

EPCs time of proliferation was represented in figure 16, showing a mean value of doubling time of 27 hours.
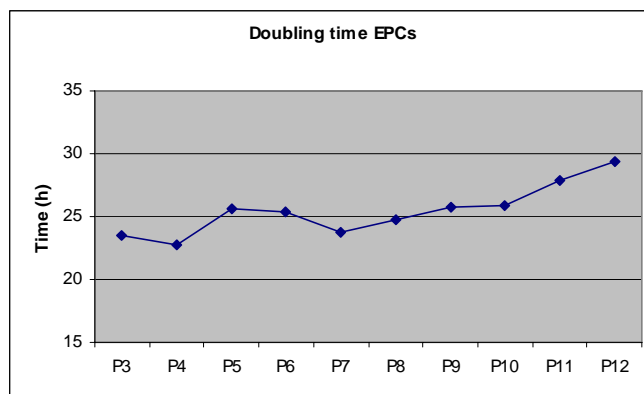

Figure 16. **Doubling time of EPCs.** Doubling time represented as the growth curve of EPCs.

Flow cytometry was used to assess the purity of EPCs and analyze the surface markers of mesenchymal, hematopoietic and endothelial lines (CD45, CD44, CD90, CD11b, CD31, Endothelium). Isolated EPCs resulted positive for CD44, CD90, (figure 17 A), Endothelium (ox43 protein expressed on vascular endothelial cells in each rat tissue), (figure 17 B). Low levels of CD31, typical of mature endothelial cells and the markers of the hematopoietic line, (CD45, CD11b), were found (figure 17 C).
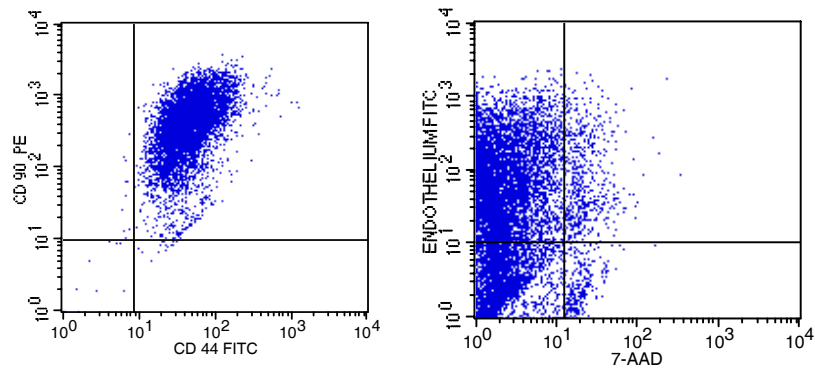
A   B
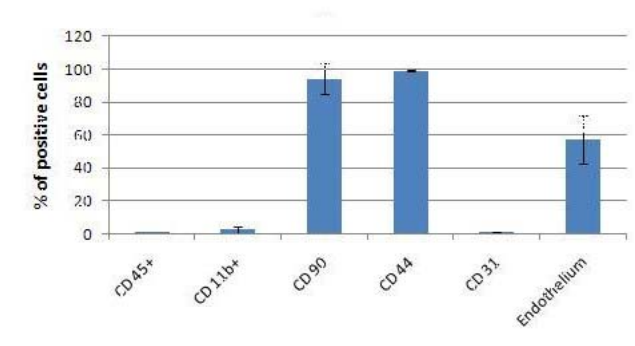
C

Figure 17. **Flow cytometric analysis of EPCs.** Expression of CD44 (**A**), CD90 (**A**), Endothelium (**B**), 7-AAD markers (**B**); total phenotypic characterization of EPCs (**C**).

The functional characterization of endothelial progenitors was obtained by means of an encapsulation test of ac-LDL-dil (Acetilated-Low density lipoprotein-dil) in immunofluorescence (figure 18).
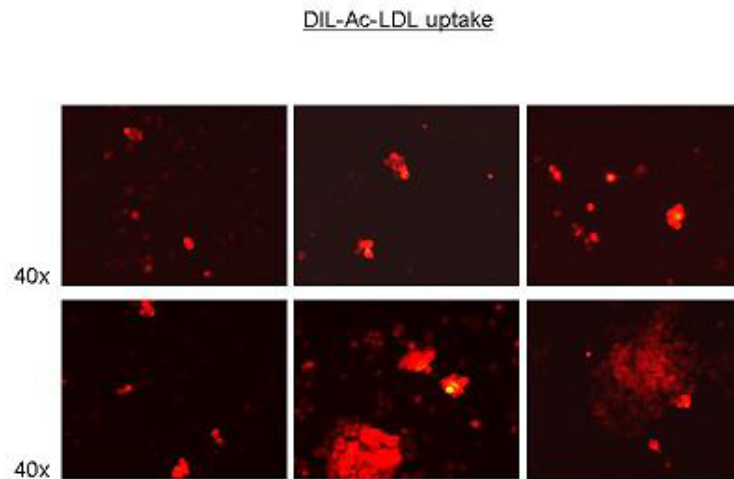
DIL-Ac-LDL uptake

Figure 18. **ac-LDL-dil (Acetilated-Low density lipoprotein-dil) immunofluorescence test.** Acetylated lipoprotein complex (red) is visible inside the cells.

4.1.3 *Assessment of angiogenic properties of EPCs*

The angiogenic ability of EPCs was assessed using an *in vitro* endothelial tube-formation assay. We performed a time-lapse experiment to investigate the formation of capillary-like structures by evaluating their growth within the first 12 hours after seeding. The first 6 hours were followed by an evaluation of EPCs GFP+ which demonstrated that EPCs can grow from a low percentage of scattered capillaries (two hours after seeding, figure 19 A) to a gradually more organized structure in discrete matrigel regions (four hours after seeding, figure 19 B). Finally EPCs GFP+ showed an ability to organize themselves successfully in regions similar to tubular structures (six hours after seeding, figure 19 C). A parallel experiment was conducted only in the bright field and showed, 12 hours from after seeding onto BD Matrigel™ Basement Membrane Matrix, the highest capacity of EPCs to form capillary-like structures (figure 19 D).
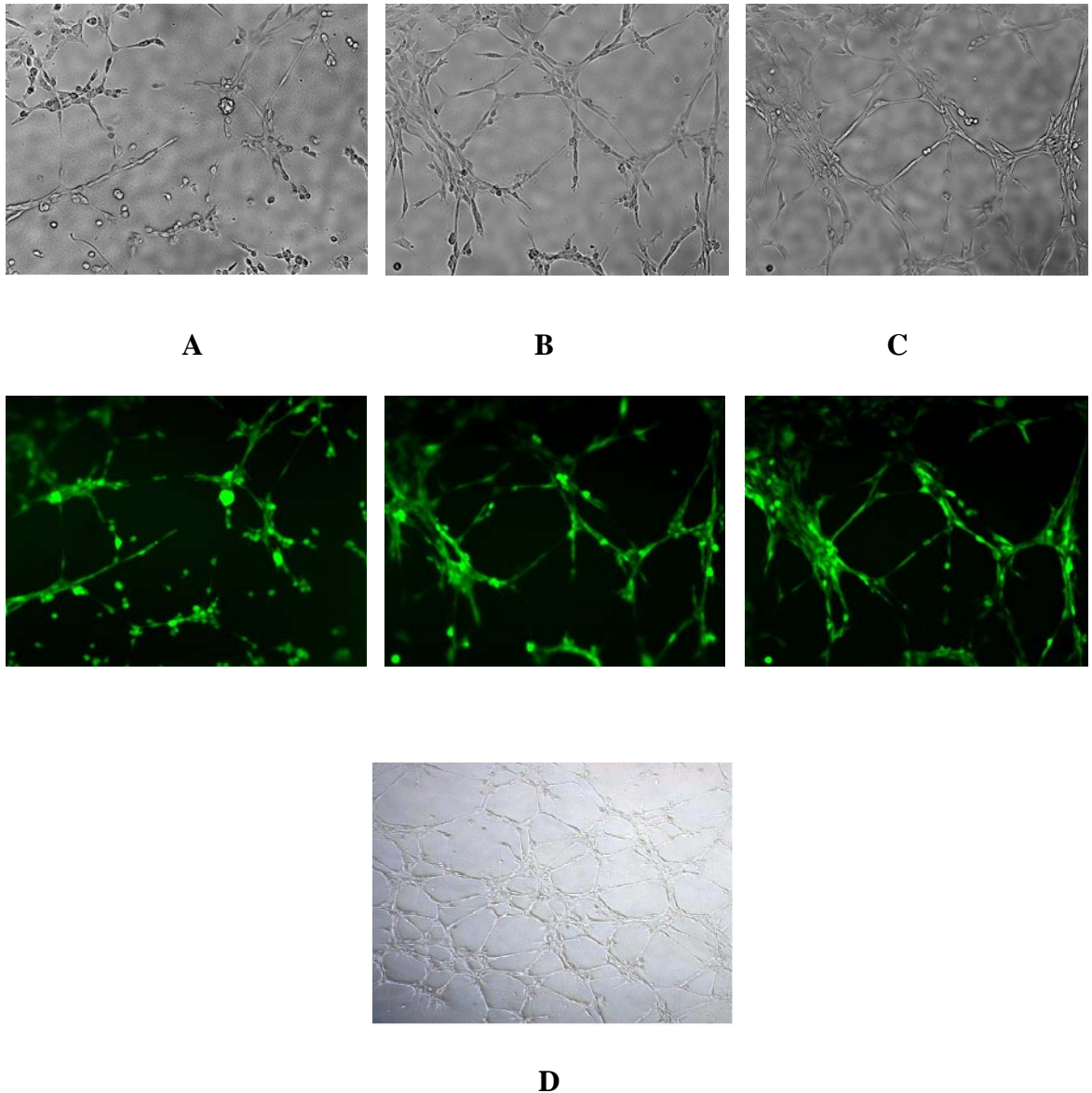
Figure 19. **Time lapse of EPCs onto BD Matrigel™ Basement**. EPCs GFP+, shown in both bright field and fluorescent images, grow gradually from a sparsely scattered capillary structure (2 hours after seeding, **A**) to a more organized (4 hours after seeding, **B**) and finally to one similar to tubular structure (6 hours after seeding, **C**). After 12 hours onto matrigel, EPCs show an excellent ability to grow in a complete capillary-like structure (bright field, **D**).

4.2 *In vivo* experiments

Islet transplants were performed in an experimental animal (rat) model in which diabetes was chemically induced by means of a single high dose of Streptozotocin (STZ). Streptozotocin, a glucosamine-nitrosourea (a DNA alkylating agent that enters cells exclusively via the GLUT2 glucose transport protein), is a diabetogenic compound that is especially toxic to pancreatic islet insulin-producing β-

cells. Chemically induced diabetes is comparable to type 1 Diabetes because most of the β-cells in the pancreatic islets are disrupted.

Syngeneic transplantations were carried out by using Lewis donors and by injecting the islets into Lewis recipients, whereas for allogeneic transplants we used Wistar Furth rats as donors and we implanted the islets into Lewis recipients.

All the animals used derived from inbred strains, created by crossing male and female siblings. The inbred offspring of this cross are again mated with each other and then again for 20 consecutive generations: the result of this manipulation is a population of animals in which only one allele of every gene is present; consequently, all animals within an inbred strain are genetically identical, akin to identical twins.

In our model a marginal mass of pancreatic islets was chosen as the minimum dose to be infused in the animals in order to decrease the blood glucose without reaching normoglycemic levels. In this context, 700 islets equivalents (IE) compared with 1400 IE, either syngeneic or allogeneic and transplanted in the portal vein of the animals, resulted in a rapid decrease in glycaemic levels: the time required for this decrease was short but not enough to re-establish normoglycaemic levels. For this reason 700 IE was chosen as the marginal mass to be transplanted alone or in combination with cell therapy in order to assess grafted islet viability and functionality.

4.2.1 *Portal vein syngeneic and allogeneic transplantation of 700 IE*

The syngeneic transplantation of a marginal mass of 700 Lewis pancreatic islets in the portal vein of diabetic Lewis rats induced a fast decrease in blood glucose, which returned to high levels (n=6) within twelve days. The values remained strongly significant until the twelfth day after transplantation (** $p<0.01$ day 3, 12 vs. day 0; *** $p<0.001$ day 5, 7 vs. day 0) with a continuous growth towards diabetic levels up to day 15, (figure 20, green line).

The group of 700 IE WF allogeneic transplanted (n=5) in Lewis rats showed a similar trend to the previous syngeneic, but the levels returned to high values 9 days after transplant. Indeed, the decrease in blood glucose levels and the return to diabetic values were more homogeneous than the syngeneic transplants. These levels were as strongly significant as the previous ones, but only up to day 7 (*** $p<0.001$ day 3, 5, 7 vs. day 0). However, the levels did not maintain their significance until the end of the monitoring time (figure 20, orange line).

There were no further significant values between day 15 of the syngeneic transplants and day 15 of the allogeneic transplants.

The animals which received 700 IE (either syngeneic or allogeneic) continued to be monitored even after 15 days, but as the glycaemic levels did not decrease, the animals were sacrificed 30 days after transplantation.

The allogeneic transplants showed worse results than the syngeneic transplants in maintaining glucose control also in the early period after transplantation.
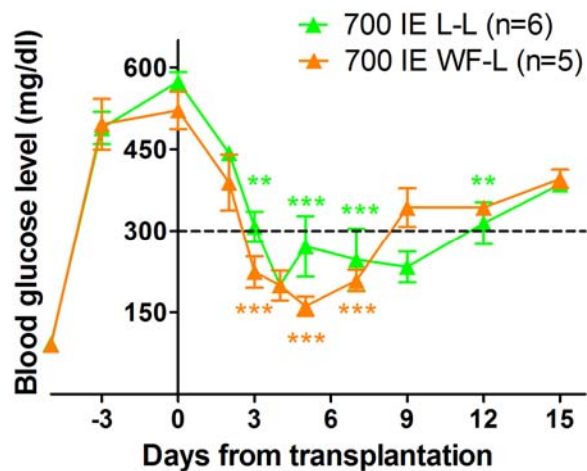

Figure 20. **Comparison between the curves relative to 700 IE syngeneic and 700 IE allogeneic transplants**. The two groups show the same major significant levels in the first week after transplantation (*** $p < 0.001$ day 7 vs. day 0 either for syngeneic or allogeneic group); 15 days after transplantation neither the syngeneic (green line) nor the allogeneic group (orange line) shows a significant decrease compared to the day of transplant.

4.2.2 *Portal vein allogeneic transplantation of 500,000 EPCs alone*

To evaluate the effect of administration of EPCs alone on blood glucose levels, an allogeneic model of transplant with a single dose of 500,000 EPCs WF was performed in the portal vein of diabetic-induced Lewis rats.

The glucose monitoring pattern was non-homogeneous, in fact the decrease in levels was fast in the early post-transplant period; this trend did not continue over time and rather showed a discontinuous growth towards diabetic levels from the fourth day after the transplant onwards.

Blood glucose levels decreased with a very significant value only at day 3 (** $p < 0.01$ day 3 vs. day 0), while one week after transplantation the significance was further decreased (* $p < 0.05$ day 7 vs. day 0), (figure 21), and after this period blood

glucose levels returned to diabetic values. It is worth noting that in this pattern the reported levels showed high values of standard error, in correspondence with low significant levels.

The animals were monitored for 30 days after transplantation because, in addition to showing no positive trend in the blood glucose levels, they gradually lost their body mass and were therefore sacrificed.
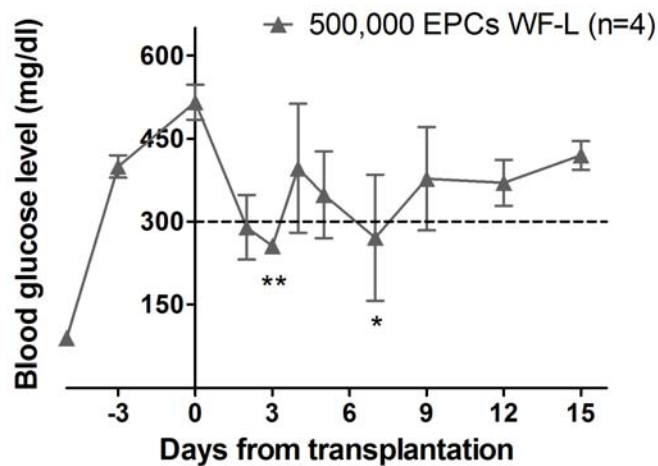

Figure 21. **Allogeneic transplantation with 500,000 EPCs**. Animals were transplanted with 500,000 EPCs WF in the allogeneic model. The trend was not homogeneous and the blood glucose levels showed a significant decrease three days after transplantation (** $p<0.01$ day 3 vs. day 0) and a slight decrease seven days after transplantation (* $p<0.05$ day 7 vs. day 0).

4.2.3 *Portal vein syngeneic and allogeneic transplantation of 700 IE + 500,000 EPCs*

In both a syngeneic and allogeneic model of a marginal dose of pancreatic islets, we studied two groups of animals which had received 500,000 EPCs in the portal vein.

The animals were checked over a duration of 180 days for the syngeneic group, and over a duration of 75 days for the allogeneic group.

The trends of the glycaemic levels of both syngeneic and allogeneic groups compared with the 700 IE alone, respectively were shown in figure 22 A and 22 B.
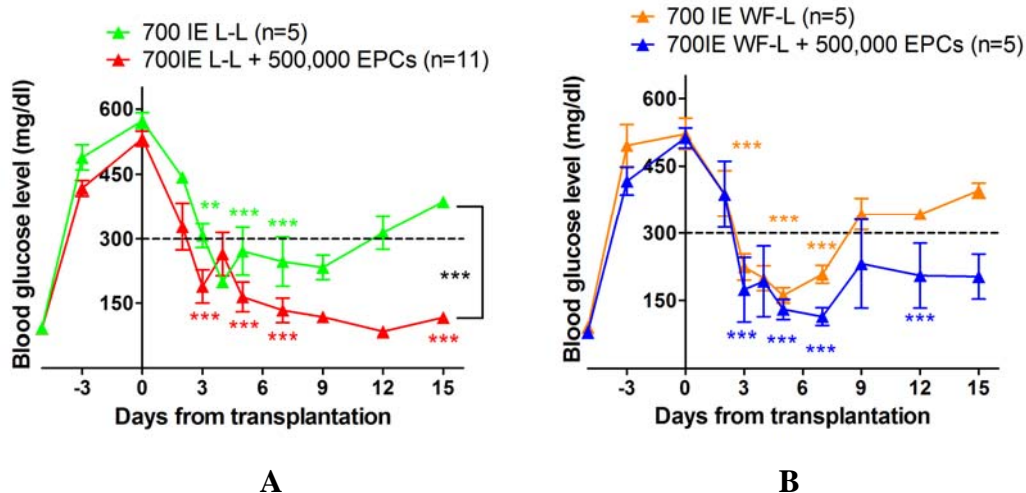
A   **B**

Figure 22. **Transplantation with 700 IE + 500,000 EPCs**. *Glycaemic levels of syngeneic 700 IE L-L compared with syngeneic 700 IE + 500,000 EPCs* (**A**) Islets alone show a return to diabetic values within 15 days after transplantation, even though the trend shows statistically significant points (green line, ** p<0.01 day 3 vs. day 0, *** p<0.001 days 5, 7 vs. day 0). The syngeneic transplants with 700 IE + 500,000 EPCs (red curve) show a considerable decrease in blood glucose values linked to continuous extremely significant levels within the first 15 days after the transplant (*** p<0.001 days 3, 5, 7, 15 vs. day 0). There is an extremely significant difference on the fifteenth day between 700 IE alone and islets plus cell therapy (*** p<0.001 day 15 700 IE vs. day 15 700 IE + 500,000 EPCs). *Glycaemic levels of allogeneic 700 IE WF-L compared with allogeneic 700 IE + 500,000 EPCs* (**B**) The trend of 700 IE islets alone (orange line) shows an increase, after the early decrease in blood glucose levels, just 9 days after transplantation. This decrease shows a good pattern in the first week (*** p<0.001 days 3, 5, 7 vs. day 0), but the levels return to diabetic levels after that time point. The curve relative to allogeneic transplants 700 IE + 500,000 EPCs (blue line) shows a non-homogeneous decrease in the first 15 days, the glycaemic levels show statistically significant values (*** p<0.001 days 3, 5, 7, 12 vs. day 0) and remain under the diabetic threshold.

In figure 22 A it was shown a comparison between animals which had received 700 IE plus 500,000 EPCs syngeneic transplant (red line) and the corresponding syngeneic 700 islets alone treatment (green line).

The group of animals transplanted with syngeneic 700 IE showed a decrease in glycaemic levels in the early days post-transplant, but the values returned to diabetic ones even though they show statistically significant points in the first 15 days (** p<0.01 day 3 vs. day 0, *** p<0.001 day 5, 7 vs. day 0). The group with syngeneic 700 IE + 500,000 EPCs showed a faster decrease in glucose levels than 700 IE alone, within 15 days after transplantation and the values remained statistically significant during this period of time (*** p<0.001 day 3, 5, 7, 15 vs. day 0).

The glycaemic levels of the islets plus cell therapy remained largely under the diabetic threshold value in the first 15 days and its curve also showed very little range of standard errors mostly from day 7 onwards, maybe due to the high number of treated animals (n=11).

It is to note an extremely significant difference between glycaemic levels of the two groups on the fifteenth day (*** p<0.001 day 15 syngeneic vs. day 15 allogeneic).

In figure 22 B it was shown a comparison between 700 IE alone (orange line) and 700 IE + 500,000 EPCs (blue line), both allogeneic. The group of animals transplanted with allogeneic 700 IE showed an increase, followed by the early decrease in blood glucose levels, just 9 days after transplantation. The values, in fact, showed statistically significant points only within 9 days post transplantation (*** p<0.001 day 3, 5, 7 vs. day 0). The curve of 700 IE + 500,000 EPCs showed a decrease within a period of 7 days and a slight increase followed by a further decrease within a period of 15 days.

Both curves showed extremely significant values in the first week following the transplant (*** p<0.001 day 3, 5, 7 vs. day 0), but, compared with 700 IE alone, the curve relative to islets plus cell therapy showed a further statistically significant level on day 12 (*** p<0.001 vs. day 0). Furthermore, the glycaemic levels remain under the diabetic threshold in the first two weeks after transplantation.

On day 15 there was no statistically significant difference between the values of the two curves. It is interesting to note that both curves referring to the islets plus cell treatment, either syngeneic or allogeneic, followed the same trend as the islets alone, but the curves relative to 700 IE + 500,000 EPCs remain under the diabetic threshold (300 mg/dl), while the curves relative to 700 IE alone return to diabetic values within 15 days.

The curves relative to cell therapies (syngeneic vs. allogeneic) were compared from day 15 to day 70 after transplantation, as shown in figure 23 A.

The animals receiving a syngeneic transplant show continuous statistically significant values until day 70 and blood glucose levels remain largely under the threshold value of 300 mg/dl (*** p<0.001 day 30, 45, 60, 70 vs. day 0).

On the other hand, the allogeneic trend showed extremely significant values only up to day 45 (*** p<0.001 day 30, 45 vs. day 0) and the glycaemic levels returned to high values from day 60 onwards, therefore the animals were sacrificed 70 days after transplantation. When comparing both curves there are very significant levels only in the last two points (** p<0.01 day 60, 70 syngeneic vs. day 60, 70 allogeneic).

The syngeneic curve of 700 IE + 500,000 EPCs was observed for longer than the allogeneic one with the monitoring period continuing up to 180 days after

transplantation. The trend of the curve is quite homogeneous, in fact there was little standard error for all of the monitoring period in addition to extremely significant values (*** p<0.001 day 90, 120 and 180 vs. day 0). Worth noting are the very low glycaemic levels until 6 months after transplant, which were even lower than 200 mg/dl, (figure 23 B).
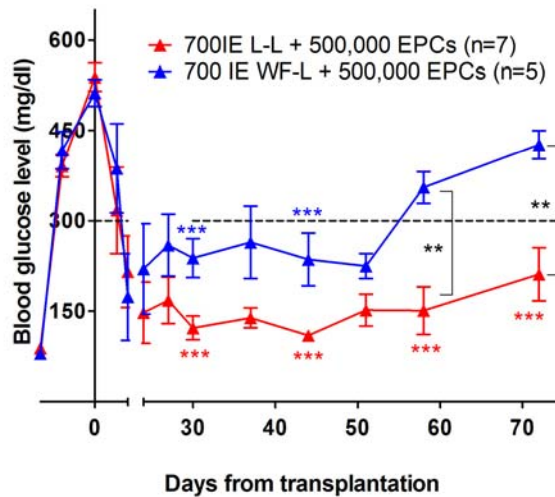
A
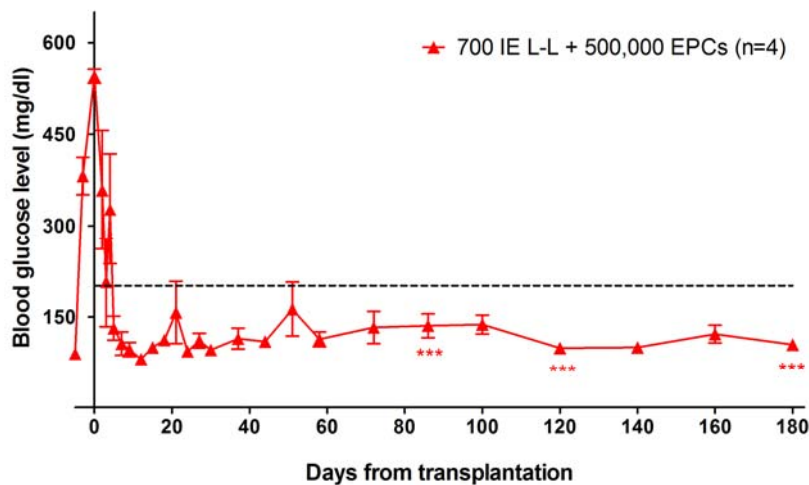
B

Figure 23. **Comparison of syngeneic and allogeneic trends in the long-term**. *Comparison of syngeneic and allogeneic transplantation curves from 15 to 70 days after transplantation* (**A**). The syngeneic transplants with 700 IE + 500,000 EPC (red curve) present a more homogeneous pattern with lower standard errors compared to the allogeneic transplants. The glycaemic values are always extremely significant in this time gap (*** p<0.001 days 30, 45, 60, 70 vs. day 0). In contrast, the curve relative to the allogeneic transplants (blue line) shows greater standard error, there are only two statistically significant values compared with the curve relative to the syngeneic transplants (*** p<0.001 days 30, 45 vs. day 0), and a return to diabetic values from day 60 onwards is shown. It should be noted that the last two time points give a very significant difference between the two curves (** p<0.01 days 60,70 syn vs. days 60, 70 allo). *Glycaemic pattern of syngeneic 700 IE + 500,000 EPC in the long-term* (**B**). The animals that received a syngeneic transplant were observed over a long period of time (up to 6 months).

The pattern shows a homogeneous decrease during the overall extension time. After 70 days from transplantation, the curve continues to show extremely significant values also at 90, 120 and 180 days (*** $p < 0.001$ vs. day 0), with low standard error values. The blood glycaemic levels remain largely under the threshold level (300 mg/dl) and even under 200 mg/dl for long-term extension time.

4.2.4 *Portal vein transplantation of 700 IE + 500,000 MSCs*

Our previous work showed the immunomodulatory effect of MSCs when these cells were administered via the tail vein into a syngeneic rat model of transplantation either in a single (150,000 MSCs) or multiple dose (3x150,000 MSCs, days 0, 2, 4 after transplant)[241]. These two types of treatment improved the glycaemic levels in diabetic animals up to 20 days after transplantation. In addition, it was also noticed that the efficacy of MSCs is dose–dependent and that the observed treatment was effective when the administered dose of the tail vein was triple compared to the portal vein dose.

We therefore decided to perform the syngeneic transplants by increasing the dose of MSCs (700 IE + 500,000 MSCs) and administering both, islets and MSCs, via the portal vein in order to obtain a long lasting effect for the glycaemic levels.

In figure 24 A it was shown a comparison between the animals transplanted with syngeneic 700 IE + 500,000 MSCs via the portal vein (violet line) and the corresponding syngeneic 700 IE alone (green line). There was a fast decrease in the blood glucose levels of syngeneic 700 IE + 500,000 MSCs transplants compared to 700 IE alone. The syngeneic transplants, 700 IE + 500,000 MSCs, showed values that remain statistically significant in the first 15 days (*** $p < 0.001$ day 3, 5, 7, 15 vs. day 0).

The group of animals transplanted with syngeneic 700 IE showed a decrease in glycaemic levels in the early post-transplant days, but the values returned to diabetic levels even though they show statistically significant points in the first 15 days (** $p < 0.01$ day 3 vs. day 0, *** $p < 0.001$ day 5, 7 vs. day 0).

It should be noted that on the fifteenth day there was a significant difference in glycaemic levels between the group transplanted with 700 IE alone and the group transplanted with 700 IE + 500,000 MSCs (* $p < 0.05$ day 15 700 IE vs. day 15 700 IE + 500,000 MSCs).

In figure 24 B it was shown the trend of the glucose levels of syngeneic transplants with 700 IE + 500,000 MSCs during the overall observation time (up to 75

days). The glycaemic levels were under the diabetic threshold value (300 mg/dl), even under 200 mg/dl, and remained so until the end of the observation period. By comparing these values with the glycaemic levels at day 0 they proved to be extremely significant (*** p<0.001 day 30, 45, 60, 72 vs. day 0).
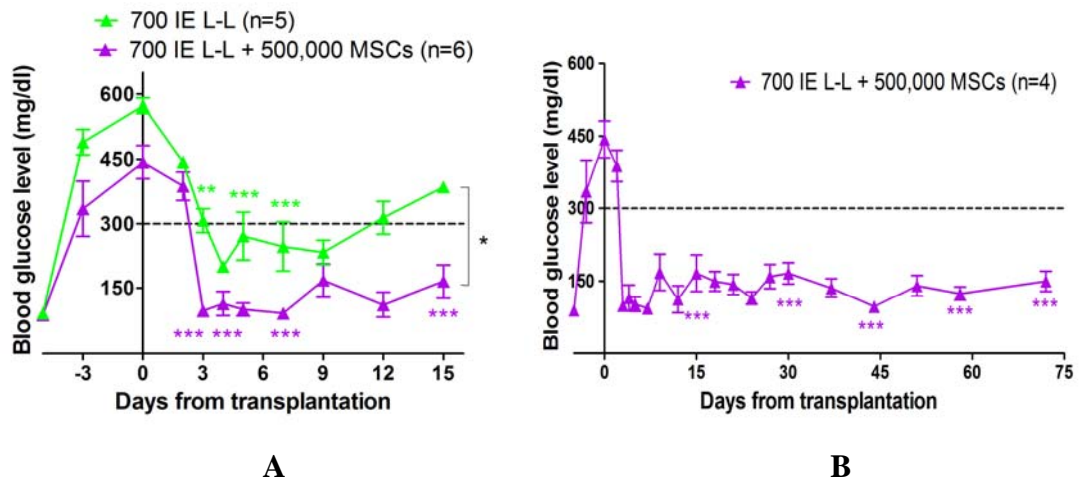

Figure 24. **Transplantation with syngeneic 700 IE + 500,000 MSCs.** *Comparison between syngeneic 700 IE and 700 IE + 500,000 MSCs via the portal vein* (**A**) Islets alone show a return to diabetic values within 15 days after transplantation, even though the trend shows statistically significant points (green line, ** p<0.01 day 3 vs. day 0, *** p<0.001 days 5, 7 vs. day 0). The syngeneic transplants with 700 IE + 500,000 MSCs via the portal vein (violet line) show a considerable decrease linked to continuous extremely significant values within the first 15 days after the transplant (*** p<0.001 days 3, 5, 7, 15 vs. day 0). There is a significant difference on the fifteenth day between syngeneic 700 IE alone and syngeneic 700 IE + 500,000 MSCs transplant (* p<0.05 day 15 700 IE vs. day 15 700 IE + 500,000 MSCs). *Glycaemic pattern of syngeneic 700 IE + 500,000 MSCs in the long-term* (**B**). The pattern shows a decrease up to 75 days. The curve continues to show extremely significant values (*** p<0.001 day 30, 45, 60 and 72 vs. day 0), with low standard error values. The blood glycaemic levels remain largely under the threshold level (300 mg/dl) and even under 200 mg/dl in the long-term.

4.2.5 Comparison between syngeneic portal vein transplantation of 700 IE + 500,000 EPCs and 700 IE + 500,000 MSCs

The curves relative to the 700 IE plus cell therapies (500,000 EPCs or 500,000 MSCs), both syngeneic, were represented in figure 25 A.

The curve relative to 700 IE + 500,000 MSCs transplant (violet line) showed a faster decreasing trend and lower levels of glycaemic values compared with 700 IE + 500,000 EPCs (red line), but both curves showed extremely significant values during the first 15 days from transplantation (*** p<0.001 day 3, 5, 7, 15 vs. day 0 either for MSCs or EPCs treatment).

Glycaemic levels for both curves remained low until 70 days after transplantation with similar patterns. Levels continued to remain significantly high in both curves even after 15 days up until the end of the monitoring time (*** p<0.001 day 30, 45, 60, 70 vs. day 0 either for MSC or EPC treatment), (figure 25 B).
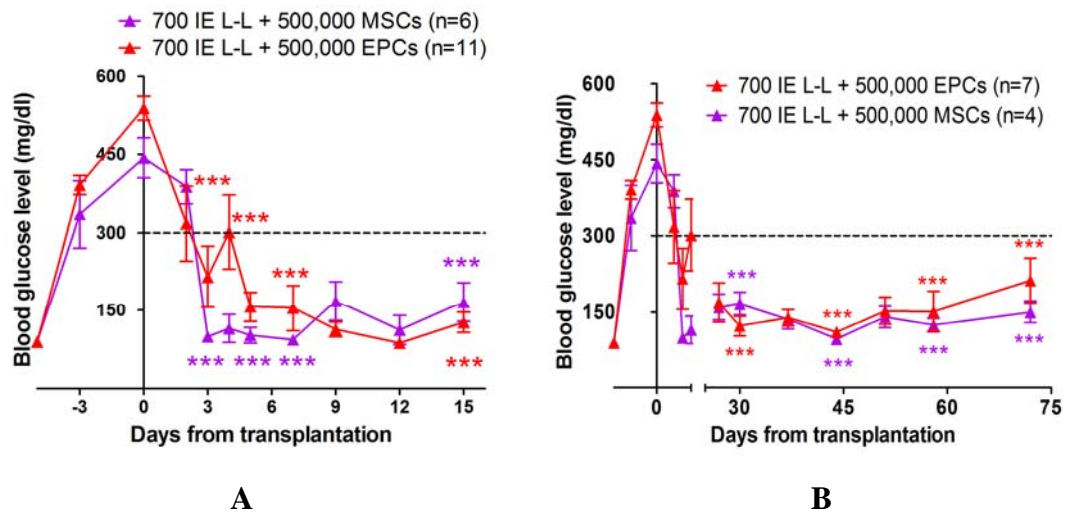

Figure 25. **Comparison between syngeneic 700 IE + 500,000 EPCs and syngeneic 700 IE + 500,000 MSCs transplants**. *Comparison between cells treatment in the first 15 days from transplant* (**A**) The syngeneic curve of 700 IE + 500,000 EPCs (red line) shows a considerable decrease, linked to extremely significant values, within the first 15 days after the transplant (*** p<0.001 days 3, 5, 7, 15 vs. day 0). The trend relative to the transplants 700 IE + 500,000 MSCs (violet line) shows a more homogeneous decrease than the previous one, with a slight increase after 7 days. Even for the MSC curve, many time points are extremely significant (*** p<0.001 days 3, 5, 7, 15 vs. day 0). *Comparison between cells treatment in the long-term* (**B**) After 15 days the two curves appear analogous in their trend of glycaemic levels over a period of up to 70 days. Indeed, both curves remain under the diabetic threshold and with the same significant time points (*** p<0.001 days 30, 45, 60, 70 vs. day 0 for both curves, syngeneic and allogeneic).

4.2.6 *Intra peritoneal Glucose Tolerance Test (IPGTT)*

In order to assess the glucose metabolism of the animals a series of intra peritoneal glucose tolerance tests (IPGTT) were performed in all transplanted groups and compared with healthy and diabetic reference curves.

A comparison between the disposal of the glucose 15 days after transplantation for animals that received syngeneic 700 IE, allogeneic 500,000 EPCs alone and syngeneic 700 IE + 500,000 EPCs was shown in figure 26 A.

The curve relative to healthy animals showed a decline in blood glucose starting 15 minutes after the D-glucose injection, while the curve relative to the syngeneic 700 IE + 500,000 EPCs transplants showed a higher increase in blood

glucose up to 30 minutes after the glucose administration followed by a decrease to non-diabetic values.

The curve relative to syngeneic 700 IE + 500,000 EPCs transplants showed a very significant value at 120 minutes when compared with diabetic values (green line, ** $p<0.01$ 120 min syngeneic vs. 120 min diabetic), while for the healthy animals an extremely significant level was observed (red line, *** $p<0.001$ 120 min healthy vs. 120 min diabetic).

The curves relative to allogeneic 500,000 EPCs alone (orange line) and syngeneic 700 IE (pink line) transplants showed higher values than the previous ones in the disposal of the glucose; both curves showed no significance levels compared to the diabetic curve.

Since we observed a fast glucose metabolism for the syngeneic 700 IE + 500,000 EPCs transplants 15 days after transplantation, we decided to compare the disposal of the glucose for that group at different time points compared to healthy animals (15, 30, 90 days after the transplant), figure 26 B.

The trends in glucose disposal of these transplants were very similar in that, they showed a similar decreasing pattern up to 90 days after transplantation which could be correlated with the long-term control of glycaemic levels for this group. The curve relative to the syngeneic transplants at 15 days post transplantation showed levels of significance at 30 and 60 minutes compared to the time of D-glucose injection (** $p<0.01$ t=30 vs. t=0; * $p<0.05$ t=60 vs. t=0).

Moreover, the trend relative to the syngeneic transplant at 30 days post transplant showed only one value of significance at 30 minutes compared to the zero point of its curve (** $p<0.01$ t=30 vs. t=0).

Finally, the syngeneic transplant curve at 90 days post transplant showed many statistically significant values at 15, 30, 60, 90 minutes compared to the time of D-glucose injection (* $p<0.05$ t=15 vs. t=0; *** $p<0.001$ t=30, 60 vs. t=0; ** $p<0.01$ t=90 vs. t=0). None of the curves relative to the syngeneic 700 IE + 500,000 EPCs at different time points showed any levels of statistical significance within the three curves.

The curve relative to the animals which received allogeneic 700 IE + 500,000 EPCs transplants was shown in figure 26 C. The curve showed a disposal of the glucose 15 days after transplantation with higher values than the syngeneic one, but there were very significant levels at 120 minutes when compared with diabetic values

(light blue line, ** p<0.01 120 min allogeneic vs. 120 min diabetic), while for the healthy animals an extremely significant level was observed (red line, *** p<0.001 120 min healthy vs. 120 min diabetic).

The curves relative to allogeneic 500,000 EPCs alone (orange line) and allogeneic 700 IE (violet line) transplants showed values even higher than the previous ones in the disposal of the glucose; both curves showed no significant levels compared to the diabetic curve.

A comparison of the glucose metabolism between the syngeneic and the allogeneic trends of 700 IE + 500,000 EPCs transplants at 15 days after transplantation compared to healthy animals was shown in figure 26 D. The values of the syngeneic curve indeed showed lower levels of blood glucose compared to the allogeneic animals but the allogeneic curve showed extremely significant levels compared to healthy ones (green line, *** p<0.001 120 min syngeneic vs. 120 min healthy).
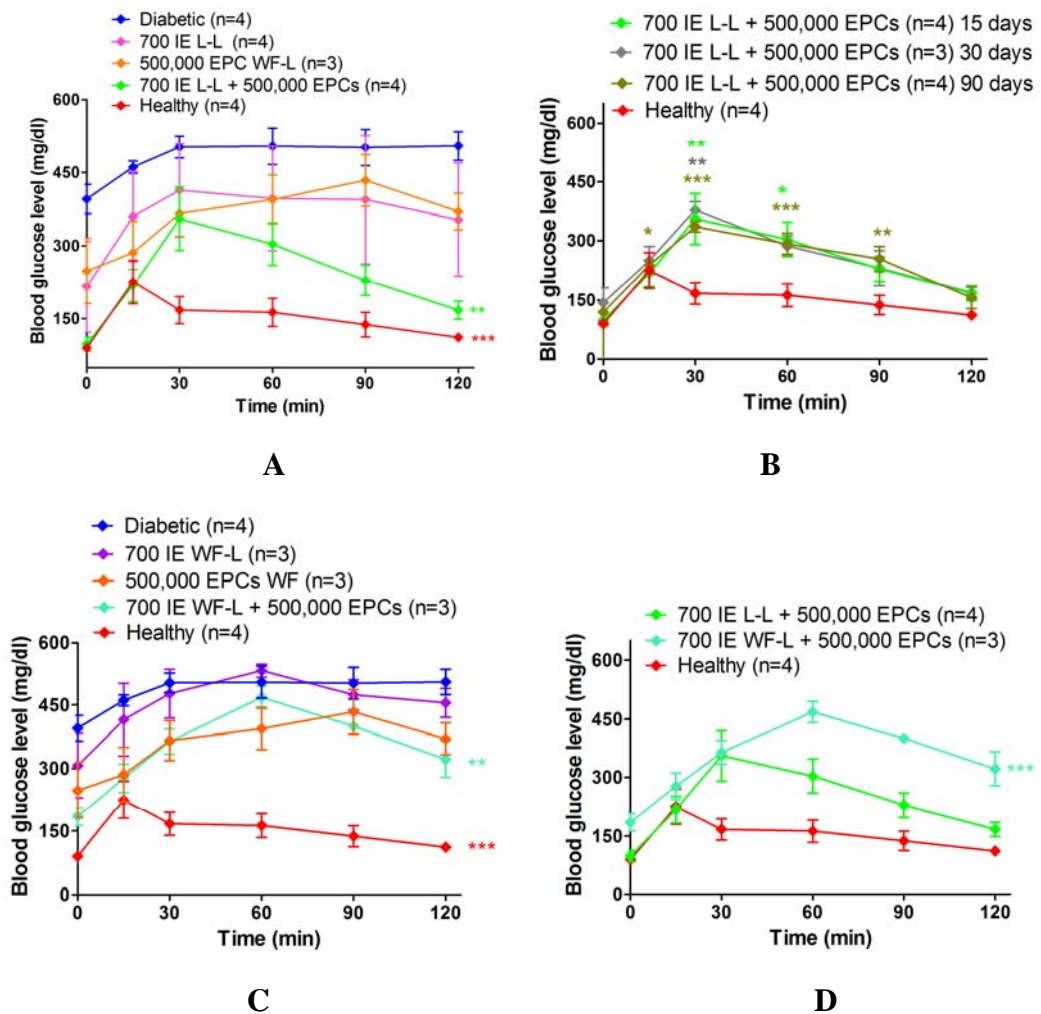
Figure 26. **Intra peritoneal glucose tolerance tests in several groups of transplanted animals**. *Comparison of syngeneic transplant curves 15 days after transplantation* (**A**). Disposal of the glucose for animals receiving syngeneic 700 IE (pink line), allogeneic 500,000 EPCs alone (orange line) and syngeneic 700 IE + 500,000 EPCs (green line) in comparison with healthy (red line) and diabetic animals (blue line). Significance of the groups: syngeneic 700 IE + 500,000 EPCs ** p<0.01 120 min syngeneic vs. 120 min diabetic; healthy animals *** p<0.001 120 min healthy vs. 120 min diabetic. *Comparison of syngeneic transplant curves at different time points* (**B**). Disposal of the glucose at 15, 30, 90 days after transplantation for animals receiving syngeneic 700 IE + 500,000 EPCs. Significance of the syngeneic 700 IE + 500,000 EPCs transplants at different time points: 15 days (green line) ** p<0.01 t=30 vs. t=0; * p<0.05 t=60 vs. t=0; 30 days (grey line ** p<0.01 t=30 vs. t=0; 90 days (brown line) * p<0.05 t=15 vs. t=0; *** p<0.001 t=30, 60 vs. t=0; ** p<0.01 t=90 vs. t=0. *Comparison of allogeneic transplant curves 15 days after transplantation* (**C**). Disposal of the glucose for animals receiving allogeneic 700 IE (violet line), allogeneic 500,000 EPCs alone (orange line) and allogeneic 700 IE + 500,000 EPCs (light blue line) in comparison with healthy (red line) and diabetic animals (blue line). Significance of the groups: allogeneic 700 IE + 500,000 EPCs transplants ** p<0.01 120 min allogeneic vs. 120 min diabetic; healthy animals *** p<0.001 120 min healthy vs. 120 min diabetic. *Comparison of syngeneic and allogeneic 700 IE + 500,000 EPCs transplants 15 days after transplantation* (**D**). Disposal of the glucose for animals receiving syngeneic 700 IE + 500,000 EPCs (green line) and allogeneic 700 IE + 500,000 EPCs (light blue line) in comparison with healthy animals (red line). Significance of the groups: *** p<0.001 120 min syngeneic 700 IE + 500,000 EPCs vs. 120 min healthy controls.

4.3 *Ex vivo* experiments

4.3.1 *Gene expression in liver tissues in IE and IE + EPCs transplantation by RT-PCR*

Quantitative RT-PCR was used to measure the expression levels of VEGF-A, SDF-1, ANG-1 and PECAM-1 in *ex vivo* liver tissues of animals transplanted with syngeneic 700 IE (IE) and 700 IE + 500,000 EPCs (IE+EPCs).

Expression levels were analyzed at different time points after transplantation (7, 15, 30 and 180 days after the transplant) and compared to the expression of the liver of healthy controls.

mRNA expression levels for VEGF-A were found to reach a peak at 15 days after transplantation, followed by a decrease at 30 days for both groups (IE and IE+EPCs) with a further decrease for IE+EPCs group at 180 days post-transplant (figure 27 A) (* $p < 0.05$ day 7 IE+EPCs vs. HEALTHY; *** $p < 0.001$ day 15 IE vs. HEALTHY; ** $p < 0.01$ day 15 IE+EPCs vs. HEALTHY).

Similar results were found also for ANG-1 where the IE group showed an increase its values of relative mRNA expression up to 15 days after the transplant and then slightly decreased at 30 days post-transplant. On the other hand, the IE+EPCs group showed an increase in ANG-1 from very low levels to levels comparable to healthy ones in the first 15 days, then the gene expression slightly decreased at 30 days and even more at 180 days after the transplant, (figure 27 B), (** $p < 0.01$ day 7 IE+EPCs vs. HEALTHY; ** $p < 0.01$ day 7 IE vs. HEALTHY; * $p < 0.5$ day 15 IE vs. IE+EPCs; * $p < 0.5$ day 30 IE vs. IE+EPCs).

It is worth noting that the expression levels of animals transplanted with 700 IE alone at 15 and 30 days after transplant remained high and also that both groups, either 700 IE or 700 IE + EPCs, showed a similar decreasing pattern from 15 to 30 days from transplant, as showed by the comparison of the ratio between the gene expression value at 15 and 30 days (1.21 IE vs. 1.36 IE+EPC).

The expression level of PECAM-1 revealed a different pattern for the two groups: the relative level of mRNA expression in IE alone markedly increased between days 7 and 15 post-transplantation and then decreased at 30 days. In the IE+EPCs group, instead, there were no relevant changes in the expression levels from days 7 to 15, but a further decrease at 30 days was observed (figure 27 C), (* $p < 0.5$ day 7 IE+EPCs vs. HEALTHY; * $p < 0.5$ day 15 IE vs. HEALTHY; * $p < 0.5$ day 15 IE vs. IE+EPCs; ** $p < 0.01$ day 30 IE vs. IE+EPCs).

The SDF-1 pattern is different from all other gene expressions: from 7 to 30 days after the transplant a reduced expression in IE alone was observed, while levels of SDF-1 relative to the IE+EPCs group revealed a small increase from 7 to 15 days post-transplant followed by a decrease at 30 days (figure 27 D).
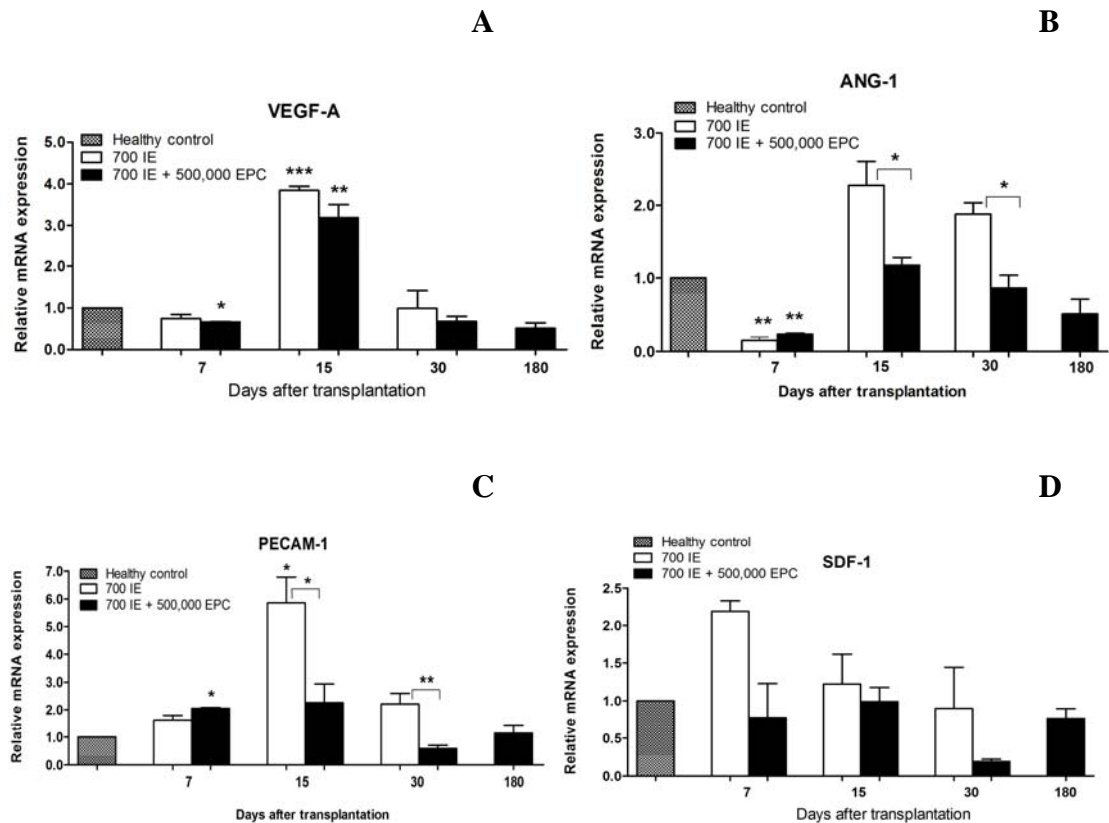
Figure 27. **Gene expression**. VEGF-A (**A**), ANG-1 (**B**), PECAM-1 (**C**), SDF-1 (**D**) expression in liver tissues of animals transplanted with IE and IE+EPCs at different time points.

4.3.2 *Comparison of gene expression in liver tissues in IE + MSCs and IE + EPCs transplantation by RT-PCR*

A comparison of the expression levels of VEGF-A, SDF-1, ANG-1 and PECAM-1 between *ex vivo* liver tissues of animals transplanted with syngeneic 700 IE + 500,000 EPCs (IE+EPCs) and 700 IE + 500,000 MSCs (IE+MSCs) was investigated by quantitative RT-PCR. Genes were studied at different time points after transplantation.

The VEGF-A gene expression showed a peak at 15 days after the transplant for the IE+EPCs group followed by a relevant decrease at 60 days; for the IE+MSCs, however, a decrease between days 7 and 60 post-transplant was observed, but the levels

remained at healthy values (figure 28 A), (* p<0.5 day 15 IE+EPCs vs. HEALTHY; * p<0.5 day 15 IE+MSCs vs. IE+EPCs).

In figure 28 B the ANG-1 gene had a peak for the IE+EPCs group at 15 days (the level was comparable to the healthy control), whereas for IE+MSCs, the gene slightly increased up to 60 days post transplantation (** p<0.01 day 7 IE+EPCs vs. HEALTHY; ** p<0.01 day 7 IE+MSCs vs. HEALTHY; * p<0.5 day 15 IE+MSCs vs. HEALTHY; * p<0.5 day 15 IE+MSCs vs. IE+EPCs; * p<0.5 day 60 IE+MSCs vs. HEALTHY; * p<0.5 day 60 IE+EPCs vs. HEALTHY).
For both IE+EPCs and IE+MSCs groups there was an increase in PECAM-1 gene expression compared to the healthy animals; all the values remained comparable during the overall monitoring time (figure 28 C), (** p<0.01 day 7 IE+EPCs vs. HEALTHY; ** p<0.01 day 7 IE+MSCs vs. HEALTHY).

Finally, the SDF-1 gene for the IE+MSCs group showed a relevant decrease from 7 to 15 days with a marked growth at 60 days post-transplant, whereas no relevant changes in the trend of the IE+EPCs group was observed for this gene expression during the observation time (figure 28 D), (* p<0.5 day 7 IE+MSCs vs. HEALTHY; * p<0.01 day 7 IE+MSCs vs. IE+EPCs; *** p<0.01 day 60 IE+MSCs vs. HEALTHY; *** p<0.01 day 60 IE+EPCs vs. IE+MSCs).

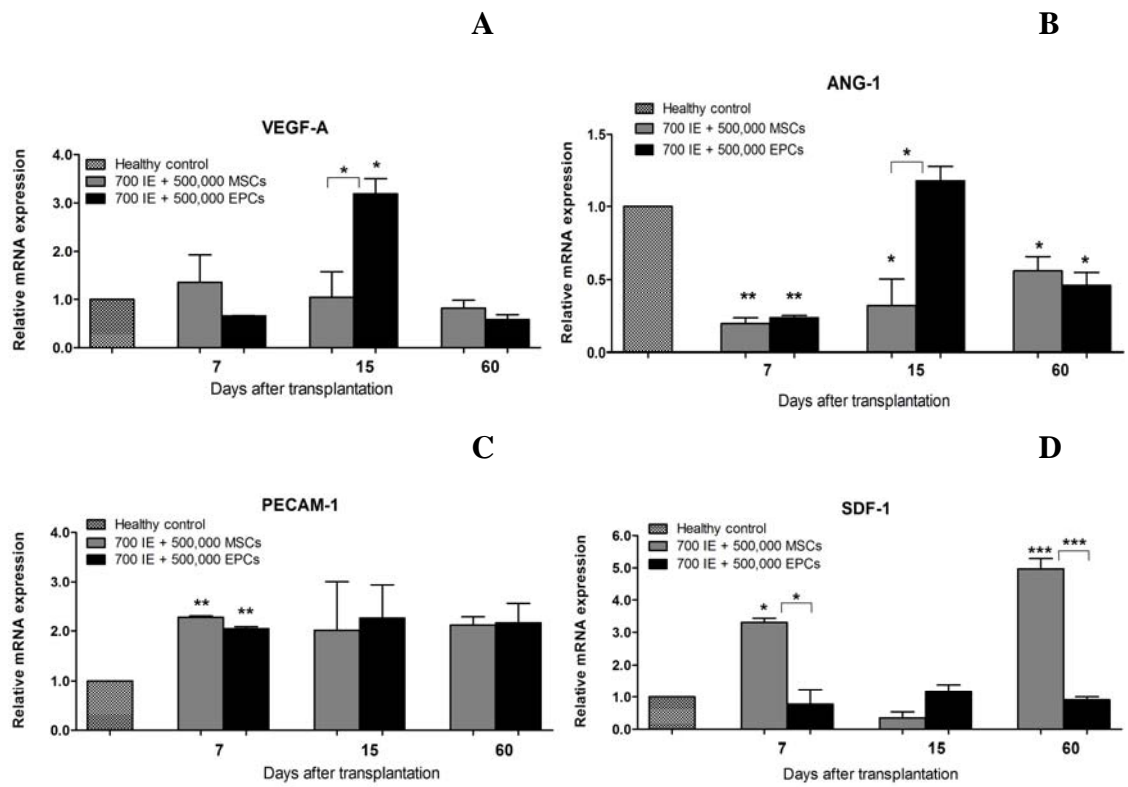Figure 28. **Comparison of gene expression**. Comparison between gene expression of VEGF-A (**A**), ANG-1 (**B**), PECAM-1 (**C**), SDF-1 (**D**) in liver tissues of animals transplanted with IE, IE+EPCs and IE+MSCs at different time points.

5. DISCUSSION

Pancreatic islet transplantation is one of the actual promising and available therapies for treating type 1 diabetes mellitus. Islet transplantation is indeed considered a promising strategy for the treatment of diabetes mainly in young patients and children with any evident insurgence of severe complications (when possibility of whole organ transplant has been discarded)[46]. Compared to the whole organ transplant and to exogenous insulin treatment, islet transplantation offers various advantages such as the a relatively simple surgical procedure (mini-invasive surgery), and consequently low post-operative risks and low morbidity; it is also possible to culture islets or cryopreserve them.

The protocols used to handle pancreatic islets for reducing their immunogenicity revealed that a short time of culture is adequate. Furthermore, for this purpose it is probable that a manipulation of pre-operative intervention of the islets is required (i.e. islet encapsulation by means of particular membranes to avoid or delay the aggressive effect from the host's immune system – immunoisolation)[242]. These procedures allow to optimize the recipient's conditioning, with a consequent easier tolerance induction and less risk of rejection. In addition, the requirement for immunosuppressive drugs treatment is low[45].

Human islet transplantation is carried out under local anaesthetic with a low infusion of islets through the insertion of a catheter into the portal vein at the transhepatic percutaneous site[243]. Due to the mini-invasive surgery, there is a reduction in complications and it enables glycaemic levels to re-establish themselves thereby avoiding any further hypoglycaemic crisis and irreversible pancreatic injuries[45].

Moreover, the islet vasculature cultured *in vitro* and their subsequent transplantation can dedifferentiate or degenerate from its original architecture. In addition, the days immediately after islet transplantation are critical since this process is characterized by substantial islet cell dysfunction and β-cells death – a more pronounced characteristic in diabetic recipients[55]. The blood flow of the transplanted islets seems to be lower in diabetic recipients[244] and could be caused by an altered regulation rather than a defect in the revascularization process[93].

Several aspects need to be overcome in order to introduce the practice of islet transplantation as a routine therapy. Besides the limits that derive from the isolation and purification of the islets, significant graft survival is required as well as the

avoidance of side effects linked to immunosuppressive drugs, which have a toxic effect on transplanted β cells and other organs and tissues[33].

In addition, in the immediate post-transplant period islets are subjected to inflammatory reactions which are either acute or chronic: macrophages (or Kuppfer's cells) that reside in the vascular hepatic sinusoid (where islets mainly tend to establish themselves) can exert harmful effects on the graft through the secretion of pro-inflammatory cytokines, digestive enzymes, reactive oxygen species[245]. Furthermore, it must be considered that a fatal, acute or chronic rejection can occur and severely impair the graft outcomes.

The aim of this project was the study of MSCs as an alternative therapy to the conventional immunosuppression in pancreatic islet transplantation given their well-known immunomodulatory functions for reducing acute rejection[62]. We also studied the role of EPCs in overcoming the critical vascularization within the pancreatic islets once they have been isolated and purified.

In our model, both types of cells (either MSCs or EPCs) were injected together with pancreatic islets and syngeneic and allogeneic transplants were performed in order to re-establish normoglycaemic levels in the long-term in chemically induced diabetic rats.

Indeed, the reduced functionality of the islets in the long-term was considered as crucial for the β-cell engraftment after transplantation into the vascular site[74].

As previously reported by Longoni et al.359, transplants performed with syngeneic and allogeneic 700 IE in the portal vein of diabetic animals have shown a decrease in glycaemic values within the first 15 days after the transplant for both types of models.

We observed that the curve relative to the syngeneic transplants (700 IE L-L) showed a decrease and then a slight growth towards diabetic levels, but the animals showed better maintenance of glucose and their significant levels by the twelfth day post transplant.

The trend of the allogeneic transplants (700 IE WF-L) showed a fast decrease in the early post transplant period but returned to diabetic values after just 9 days from transplant with significant levels only in the first week after the transplantation (figure 20).

These data reflect the key role of the implantation site for pancreatic islets because the intraportal site is considered to provoke a reduction in islet function, mostly in the long-term[74].

As shown by Jacobson, cell therapies based on the use of MSCs and pancreatic islets resulted in a lower grade of rejection compared to an islet-alone transplant when both were administered under the kidney capsule and they prolonged islet survival in order to obtain better control of blood glucose levels[212]. Moreover, MSCs were reported to enhance the survival of insulin-secreting β-cells when they were injected together with islets into the omental pouch[215].

The protective role exerted by MSCs in islet transplantation was further assessed by a model of a transplant via the portal vein in diabetic-induced animals; it was indeed reported that the islets transplanted with MSCs maintained a normal, undisturbed structure, whereas islet-alone transplants appeared fragmented[218].

On the basis of data obtained in our previous work, in which islets were injected in the portal vein of diabetic animals which simultaneously received a triple dose of MSCs in the tail vein (at days 0, 2, 4 after the transplant)[241], we decided to perform a pancreatic islet transplantation in a syngeneic model by increasing the dose of MSCs administered via the portal vein in order to prolong the blood glucose control. We observed that the MSCs were able to improve glucose level control during the overall monitoring time (figure 24), as confirmed also by recent studies on a model of islet and MSC co-transplant[246].

As shown by Rackham, the transplantation of MSCs together with IE under the kidney capsule improved the ability of islets to reverse hyperglycaemia in streptozotocin-treated rodents[246].

Moreover, several papers demonstrated also that the immunosuppressive properties of MSCs highlighted the allogeneic islet survival after transplantation by secreting cytokines or metalloproteinases[247]. In the syngeneic islet transplantation model, the advantageous role of co-transplanted MSCs could be due to trophic factors on islet cell viability and function[213].

Since the altered vascularization of pancreatic islets in impaired tissue is considered to be fundamental for the success of islet transplantation, we decided to improve islet revascularization in our experimental model by exploiting the angiogeneic properties of EPCs. An impaired endothelium can be repaired by being partially covered by resident endothelial cells together with the contribution of circulating EPCs[118]. The

typical properties of EPCs could also heal the endothelial damage and form new blood vessels in ischaemic tissues. Studies on experimentally induced mechanical endothelial damage showed that EPCs were able to repair the vessel wall, re-establishing anatomical and functional endothelial integrity[125]. Several studies showed the minor recruitment of EPCs *in vivo* at sites of ischemia in diabetic models and also that reendothelization is less likely to take place in the presence of EPC dysfunction[160].

On the basis of evidence reported in literature, in this project we firstly evaluated the effect of the administration of EPCs alone on blood glucose levels in an allogeneic transplant model via the portal vein of diabetic-induced Lewis rats. The resulting curve showed a non-homogeneous trend, so that within the first week after transplant the animals returned to diabetic values. The significant levels were observed only at days 3 and 7 after the transplant, exactly in correspondence with a decrease in the glucose values due to remaining β-cell function, (figure 21). The EPCs alone were effective in the early post-transplant days as confirmed by low glycaemic levels. We hypothesized that this temporary effect was due to an improvement in the vascularization of the residual pancreatic islets. In addition, this was not enough to re-establish the complete functionality and glucose homeostasis for the entire observation time, because the temporary effect was not associated with a recovery of islet mass.

Furthermore, we decided to combine the effect of EPCs with pancreatic islets: both a syngeneic and allogeneic model of a marginal mass of 700 IE was transplanted together with 500,000 EPCs in the portal vein of diabetic animals, (figure 22). Both groups reached normoglycaemic levels; the animals which received allogeneic therapy were found to re-establish levels under the diabetic threshold over a period of up to 75 days, (figure 23 A), whereas the syngeneic ones remained normoglycaemic during the overall monitoring time (6 months after the transplant), (figure 23 B). Thus, we hypothesized that the animals transplanted with allogeneic cell therapy were reported to become normoglycaemic only up to 75 days because they could incur allograft rejection due to a lack in the immunosuppressive treatment.

These data were in accordance with the results obtained with IPGTT performed on syngeneic transplanted animals, while the allogeneic ones were reported to have a worse trend in the curve of IPGTT as also confirmed by glycaemic levels, (figure 26). These data need to be further confirmed by histological analysis on hepatic and pancreatic tissues.

We subsequently compared the group of animals transplanted with 700 IE + 500,000 EPCs and 700 IE + 500,000 MSCs: a similar decreasing pattern was observed for both groups and they were also shown to be under the diabetic threshold value until the end of the monitoring time, (figure 25). We did not observe relevant statistically significant differences between the two curves.

Our data based on the detection of glycaemic levels appeared to improve the vascularization of the graft. This was further investigated by *ex vivo* experiments on hepatic samples of transplanted animals by quantitative evaluation of gene expression with a molecular biology assay.

Since the angiogenesis issue is a very complex argument to deal with regarding endothelial proliferation and differentiation, we found that a fine series of mechanisms are involved in the regulation of angiogenesis and a molecular control of endothelial cell behaviour during cell migration and proliferation for the blood vessel formation is equally implicated[229].

To this aim, the most important gene involved in the regulation of blood vessel sprouting during development, growth and disease, is VEGF, also known as the vascular permeability factor. In particular, VEGF-A is the main member of a family of homodimeric glycoproteins whose expression is positively regulated by hypoxia[248].

Our data showed that syngeneic transplants with 700 IE caused a marked increase in the VEGF-A level in liver tissues of animals in the first 15 days after transplantation, (figure 27 A). This could be due to the incomplete vascular network formation and a partial recovery in functionality when the islets alone are transplanted via the portal vein in diabetic recipients. In agreement with data demonstrating that VEGF in the diabetic group at 5, 7, and 14 days showed a marked gene expression, the vascularization of transplanted islets is delayed by the presence of hyperglycemia, deriving from an increase in local oxygen consumption[103].

In comparison with animals treated with syngeneic 700 IE, a similar pattern for VEGF-A gene expression was observed for 700 IE + 500,000 EPCs. A peak was observed at 15 days also for this group with a consequent and relevant decrease up to healthy values, and even under the healthy threshold for the overall monitoring time (up to 6 months), (figure 27 A).

We found that 15 days after transplantation the EPCs were able to regulate the revascularization process already begun by the islets; this result was correlated with the decreased level of VEGF-A expression compared to the islets alone at the same time

point. This could be in accordance with other *in vivo* studies showing that diabetes leads to an inability to mobilize EPCs from the bone marrow to the peripheral circulation in response to ischaemia and that post ischaemic angiogenesis was significantly compromised in diabetic animals compared to non-diabetic ones[157]. However, we found a full recovery in the glycaemic pattern in the long-term for the syngeneic 700 IE + 500,000 EPC group, thus we supposed that our observed trend in VEGF-A expression for the long period was consistent with these data.

The analysis of VEGF-A expression for the syngeneic 700 IE + 500,000 MSC group revealed no relevant changes, (figure 28 A).

Another key gene involved in the cellular mechanism of angiogenesis is ANG-1, which has been extensively reviewed by many authors[249]. ANG-1 which is produced by mural cells, can stabilize vessels and promote pericytes adhesion by making them leakage-resistant and by tightening endothelial junctions to maintain of endothelial cell quiescence[250]. This gene is also able to promote basement membrane deposition and vascular maintenance[249]. Recently, this traditional aspect of the angiopoietin family was changed by a study of the Jeansson's group who demonstrated that ANG-1 was not necessary in the quiescent mature vasculature but had a protective factor in the regulation of responses to tissue injury and microvascular disease in diabetes[251]. In this context Brissova found that the importance of revascularization after islet transplantation was expressed by a reciprocal endocrine cell–endothelial cell communication, since early differentiating endocrine cells produced angiogenic factors also including ANG-1[106].

We observed an increase in ANG-1 expression in the syngeneic 700 IE alone group at 15 days after transplantation, the critical time point for islet revascularization; at 30 days after the transplant, we observed only a slight decrease in the genetic value of ANG-1, thus suggesting the weak ability of islets to tighten and mature blood vessels in a diabetic environment, (figure 27 B).

A similar pattern was observed for the syngeneic group transplanted with 700 IE + 500,000 EPCs where a decreasing trend occurred from 15 days after the transplant up to the end of the monitoring time of 6 months, (figure 27 B). Gene levels concerning this group were much lower than the previous ones, indicating that the revascularization was supposed to be completed for animals treated with EPCs.

As previously studied by Ward et al.[252], a marked expression in ANG-1 in the liver of developing mice induced vascular remodelling effects including hepatic

arterial sprouting and enlargement, while in adult mice a reduced effect was observed in liver with regard to vessel size. The data we obtained were in line with that of Jeansson's group because ANG-1 was essential for vascular development, while it was negligible in quiescent vessels[251]; furthermore, the developing vasculature did not involve the regulation of pericyte recruitment or smooth muscle cell investment, as previously believed, thus conferring a more protective role for ANG-1.

In the group of animals treated with 700 IE + 500,000 MSCs we observed an irrelevant increase in ANG-1 expression from 7 to 60 days post-transplant, (figure 28 B). This could be correlated with a study by Zacharek and colleagues who observed that treating strokes with MSCs promoted angiogenesis and vascular stabilization by increasing Ang1/Tie2 expression and up-regulating ischemic brain occludin expression[228]. This evidence needs to be carefully understood also because another disease model was used, thus gene expression could vary according to the observed tissue[101].

We also investigated the function of PECAM-1 whose expression is restricted to cells of the vascular system, namely platelets, monocytes, neutrophils, selected T cells and endothelial cells. It was suggested that this gene was involved in transendothelial migration of neutrophils, monocytes, and natural killer cells in both *in vivo* and *in vitro* models, since transmigration and inflammation could be significantly reduced when antibodies directed against PECAM-1 were used[253].

In our transplant groups we observed a marked signal for PECAM-1 in the liver tissue of animals transplanted with 700 IE alone increasing from 7 to 15 days after surgery and a further reduction to low levels at 30 days. In the group of animals which received 700 IE + 500,000 EPCs, a different pattern was observed: from 7 to 15 days after the transplant the trend of the gene was similar, at 30 days there was a decline, while at the end of the monitoring time of 6 months a slight increase appeared again, (figure 27 C). Moreover, in the group of animals treated with 700 IE + 500,000 MSCs we found no great deviations in PECAM-1 expression during the overall monitoring time in our *ex vivo* liver tissues, (figure 28 C).

We therefore hypothesized that the role of transmigration attributed to PECAM-1 had to be considered for animals receiving 700 IE alone where a peak was observed at 15 days post-transplant, whereas we did not find a marked regulation of this gene for the other groups, either 700 IE + 500,000 EPCs or 700 IE + 500,000 MSCs. We speculated that this trend could be due to a lack of migration of cells such as

neutrophils, monocytes, and natural killer cells because we administered either EPCs or MSCs exogenously, so no relevant changes in the expression profile of this gene were observed.

Finally, we observed the gene expression of SDF-1 which has an important role in the regulation of a variety of cellular functions of endothelial progenitor cells, such as cell migration, proliferation, survival and angiogenesis. SDF-1 was also found to exert its essential role in stem cell homing toward the ischemic/hypoxic myocardium by recruiting the progenitor cells that express its cognate receptor, CXC chemokine receptor 4 (CXCR4)[254].

Since β cell injury was recently found to induce SDF-1 production[254], we observed, in accordance with this evidence, that a peak at 7 days post transplant was detected in the group of animals transplanted with 700 IE alone, due to islet damage in the first few days and a recruitment of endothelial cells, (figure 27 D).

Moreover, in the syngeneic group of 700 IE + 500,000 EPCs transplants a lower value for SDF-1 gene expression was observed when compared to the previous one. Since the effect of SDF-1 on neovascularization appears to enhance the recruitment and incorporation of transplanted EPCs[255], we supposed that a steady state in the gene expression up to 15 days, followed by a decrease and a further increase, could be due to a recovery in the neovascularization and also to an improvement in β cell function for animals who received this cell therapy, (figure 27 D).

Finally, we observed that animals treated with syngeneic 700 IE + 500,000 MSCs showed reduced SDF-1 expression from 7 to 15 days after the transplant, whereas at the end of the monitoring time a relevant increase in the gene was observed again, (figure 28 D). The increased expression of this gene at 60 days after transplantation could be in accordance with an *in vivo* study on a rat model of myocardial infarction[256] which showed that MSCs differentiated into endothelial cells were due to the presence of SDF-1α and also that an over-expression of SDF-1α can produce effective angiogenesis with a resulting prevention of progressive heart dysfunction after a myocardial infarction.

On the basis of our results, we hypothesized that EPCs were able to re-establish the impaired vascularization of transplanted islets by inducing a full recovery in islet functionality in the syngeneic transplant model. In the allogeneic model we observed a control of islet function up to 75 days post-transplant, thereby confirming

the outcome of allorejection as we did not administer either pharmacological or MSC-based immunosuppressive treatment.

Furthermore, from our molecular biological results we observed that the critical time point for the revascularization was the fifteenth day when the angiogenic and revascularizing genes were observed (VEGF-A, ANG-1, PECAM-1). After this time point there was a decrease in all gene expressions, except for SDF-1 which was reported to have a different pattern.

In the group transplanted with 700 IE alone, a relevant expression in VEGF-A, ANG-1 and PECAM-1 genes was observed which could be compared to β-cell injury; this cell therapy needs to be further vascularized.

On the other hand, the group of animals transplanted with 700 IE + 500,000 EPCs showed a lower expression in the same genes when we compared the same time points with 700 IE alone. In addition, angiogenic gene expression for the 700 IE + 500,000 EPC group showed that at the end of the monitoring time the levels were comparable to the healthy control. These results suggested that EPCs were considered to be able to regulate the process of islet revascularization.

The SDF-1 gene for the 700 IE alone showed a peak at 7 days post-transplant, indicating that a β cell injury occurred and endothelial recruitment was necessary. The same gene expressed by animals treated with the 700 IE + 500,000 EPCs group was found to be different, because the levels were close to the healthy control. We suggested that endothelial recruitment did not occur, also because the endothelial cells were administered exogenously.

With regard to cell therapy administered with 700 IE + 500,000 MSCs via the portal vein, we observed that all genes, except for SDF-1, were down-regulated when compared to IE and EPCs treatment. We speculated that after this down-regulation MSCs started their revascularization function. This could be in accordance with the immunomodulatory property of MSCs as observed in the treatment of GVHD[62].

The only gene which was not down-regulated for MSCs treatment was SDF-1, which was reported to have a peak value in the first few days after the transplant and to recruit endothelial cells; it then decreased and finally a high value was observed again. This could be explained with the hypothesis that a differentiation process is ongoing, as confirmed by Tang in a model of myocardial infarction[256].

Nevertheless, all these data need to be confirmed by further histological evaluation that is currently in progress in collaboration with the University of Cagliari. The histological analysis will supply evidence of the supposed presence of lymphocytic infiltration, as a supporting hypothesis of rejection outcome in allogeneic models, or vascularization which could occur due to a supposed new vessel formation in grafted tissues in syngeneic models.

Furthermore, experiments on vessel density, the hypoxia and the VEGF receptors assessment as well as the detection of soluble factors released by either EPCs or MSCs in our pancreatic islet transplants, will be investigated. In addition, we are also examining, by means of biochemical *in vitro* assays, our hypothesis that MSCs begin the revascularization process and finally differentiate into endothelium.

In conclusion, in this project we observed for the first time that EPCs were able to revascularize pancreatic islets and that these cells could improve syngeneic graft survival up to the complete healing of diabetic patients (6 months), as shown also by complete glycaemic recovery and consistent intraperitoneal glucose tests. Thus, EPCs could act through a regulatory mechanism as shown by their angiogenic gene expression.

Furthermore, we suppose that the cell therapy based on MSCs showed that these cells were able to improve graft vascularization as supported by glycaemic values. Nevertheless, the mechanism of action of MSCs could be different to that of EPCs because MSCs exerted their ability to be immunomodulatory. We hypothesized that MSCs could be considered as the beginner of a new vascularization process and that they completed their action with a final differentiation development.

In order to have a more complete spectrum of our evidence, further studies need to be carried out with regard to the allogeneic model such as the administration of immunosuppressive treatment, either pharmacological or MSC-based, to obtain a full recovery of pancreatic islet function.

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7. ACKNOWLEDGEMENTS