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ADIPOSE-DERIVED STEM CELLS AND TISSUE REVASCULARIZATION: ENHANCING ISLET SURVIVAL AND PERFORMANCE FOR DIABETES CARE

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CONTENTS

Abbreviations

α-SMA: alpha-Smooth Muscle Actin

ACTB: beta-Actin

ASC: Adipose-derived mesenchymal Stem Cell

BDNF: Brain-Derived Neurotrophic Factor

bFGF: basic Fibroblast Growth Factor

BMI: Body Mass Index

BM-MSC: Bone Marrow-Mesenchymal Stem Cell

BSA: Bovine Serum Albumin

BU: Butyric acid

cGMP: current Good Manufacturing Practice

CITR: Collaborative Islet Transplant Registry

DC: Dendritic Cell

DCCT: Diabetes Control and Complication Trial

DM: Diabetes Mellitus

DMEM: Dulbecco's Modified Eagle Medium

EBM: Endothelial Basal Medium

EC: Endothelial Cell

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

ELISA: Enzyme-Linked Immunosorbent Assay

eNOS: endothelial Nitric Oxide Synthase

EPC: Endothelial Progenitor Cell

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

GAGs: Glycosaminoglycans

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green Fluorescent Protein

G-CSF: Granulocyte Colony-Stimulating Factor

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HA: Hyaluronic Acid

HBSS: Hank’s Balanced Salt Solution

HDAC: Histone deacetylase

hESC: human Embryonic Stem Cell

HFF: Human Foreskin Fibroblast

HGF: Hepatocyte Growth Factor

HPRT1: Hypoxanthine phosphoribosyltransferase 1

HUVEC: Human Umbilical Vein Endothelial Cell

IBMIR: Instant Blood-Mediated Inflammatory Reaction

IBMX: Isobutyl-methyl xanthine

IDO: Indoleamine 2,3-deoxygenase

IEQ: Islet equivalents

IGF: Insulin-like Growth Factor

IL: Interleukin

IPGTT: Intraperitoneal Glucose Tolerance Test

IPC: Insulin Producing Cell

iPS: induced Pluripotent Stem

ISL1: Islet-1

KDR: Kinase insert Domain Receptor

KGF: Keratinocyte Growth Factor

M-CSF: Macrophage Colony-Stimulating Factor

MHC: Major Histocompatibility Complex

MSC: Mesenchymal Stem Cell

NGS: Normal Goat Serum

NK: Natural Killer

NMS: Normal Mouse Serum

PAK: Pancreas-After-Kidney

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDGF-R: Platelet-Derived Growth Factor Receptor

PGE2: Prostaglandin E2

PTA: Pancreas Transplant Alone

RA: Retinoic Acid

SD: Standard Deviation

SEM: Standar Error of the Mean

SPK: Simultaneous Pancreas-Kidney

SVF: Stromal Vascular Fraction

T1DM: Type 1 Diabetes Mellitus

TCA: Trichloroacetic Acid

TF: Tissue Factor

TGF-β: Transforming Growth Factor Beta

TNF-α: Tumor Necrosis Factor Alpha

VEGF: Vascular Endothelial Growth Factor

vWF: von Willebrand Factor

WJ: Wharton's jelly

ABSTRACT

Pancreatic islet transplantation represents a fascinating procedure that, at the moment, can be considered as alternative to standard insulin treatment or pancreas transplantation only for selected categories of patients with type 1 diabetes mellitus. Among the factors responsible for leading to poor islet engraftment, hypoxia plays an important role.

Mesenchymal stem cells (MSCs) were recently used in animal models of islet transplantation not only to reduce allograft rejection, but also to promote revascularization. Currently adipose tissue represents a novel and good source of MSCs. Moreover, the capability of adipose-derived stem cells (ASCs) to improve islet graft revascularization was recently reported after hybrid transplantation in mice.

Within this context, we have previously shown that hyaluronan esters of butyric and retinoic acids can significantly enhance the rescuing potential of human MSCs. Here we evaluated whether *ex vivo* preconditioning of human ASCs (hASCs) with a mixture of hyaluronic (HA), butyric (BU), and retinoic (RA) acids may result in optimization of graft revascularization after islet/stem cell intrahepatic cotransplantation in syngeneic diabetic rats. We demonstrated that hASCs exposed to the mixture of molecules are able to increase the secretion of vascular endothelial growth factor (VEGF), as well as the transcription of angiogenic genes, including VEGF, KDR (kinase insert domain receptor), and hepatocyte growth factor (HGF). Rats transplanted with islets cocultured with preconditioned hASCs exhibited a better glycemic control than rats transplanted with an equal volume of islets and control hASCs. Cotransplantation with preconditioned hASCs was also associated with enhanced islet revascularization *in vivo*, as highlighted by graft morphological analysis. The observed increase in islet graft revascularization and function suggests that our method of stem cell preconditioning may represent a novel strategy to remarkably improve the efficacy of islets-hMSCs cotransplantation.

1 - INTRODUCTION

1.1 Diabetes

Diabetes mellitus (DM) is a systemic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (*Diabetes Care, 2008*).

This pathology, long considered a disease of minor significance to world health, is now taking its place as one of the main threats to human health in the 21st century. Indeed, diabetes incidence appears to be rapidly rising in many parts of the world (fig. 1.1): in the year 2000, 150 million people worldwide were diagnosed with DM, and this number is considered to double in 2025 (*Zimmet et al., 2001*).

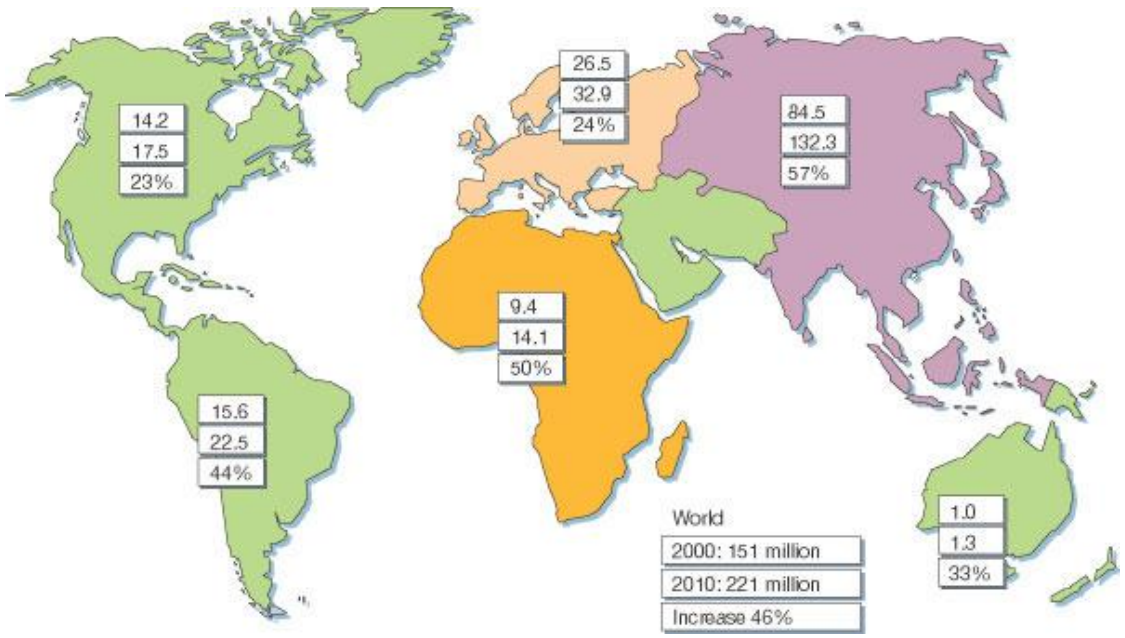


Figure 1.1 - Numbers of people with diabetes (in millions) for 2000 and 2010 (top and middle values, respectively), and the percentage increase (*Zimmet et al., 2001*).

Pronounced changes in the human environment, behaviour and lifestyle have accompanied globalization, and these have resulted in escalating rates of both obesity and diabetes. In general, as countries become richer, people eat a more sugar- and fat-

rich diet and are less physical active. On average, nearly 8% of adults living in high-income countries have diabetes. It is, however, upper-middle and middle-income countries that have the highest prevalence of diabetes (fig. 1.2); over 10% of adults in these countries have the condition. The mortality rate of diabetes varies sharply with the prosperity of the country. In 2011, the disease caused more than 3.5 million deaths in middle-income countries, of which more than 1 million were in China and just less than a million were in India. Approximately 1.2 adults died of a diabetes-associated illness per 1,000 cases in 2011 in low- and middle-income countries: more than double the mortality rate of high-income countries, where the healthcare recourses are greater (*Scully, 2012*).

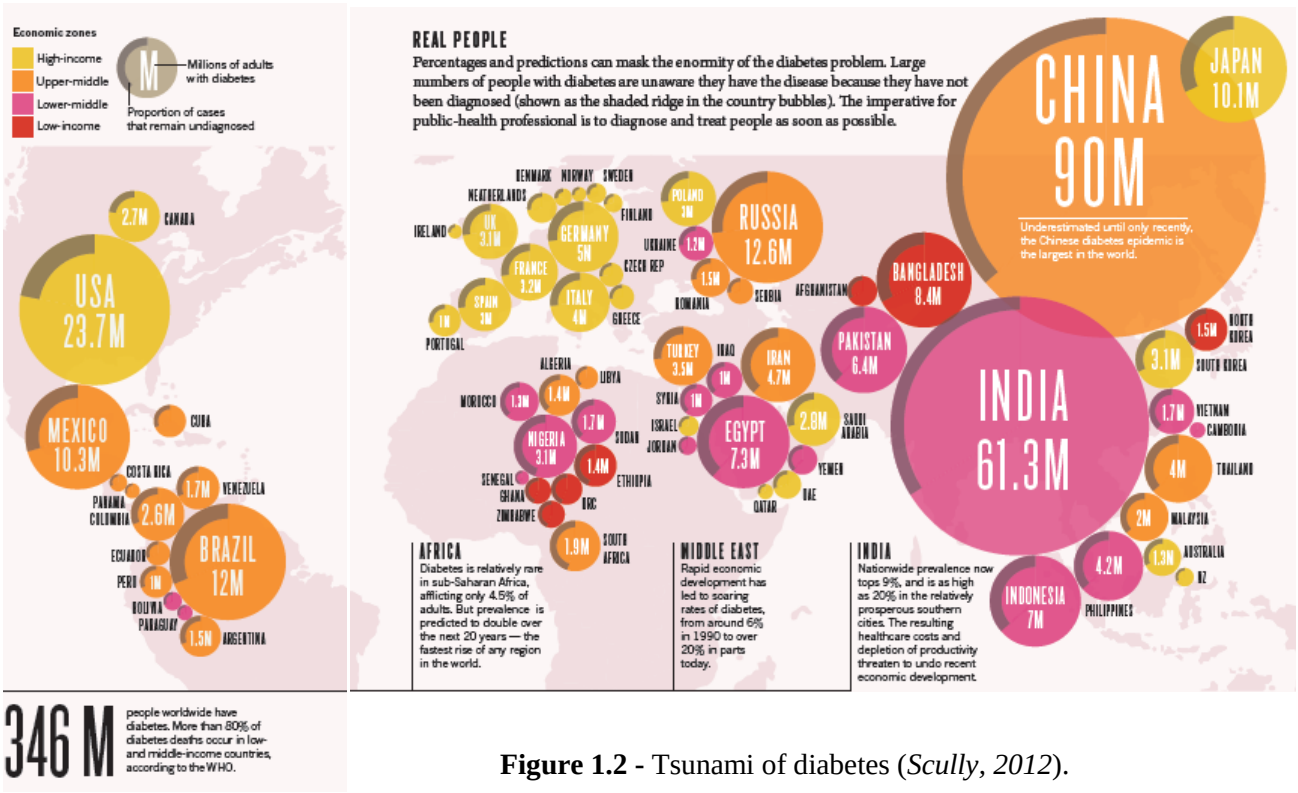


Figure 1.2 - Tsunami of diabetes (*Scully, 2012*).

Several pathogenic processes are involved in the development of DM. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is the deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action.

Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease.

The diagnosis of diabetes is based on measurement of fasting plasma glucose level, but also on measurement of plasma glucose concentration after an oral glucose load (glucose tolerance test).

There are three main forms of diabetes:

1. Type 1 diabetes (T1DM) is due primarily to autoimmune-mediated destruction of pancreatic β-cell islets, resulting in absolute insulin deficiency. It was previously referred to as "insulin-dependent diabetes mellitus" or "juvenile diabetes". Indeed, it is predominantly a disease of the young, usually developing before 20 years of age. T1DM makes up approximately 10% of all cases of diabetes. Although it is numerically less prevalent than type 2 DM in the general population, it is one of the most common chronic diseases of children, developing in approximately 1 in 600 children. The incidence is relatively low for children under the age of 5, increases between 5 and 15, and then tapers off. The most severe forms of DM are generally linked to this variant.

2. Type 2 diabetes (T2DM), which accounts for almost 90% of all cases of diabetes in adults worldwide, is characterized by insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with abnormal insulin secretion. This form was previously referred to as “non insulin-dependent diabetes mellitus” or "adult-onset diabetes". A degree of hyperglycemia sufficient to cause pathologic and functional changes in various target tissues, but without clinical symptoms, may be present for a long period of time before diabetes is detected.

3. The third main form, gestational diabetes, occurs when pregnant women without a previous diagnosis of diabetes develop a high blood glucose level. It may precede development of T2DM.

In some individuals with diabetes, adequate glycemic control can be achieved with weight reduction, exercise, and/or oral glucose lowering agents. These individuals therefore do not require insulin. Other individuals who have some residual insulin secretion are not dependent on exogenous insulin, but may require it for adequate glycemic control if this is not achieved with diet alone or with oral hypoglycemic agents. Individuals with extensive β-cell destruction require insulin for survival to prevent the development of ketoacidosis. The severity of the metabolic abnormality can progress, regress, or stay the same. Thus, the degree of hyperglycemia reflects the severity of the underlying metabolic process and its treatment more than the nature of the process itself.

1.2 Type 1 diabetes mellitus

As already stated, T1DM is characterized by the autoimmune selective destruction of the insulin-producing β-cells of the pancreas, termed islets of Langerhans (*Eisenbarth, 1986*). The absolute deficiency of insulin results in a wide spectrum of metabolic dysfunction, particularly impaired glucose homeostasis. T1DM pathogenesis involves environmental triggers that may activate autoimmune mechanisms in genetically susceptible individuals (table 1.1).

Table 1.1 - Possible aetiological factors in type 1 DM (*Zimmet et al., 2001*).

Non-genetic*	Genetic†
Viral infections (for example, coxsackie, cytomegalovirus)	Human leukocyte antigen (HLA) associated
Early infant diet (early cessation of breast feeding/early introduction of cow's milk)	Non-HLA associated
Perinatal infections	
Toxins (for example, dietary nitrosamines, bafilomycin, concanamycin A)	
Vaccine administration	

*No clear evidence for the role of any of these agents has been established.
†Consistent evidence for both HLA- and non-HLA-associated genes has been established.

The discovery of insulin in 1922 was a monumental achievement, transforming type 1 diabetes mellitus from a death sentence to a manageable, chronic condition. The Diabetes Control and Complications Trial (DCCT) confirmed that tight glucose control prevents or even reverses long-term complications of type 1 diabetes (*New Engl J Med,*

1993). However, insulin therapy itself can be life-threatening, as an overdose can result in severe hypoglycemia. In fact, tight glucose control is associated with more frequent episodes of hypoglycemia, especially in patients during acute illnesses (*Krinsley et al., 2011*). Many patients experience wide excursions in plasma glucose levels that lead to the secondary complications of diabetes, since most existing insulin formulations cannot mimic the natural regulatory ability of the insulin-producing β-cells of the endocrine pancreas. Worthy to note, more recent innovations in insulin delivery and therapy, such as insulin degludec (an ultra-long-acting basal insulin analog that can last up to 40 hours) has been shown to potentially mimic normal body glycemic control better than existing 24 hour-based regimens using long-acting insulin analog glargine.

Many insulin-dependent type 1 diabetes patients have some degree of hypoglycemia unawareness, the inability to sense low blood glucose, thus the benefits of stringent glycemic control may be outweighed by the risk of a potentially fatal insulin overdose (*Cryer, 2005*). Up to 10% of mortality in patients with T1DM is the result of hypoglycemia unawareness (*Cryer, 2008*), which potentially causes loss of consciousness or inability to awaken from sleep ("dead-in-bed" syndrome) (*Ragnar, 1997*). Despite advances in insulin therapy, the frequency of hypoglycemia unawareness has not declined in the past two decades (*McCrimmon et al., 2010*), thus alternative therapies are required (*Jamiolkowski et al., 2012*).

1.3 Whole pancreas transplantation

The first successful pancreas transplantations were simultaneous pancreas-kidney (SPK) transplants performed in 1966 in two patients with end-stage diabetic nephropathy (*Kelly et al., 1967*). One of the two recipients achieved near-normal glycemia for approximately two months. T1DM patients that have undergone a successful kidney transplant are candidates for pancreas-after-kidney (PAK) transplantation. SPK and PAK transplants have continued to improve quality of life and reduce hyperglycemia-related complications, while peri- and post-operative morbidity and mortality have steadily declined (*Nathan, 2003*).

However, whole pancreas transplantation is intrinsically a major surgical procedure with a high procedure-related morbidity leading to reoperation rates of >40% and mortality rates of up to 4% (*Johnson et al., 2012*). Major complications include chronic immunosuppression, surgical complications, and graft rejection (*Venstrom et al., 2003*),

and are related to the transplanted pancreatic exocrine tissue rather than the endocrine component. As islets of Langerhans only comprise 2% of the pancreas, it could be argued that 98% of a whole pancreas transplant is surplus to requirement. Standard medical therapy with insulin and close glucose monitoring are safe and successful in most patients with type 1 diabetes, thus the American Diabetes Association believes that pancreas transplant alone (PTA) carries unjustified risks (*JAMA, 1996; Robertson et al., 2004*).

1.4 Pancreatic islet transplantation

The aim of islet transplantation is to "reverse" diabetes by transplanting only the endocrine component of the pancreas (about 2% of the pancreas mass). The advantage of this procedure, that has always aroused attention from researchers, is avoidance of the major surgery needed for whole pancreas transplantation. As a cellular transplant it is minimally invasive, and it is associated with low morbidity and almost no mortality. However islets, though only clusters of 100-1,000 hormone-secreting cells with a diameter of 0.3-0.7 mm, obey the same immunological laws that govern solid organ transplantation, i.e. allogeneic islets trigger immune-mediated rejection that must be controlled with immunosuppressive drugs (*Cardani et al., 2007*). Noteworthy, isolated islets are potentially amenable to various types of manipulation/preconditioning that will enable these transplants to be performed in the future using minimal or even no immunosuppression. Islet transplantation therefore fulfills the criteria for an ideal treatment for reversing T1DM. The relative advantages and disadvantages of islet and whole pancreas transplantation are compared in table 1.2. It should be emphasized, however, that these two therapies are complementary rather than in competition, and that each form of β-cell replacement has merits for different patients (*Johnson et al., 2012*).

The modern era of islet transplantation began in 1972 with reports from two laboratories demonstrating successful reversal of diabetes in rodents by isolated islet transplantation (*Ballinger et al., 1972*; *Reckard et al., 1973*). Since then, a number of critical milestones have been achieved, including: 1) selection of the portal vein and liver as a site for inoculation of isolated islets (*Kemp et al., 1973*); 2) an automated method for isolation of human pancreatic islets (*Ricordi et al., 1988*); and 3) improvements in collagenase enzyme blend for isolation and purification of islets.

According to the Collaborative Islet Transplant Registry (CITR) report, the peri-procedural complications of islet transplantation have an estimated 20-fold lower morbidity risk than pancreatic transplants (*Sà et al., 2008*). Another advantage of islet transplantation is that healthy islets can be isolated from pancreases that may not be used for whole organ transplantation, which is extremely valuable given the shortage of donors (*Rother et al., 2004*).

Table 1.2 - Comparison of islet and whole pancreas transplantation (*Johnson et al., 2012*).

	Islet transplant	Pancreas transplant
First performed	1974 (Minneapolis)	1966 (Minneapolis)
Total number of cases	>1400	>24,000
Donor:recipient ratio	2:1-4:1	1:1-2:1
Number of transplants required to achieve therapeutic targets	1-4	1-2
Pretransplant graft testing	Extensive testing possible	No means at present
Preferred mode	IA	SPK
Transplantation procedure	Percutaneous	Laparotomy
Amount of tissue transplanted	0.5-5 g	~100 g
Procedure-related complications	Minimal: Bleeding Portal vein thrombosis	Significant: Graft thrombosis Graft pancreatitis Anastomotic breakdown Collections Pseudocysts Fistulae Sepsis and peritonitis
Mortality risk	Negligible (~0%)	Moderate (4%)
Insulin independence		
1 year	75%	85
5 years	15% (>60%)	~70%
Graft function at 5 years	70%	70%

1.4.1 Historical outlines

The history of pancreatic islet transplantation is long. The first transplant of fragments of the pancreatic gland in order to cure diabetes dates back even to the 20th December 1893, 28 years before the discovery of the insulin. Two English medical doctors from the Bristol Royal Infirmary Hospital, Dr. Watson-Williams and Dr.

Harsant, harvested a pancreas from a deceased sheep and transplanted three pieces of the gland into the subcuticular tissue of a 15-year-old boy with uncontrollable diabetic ketoacidosis. Obviously, despite a temporary improvement of the clinical situation, the xenotransplant failed after three days for acute rejection.

After that first experiment almost one century passed before Paul Lacy and colleagues in 1967 developed the technique of islet isolation in rats. Two important innovations made this procedure possible: the injection of digestion enzyme solution in the pancreatic duct and the centrifugation with different density gradients to separate the islets from the discarding tissue.

After few years the improvement of the glycemic control by intraperitoneal transplantation of islets was demonstrated in diabetic rats (*Younoszai et al., 1970*), and subsequently the liver, using the same experimental model, was selected as preferable implantation site (*Kemp et al., 1973*).

The first clinical series of islet transplantation was reported in the late seventy using azathioprine and steroids as immunosuppressive therapy (*Najarian et al., 1977*). Although these first cases did not experience any complications, the efficacy of the transplant was very limited. Seven patients over seven failed to reach insulin independence after intraperitoneal or intraportal islet transplantation even though some of them reduced the need of exogenous insulin for a period.

Interestingly the first real clinical success in the field of islet transplantation was reported in one case of combined islet-kidney transplantation performed on a T1DM uremic patient in Zurich in 1978. This patient reached insulin independence and maintained it almost one year after the embolization in the spleen of fragments of donor pancreas (*Largiader et al., 1979*).

1.4.2 The Edmonton protocol

Until the year 2000, islet transplantation was a highly successful procedure in animal models, with almost universal reversal of diabetes. However, translating these experimental results into human clinical trials was rather unsatisfying, especially for what concerned the long-term efficacy of the graft. In the 1990s, almost 500 islet transplants were performed in adults with severe T1DM, but the overall insulin-independence rates achieved were only 11%, and the 3-year incidence of sustained graft function was 19% in selected patients (*Brendel et al., 2001*).

Surprisingly, in 2000 the team from the University of Edmonton (Canada) reported 100% of insulin independence in seven consecutive T1DM patients at 1 year from islet transplantation (*Shapiro et al., 2000*). This landmark paper led to a resurgence of islet transplantation activity across the world, with centers using the "Edmonton Protocol" with 1-year insulin independence rates of 80-85% being achieved in leading centers. The "Edmonton Protocol" involved numerous small changes to existing protocols, but the principal factors that seem to have been responsible for the dramatically improved clinical outcomes include: firstly a second "top up" transplant being given to all patients within 3-6 months from the initial transplant, thereby ensuring that the recipient received a total of at least 11,000 islet equivalents (IEQ) per kg; secondly the use of a maintenance immunosuppressive regimen without steroids.

Later, with the aim to assess the applicability and reproducibility of the results obtained from Edmonton group, a multicentric study started using the Edmonton protocol involving 9 transplant centers, 6 of which were American and 3 European. Data from this study reported in 2006 did not confirm however the Edmonton results, showing a high variability in transplantation outcome according to the isolation center (*Shapiro et al., 2006*).

The critical passage in the procedure of islet transplantation is exactly the phase of organ processing in order to isolate the islets of Langerhans. These considerations lead to the development of several networks in which the isolation procedure is performed by few specialized and experienced centers and the islet transplantations are spread to several different centers even at long distance (*Kempf et al., 2005*).

In 2008 the Collaborative Islet Transplant Registry reported approximately a 30% of insulin independence by 3 years post-operatively over 325 adult recipients which were treated between 1999 and 2007 with an islet alone or a combined kidney-islet transplantation. However, more than 75% of the recipients maintained a residual graft functionality detected by the endogenous secretion of C-peptide at 3 years from the transplantation.

This apparently disappointing outcome showed anyway an important improvement in the quality of life of the patients reducing the episodes of severe hypoglycemia and the development of complications related to the diabetic disease (*Alejandro et al., 2008*).

1.4.3 Methodology of islet transplantation

Islet transplantation involves four principal components (fig. 1.3), namely pancreas donation and retrieval, islet isolation, islet culture, and the islet transplant itself (including patient selection and the pre- and post-transplant patient management). Although a number of aspects of this treatment are readily available in many hospitals, islet isolation has remained a challenging procedure requiring considerable expertise, and, therefore, this has been centralized to a small number of highly specialized centers. Islets are then either transplanted at those centers or transported to "satellite" centers for infusion into patients (*Johnson et al., 2012*).

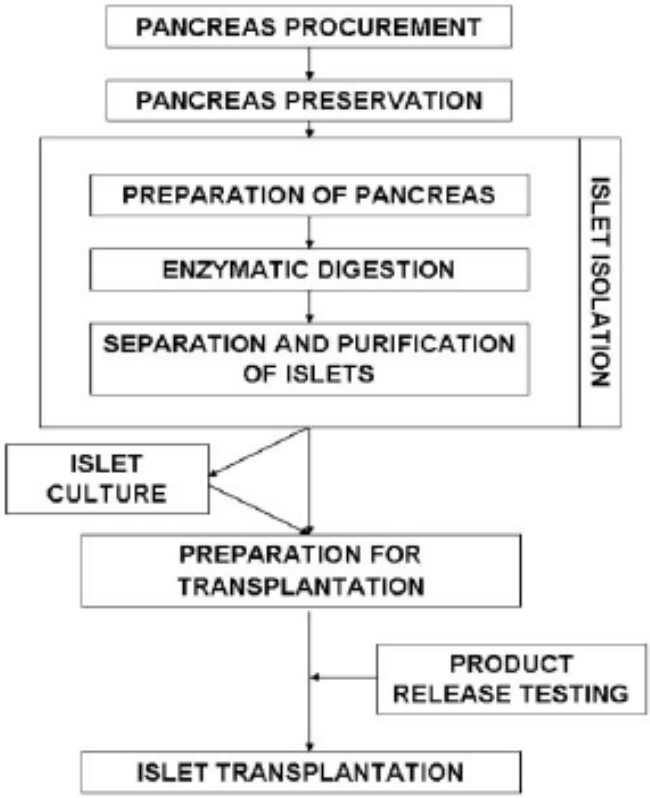


Figure 1.3 - Essential steps in islet transplantation (*Onaca et al., 2007*).

1.4.3.1 Pancreas retrieval

The organs used for the islet isolation generally are pancreas previously proposed and not utilized or excluded for the whole pancreas transplantation. This strategy, adopted in order to decrease the competition between the two types of transplantation, allows in the case of pancreas for islets isolation the use of organs from donors with high BMI or >50 years old. Moreover, with the aim to expand the pool of donors, some

authors reported positive experience of islet transplantation using donors after cardiac death (*Saito et al., 2010*).

The harvesting procedure of the gland is similar to the procedure for the whole pancreas transplantation, and need the same meticulous attention. After perfusion with cold preserving solution, the pancreas is collected paying particular attention not to section the pancreatic capsule, event that may impair the enzymatic digestion.

The pancreas is harvested en bloc with a portion of duodenum and the spleen. After procurement the pancreas is kept in cold preservation solution at 4°C, and transported to the Islet Isolation Facility. At the moment, the pancreas preservation is obtained thanks to a double-layered system: the organ is placed within a superior layer of preservation solution and an inferior layer of perfluorocarbons which are constantly oxygenated and help maintaining a high oxygen solubility coefficient. This method allows the development of a highly oxygenated environment for the pancreas which is of great importance for the protection of the islets (*Hering et al., 2002*). The gland can be kept in this solution for several hours although it was reported that the best results were achieved when the cold ischemia time was <16 hours (*Tsujimura et al., 2004*).

1.4.3.2 Islet isolation

Human islet isolation remains a challenging process, with only about 50% of optimal pancreases achieving transplantable islet yields in the leading isolation centers worldwide. The technical hurdles, together with the new stringent regulations introduced in most countries regarding human tissue processing, have meant that islet isolation is no longer performed in every center. Indeed, a number of "hub and spoke" networks have been created worldwide, in which islets are isolated at a central Islet Isolation Facility, and isolated islets then transported to different satellite centers for infusion into local patients. This model is more cost-effective, and it also enables consolidation of islet isolation expertise.

With regards the technical side of islet isolation, the procedure currently used to extract the intact islets of Langerhans while removing the exocrine/acinar tissue is the so-called automated method established in 1987 by Ricordi and colleagues. This is performed in two main stages: first, the pancreas is broken down by a combination of enzymatic digestion and mechanical dissociation, resulting in the islets being released from the surrounding tissue and the pancreas being broken down into a liquid digest

containing both the islets, and the exocrine and ductal tissue (pancreas digestion). Second, the pancreatic digest is subjected to density-gradient purification during which the lower density islets are separated (purified) from the other tissue (islet purification).

Pancreas digestion

Once retrieved, the pancreas is disinfected with a dilute iodine solution. The surrounding fat is removed by careful dissection, making sure that the pancreatic capsule is kept intact. The pancreatic duct is then cannulated using a standard intravenous cannula. A solution containing commercially available bacterial collagenase, derived from Clostridium histolyticum, is perfused through the cannula into the pancreatic ductal network, thereby distributing collagenase throughout the pancreatic parenchyma. The distended pancreas is then placed in a stainless steel or a disposable Ricordi chamber, which is situated within the isolation circuit. The digestion process is a combination of enzymatic digestion by collagenase, and mechanical dissociation of the pancreas by the manual or automated agitation of the ball bearings/marbles within the digestion chamber. Minimal essential media containing antibiotics and a number of other required additives are circulated around the circuit while digestion proceeds. Digestion is monitored throughout by analysis of fluid "biopsies" taken every 3-5 minutes. Each "biopsy" is stained with dithizone, a compound that stains the zinc granules within islets red, while leaving exocrine tissue unstained. At the point where sufficient freed islets are seen completely devoid of exocrine tissue, the isolation circuit is switched to an "open" circuit, and collection of the pancreatic digest undertaken. The collected pancreatic digest is centrifuged, and the packed cell volume resuspended in University of Wisconsin solution.

Islet purification

The pancreatic digest contains both released islets and the broken down exocrine tissue. The aim of islet purification is to separate these two different components, thereby producing pure islets. This enables the transplanted tissue volume to be kept to a minimum. Islet purification is performed by density-gradient centrifugation, which makes use of the fact that islets are less dense than their exocrine counterpart. Once the islets have been purified, they are counted and assessed for overall purity and viability percentages. The exocrine tissue is then discarded, and the islets placed in culture media.

1.4.3.3 Islet culture

Previous islet transplant protocols used "fresh" islets immediately after isolation. However, the original Edmonton protocol has been modified in several ways so that most centers now culture isolated islets for 48 to 72 hours to obtain a cleaner islet suspension with less immunogenic and thrombogenic collagen and debris, and administer anti-inflammatory therapy peri-transplant (*Harlan et al., 2009*). A period of at least 24 hours of culture is encouraged for a number of reasons: first, there are data to suggest that islets benefit from a "recovery" period after the metabolic insults endured during islet isolation, before being implanted. Second, the culture step enables the islets to be subjected to detailed quality testing before the transplant is performed, including tests of islet function, viability and sterility. Third, before the culture step was routinely used, the necessity to use "fresh" islets rendered islet transplantation an emergency procedure that had to be performed whenever the islets had been isolated. Islet culture has enabled this procedure to be planned, benefitting both patient and health care resources (*Johnson et al., 2012*).

1.4.3.4 Islet transplant procedure

The islet transplant procedure itself is the culmination of a time-consuming process of careful patient selection and detailed patient work-up (fig. 1.4). The immediate post-transplant management is also critical to ensure optimal islet graft function.

Islet transplantation is a quite safe procedure, anyway the requirement for long-term immunosuppression, which has as well known important side effects, means that this treatment is currently reserved for people with the most severe forms of T1DM. This limitation is a consequence of the benefit/cost ratio between improvement of the glycemic control and the necessity for recipients to be treated with chronic immunosuppressive therapy.

These considerations and the availability of new generation basal insulin and sophisticated micro insulin pumps lead the majority of European centers to perform islet transplantation almost exclusively in combination with kidney transplant in T1DM patients candidate (simultaneous islets-kidney transplant), or just subjected (islet after kidney transplant), to a kidney transplantation for end-stage diabetic nephropathy. These patients therefore would anyway be treated with immunosuppressive drugs (*Cavallari et al., 2012*).

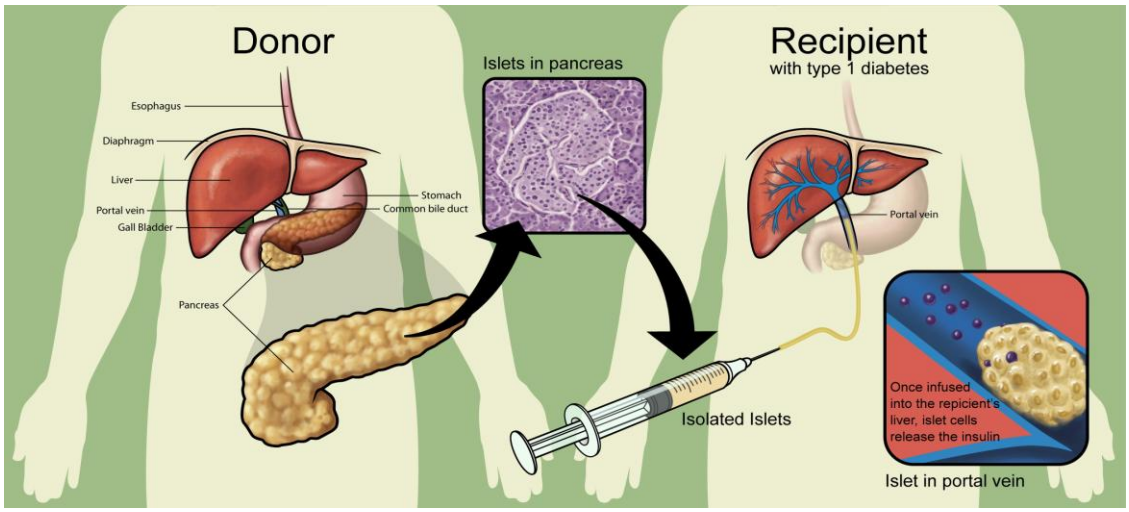


Figure 1.4 - The main idea of islet transplantation is to process the donor's pancreas so as to remove the exocrine tissue and isolate the portion of the gland responsible for the endocrine hormone secretion. The medical team infuses the isolated islets into the portal vein. The islets are transported by the bloodstream into the liver, where they lodge, starting to produce the right amount of insulin to regulate the blood sugar (*Naftanel et al., 2004*).

Although a number of anatomical sites have been investigating for hosting the islet transplant, nowadays the liver, by islet injection via the portal vein, remains the site preferably used in the clinical setting for several reasons: the position of the liver and portal vein lend themselves to percutaneous access; the structure of the sinusoids prevents islets from flowing straight through the liver and is ideal for islet entrapment; the total blood flow within the liver facilitates islet engraftment and maximal function. Alternative sites include the spleen, the renal subcapsule, the omental pouch, the peritoneum, the skeletal muscle, and the ovary or testis (both immune-privileged sites but with practical drawbacks) (*Johnson et al., 2012*).

The adequate amount of islets obtained is calculated with respect to the body weight of the recipient and resuspended immediately before the intrahepatic transplantation in an adequate solution (*Cavallari et al., 2012*).

In the face of organ shortage and procedure-related costs, these findings may lead to a new paradigm in islet transplantation, where the primary aim is not necessary to achieve the same insulin independence as in whole organ transplantation, but to improve the glycemic control of the patient through a less invasive procedure.

Promising fields of research are nowadays focused on increasing the engraftment and survival of the islets after transplantation. If these studies will give positive results, it will be possible in future to extend the actual indications of the combined kidney-islet transplantation procedure.

1.4.4 Etiologies of graft dysfunction

Multiple reasons have been cited as underlying etiologies of graft failure (fig. 1.5). The period surrounding transplantation is marked by a rapid loss of approximately 50-70% of donor islets (*Korsgren et al., 2005*). This large-scale islet loss is partly caused by the quality of donor pancreata as well as the isolation process itself, which includes enzymatic digestion, cold-storage time, and exposure to hypoxia during isolation and culture (*Nanji et al., 2006*). These combined stresses generate inflammatory cytokines and initiate pro-apoptotic NF-κB, mitogen-activated kinase, and poly(ADP-ribose) polymerase stress pathways before transplantation has even occurred (*Abdelli et al., 2004*).

Another major culprit, detrimental to islet survival, is the innate immune system, which launches a large-scale inflammatory reaction, initiating massive β-cell death immediately after intravascular infusion (*Korsgren et al., 2005*). This instant blood-mediated inflammatory reaction (IBMIR) occurs secondary to the brisk activation of coagulation and complement cascades, which are triggered by exposure of tissue factor (TF) expressed by islets to human blood.

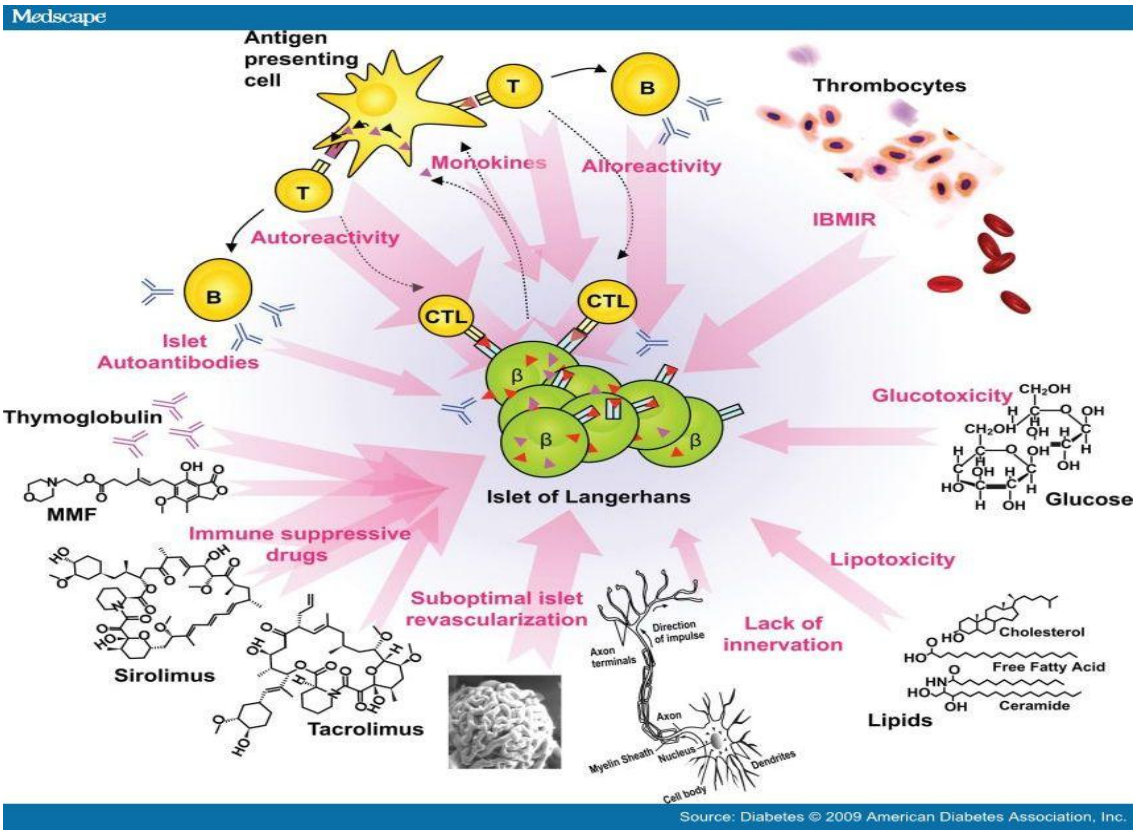


Figure 1.5 - Factors limiting islet graft function and/or survival (*Harlan et al., 2009*).

Although the innate immune response causes early β-cell death, long-term deterioration in graft function is also related to allo- and autoimmunity (*Ryan et al., 2005*). Similar to solid organ transplants, islet grafts are susceptible to the development of allograft rejection via sensitization to alloantigens presented by antigen presenting cells and subsequent activation of a T-cell-dependent immune response. The rejection process can be mitigated by immunosuppressants, but once drug levels are decreased, allosensitization occurs (*Cardani et al., 2007*).

Given that T1DM is an autoimmune disease, recurrent autoimmune destruction involving donor islet antigens may also play a role in graft failure.

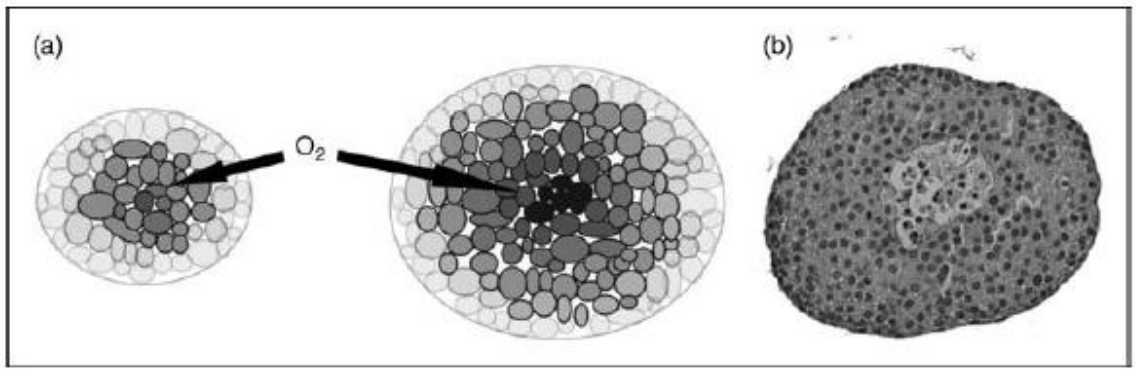


Figure 1.6 - (a) Schematic drawing of oxygen supply to pancreatic islets of different sizes during islet culture and early post-transplantation. Darker colour indicates less oxygenation. (b) Micrograph of islet with developed central necrosis due to poor oxygenation of the islet core (*Lau et al., 2009*).

Another hurdle that has emerged as a limitation in graft viability and function is the development of an optimal vascular network (*Menger et al., 2001*). Normal pancreatic islets are microorgans, which contain a dense capillary network, approximately 10 times higher than that of the surrounding exocrine tissue. Although the islet cells comprises only 1% to 2% of the total pancreatic mass, islets receive 5% to 10% of the pancreatic blood flow (*Lifson et al., 1985*). The blood vessels within the islets are lined with fenestrated endothelial cells (ECs), suggesting an increased importance of perfusion and sensitivity to hypoxia (*Lau et al., 2009*). Unfortunately, this network is interrupted during the islet isolation process, and as a consequence β-cells become necrotic in culture, starting from the core region of larger islet (fig. 1.6). Transplanted islet oxygen, nutrient supply, and exposure to intraislet paracrine signaling are limited by rate of neovascularization and alterations in the vascular development that differ compared to the vascular networks seen in native islets (*Carlsson, 2011*). The reestablishment of an

adequate blood flow to transplanted islets depends on an angiogenesis process that requires several days, and, even when complete (generally by 10-14 days after transplant), is suboptimal.

The site of transplantation may also have important implications. Concerning the liver, often the standard organ of choice, one should consider the drawbacks of the portal vein, including the IBMIR, higher levels of toxic immunosuppressants in the portal circulation, periportal steatosis, and an inability to routinely biopsy the transplanted islets because they are dispersed within the liver (*Harlan et al., 2009*).

1.5 Adult stem cells

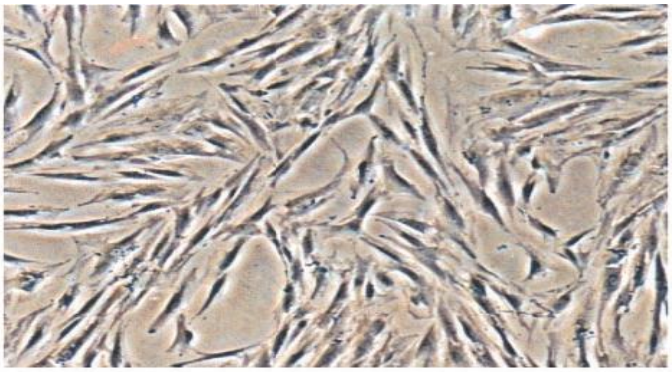
Several studies have revealed that a population of adult stem cells and supporting cells reside in most of adult mammalian tissues/organs, including bone marrow, heart, kidneys, brain, skin, eyes, gastrointestinal tract, liver, pancreas, lungs, breast, ovaries, prostate, and testis. In general adult stem cells are thought to be localized in specific areas of each tissue, within a specialized microenvironment called "stem cell niche", consisting of the neighboring cells such as fibroblasts, endothelial cells and/or stromal components that tightly regulate their functions through direct interactions and release of specific soluble factors (*Li et al., 2005*).

All the multipotent or bipotent adult stem cell types display a long-term self-renewing capacity and in appropriate conditions (including the onset of an intense injury) give rise to all the mature and specialized cell types of distinct lineages in the tissues/organs from which they originate, or sometimes even to cell lineages of distant sites. Despite some adult stem/progenitor cells, found in bone marrow, skin and gastrointestinal tract, usually show a rapid turnover, to replenish the cells lost along lifespan other adult stem cell types remain quiescent and rarely divide in normal conditions, undergoing a sustained proliferation only after tissue injury (*Mimeault et al., 2008*).

1.5.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent progenitor cells found in perivascular spaces of many adult tissues. They have been known since 15-20 years, and were identified for the first time in bone marrow. To isolate human MSCs, bone

marrow aspirate is harvested from the iliac crest and processed. Bone marrow-MSCs (BM-MSCs) are relatively easy to expand in culture under conditions in which they retain some of their potential to differentiate into multiple cell lineage that include osteoblasts, adipocytes, chondrocytes, myoblasts, and early progenitors of neural cells. They are enriched and expanded in presence of fetal calf serum by their tight adherence to plastic tissue culture dishes. A culture of human MSCs, unlike murine cells, can become free of hematopoietic precursors after one or two passages, and can be extensively expanded before reaching senescence (*Sekiya et al., 2002*).



hMSC surface markers

Positive	Negative
CD13, CD29, CD44, CD49a, b, c, e, f, CD51, CD54, CD58, CD71, CD73, CD90, CD102, CD105, CD106, CDw119, CD120a, CD120b, CD123, CD124, CD126, CD127, CD140a, CD166, P75, TGFb1R, TGFbIIR, HLA- A,B,C, SSEA-3, SSEA-4, D7	CD3, CD4, CD6, CD9, CD10, CD11a, CD14, CD15, CD18, CD21, CD25, CD31, CD34, CD36, CD38, CD45*, CD49d, CD50, CD62E,L,S, CD80, CD86, CD95, CD117, CD133, SSEA-1

Figure 1.7 - Culture of human MSCs (*Pittenger et al., 2004*).

Human MSCs in culture appear fibroblast-like and homogeneous in size and morphology (fig. 1.7) starting from the second passage. MSCs haven't a unique peculiar marker but, in recent years, a panel of cell surface antigens emerged to characterize them. Negative and positive selection markers of MSCs have been so defined: MSCs do not express CD14, CD34, CD45, CD117 (cKit), HLA class I, and HLA-DR antigens, whereas they are positive for CD13, CD29, CD44, CD73, CD90, CD105, and CD166. These markers are located in the cell membrane of the MSCs, and antibodies are readily available for FACS analysis and sorting (*Maurer, 2011*).

1.5.2 Alternative sources of hMSCs

Bone marrow represents the main source of MSCs for both experimental and clinical studies. However, the number of bone marrow MSCs significantly decreases with age, which makes necessary the search for adequate alternative sources for autologous and allogeneic use. Most attention should be paid to tissue containing cells with higher proliferative potency, capability of differentiation, and lower risk of contamination. In 2003 scientists saw in the umbilical cord/placenta vessels the presence of MSCs, in particular Romanov and colleagues found a type of cell ascribable to MSCs for the presence of surface markers, morphology and the possibility to differentiate into adipocytes and osteoblasts. Other research groups have found multipotent stem cells in tissues like mobilized peripheral blood, umbilical cord blood and fetal membranes (*Ventura et al., 2007*), deciduous teeth, umbilical cord mesenchyme (Wharton's jelly, WJ), and adipose tissue.

1.5.2.1 Adipose-derived stem cells

Adipose tissue derives from embryonic mesoderm, and has emerged as a promising source of adult stem cells with regenerative capacity. As mesenchymal stem cells, the isolated cells are capable to differentiate into mesodermal and potentially ectodermal and endodermal lineages, but again the ability to differentiate into all lineages is somewhat controversial (*Mizuno et al., 2012*). These stem cells have been variously termed, anyway a recent consensus was reached by investigators at the 2004 conference of International Fat Applied Technology Society, which has settled on the term "adipose-derived stem cells" (ASCs).

Abundant numbers of ASCs can be derived from lipoaspirate, the waste product of liposuction surgery. Processing 300 ml of lipoaspirate routinely yields between 1×10^7 and 6×10^8 ASCs with >90% cell viability. The yield compares favourably with a bone marrow aspirate. Compared with BM-MSCs, ASCs are more easily cultured and grow more rapidly. They can also be cultured for longer than BM-MSCs before becoming senescent. All of these qualities make ASCs a useful source of mesenchymal stem cells (*Locke et al., 2009*).

Adipose stem cells can be found in any type of adipose tissue, including subcutaneous and omental fat. In recent years obesity has become a huge problem in the Western world, and the number of liposuctions has increased significantly. Liposuction

is considered a safe and well-tolerated procedure, with a complication rate of around 0.1%. Research shows that 98-100% of the adipose cells in the lipoaspirate are viable. Given the safety and popularity of liposuction, lipoaspirate seems an ideal source of ASCs, and a safe autologous cell source for clinical use.

ASCs are obtained from the adipose stromal vascular fraction (SVF), a population of cells harvested after enzymatic dissociation of adipose depots followed by density separation from adipocytes. After standard condition cells develop a fibroblast-like morphology.

A comparative analysis of MSCs obtained from bone marrow, adipose tissue, and umbilical cord clearly showed that ASCs were not different regarding morphology, immune phenotype, success rate of isolation, colony frequency, and differentiation capacity. However, some characteristics, such as the colony frequency and the maintenance of proliferating ability in culture, seem even to be superior in ASCs.

Table 1.3 - Molecular phenotype of adipose-derived stromal cells (modified from *Schaffler et al., 2007*).

ASC-positive cellular markers and genes	ASC-negative cellular markers and genes
CD9	**CD11b**
CD10	**CD14**
CD13	**CD19**
CD29	CD31
CD44	**CD34**
CD49 (d)	**CD45**
CD49 (e)	**CD79α**
CD54	CD80
CD55	CD117
CD59	CD133
CD73	CD144
CD90	**HLA-DR**
CD105	**c-kit**
CD106	MyD88
CD146	STRO-1
CD166	Lin
HLA I	HLA II
Fibronectin	
Endomucin	
ASMA	
Vimentin	
Collagen-1	

There isn't a clear discrimination between ASCs (fibroblast-like stem cells) and fibroblasts, but observing the set of criteria to define human MSCs (i.e. plastic adherence under standard culture condition, ability for differentiation, expression of CD73, CD90, CD105, lack of expression of c-Kit, CD14, CD11b, CD34, CD45, CD79α

and the known ASC expression profile of surface markers in table 1.3) we could support the hypothesis that ASCs and BM-MSCs have originated from identical precursor cells (*Schäffler et al., 2007*) .

ASCs, similarly to BM-MSCs, have the potential to differentiate into bone, cartilage, tendons, skeletal muscle, and fat when cultivated under lineage-specific conditions. Moreover ASCs demonstrated to have the potential for "cross differentiation" in neurons, endocrine pancreatic cells, hepatocytes, endothelial cells and cardiomyocytes (cells of non-mesodermal origins) (table 1.4).

Table 1.4 - Experimentally used factors triggering the differentiation of adipose-derived stem cells (*Schaffler et al., 2007*).

Type of differentiation	Differentiation factors
Adipogenic	Insulin, IBMX, dexamethasone, rosiglitazone, indomethacin
Chondrogenic	BMP-6, BMP-7, FGF-2, TGF-β_1, TGF-β_2, TGF-β_3, dexamethasone, IGF-1
Osteogenic	$1,25(OH)_2D_3$, β-glycerophosphate, ascorbic acid, BMP-2, dexamethasone, valproic acid
Myogenic differentiation	Specific microenvironment?
Cardiomyogenic differentiation	IL-3, IL-6, SCF
Vascular/endothelial	Specific microenvironment?
Neurogenic	Valproic acid, insulin, hydroxyanisole, hydrocortisone, EGF, FGF
Pancreatic/endocrine	Activin-A, exendin-4, pentagastrin, HGF, nicotinamide, high glucose concentration
Hepatic	HGF, OSM, DMSO
Hematopoietic	Specific microenvironment?

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxy-cholecalciferol; BMP, bone morphogenetic protein; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IBMX, 3-isobutyl-1-methylxanthine; IGF, insulin-like growth factor; IL, interleukin; OSM, oncostatin M; SCF, stem cell factor; TGF, transforming growth factor.

1.5.2.2 Lipogems: a highly enriched stromal cell fat-derivative

Regulation of hMSC usage requires that extensively manipulated cells for clinical use have to be manufactured in accordance with current Good Manufacturing Practice (cGMP) guidelines. However, these restrictions are not applied in case of minimal manipulation [Regulation (EC) No 1394/2007 of the European Parliament and of the Council]. Therefore, developing devices and strategies encompassing a minimal manipulation to make available a ready-to-use, autologous fat product would have obvious biomedical and clinical relevance to translate cell therapy promises into a clinical practice. Akin to this perspective, Lipogems is an innovative system that isolates a highly enriched ASC-containing product from minimal amounts of lipoaspirates (*Tremolada et al., 2010*). Lipogems simply uses mild mechanical forces, avoiding the use of enzymes, additives, and other inherent manipulation (i.e. separative centrifugation and subfractional harvesting). The clusters of fat obtained from a liposuction is reduced by using filters and spheres, contained in a completely closed

system, designed for total washout of oil and blood. Oil removal also makes inflammation negligible while increasing the chances for cell engraftment. The isolation is really fast, taking 15-20 minutes for each sample.

The Lipogems product is very fluid and exhibits a number of clinically relevant features:

1. The Lipogems product can be reinjected immediately into the donor patient itself. This autologous strategy is safe, it does not require stem cell expansion or manipulation, and therefore it is not subjected to any of the regulatory restriction imposed by the cGMP guidelines.
2. Differently from unprocessed lipoaspirate, the non-expanded Lipogems product has a remarkably preserved vascular/stromal architecture, and can be stored frozen without losing neither the architecture nor the ability to release highly functional and viable hASCs after thawing.
3. Lipogems is highly enriched in MSCs and pericytes, with a low amount of hematopoietic-like elements (*Bianchi et al., 2012*).
4. When placed in cell culture medium, the Lipogems product spontaneously releases its stem cell pool, with subsequent attachment of a highly homogeneous hMSC population to the plastic dish. These cells can be mechanically harvested and pooled prior to any cell expansion, being equivalent to a "time zero, non-proliferating condition" that does not involve any substantial manipulation.

1.5.3 MSC immunogenicity

Immunomodulatory and anti-inflammatory properties are increasingly viewed as key properties of MSCs isolated from multiple organ sources, which can extend (or even supersede) their importance as versatile multipotent cells. While the differentiation properties of MSCs seem to be dependent on microenvironmental clues *in vivo*, the immunomodulatory effects appear to be rather intrinsic.

Many reports have evidenced, first *in vitro* and then *in vivo*, the ability of MSCs to express molecules that can interact with both innate and adaptive immunity processes, in a cell contact-mediated fashion and/or in experimental settings where cell-cell interaction is precluded, thus suggesting the importance of soluble factors in mediating these processes (*Vija et al., 2009*).

1.5.3.1 *In vitro* evidence of immunomodulation

Adult human MSCs express intermediate levels of major histocompatibility complex (MHC) class I molecules on their cell surface, but not MHC class II (HLA-DR), properties that make them hypoimmunogenic and immunoprivileged, allowing transplantation across allogeneic barriers. In addition MSCs can synthesize trophic mediators such as growth factors and cytokines, involved in cell signaling and modulation of immune response. Recent studies demonstrated that the secretion of key soluble factors is not constitutive, but is often a consequence of cross-talk between MSCs and T lymphocytes, the latter being able to trigger this *de novo* expression (*Nauta et al., 2007*).

In vitro experiments show that MSCs can modulate the immunological activity of different cell populations, the most important being their inhibitory effect on T-cell proliferation and dendritic cell (DC) differentiation. MSCs are also able to inhibit proliferation of $CD4^+$ helper and $CD8^+$ cytotoxic T-cells, as well as memory and naïve T-cells, B-cells and Natural Killer (NK) cells (fig. 1.8).

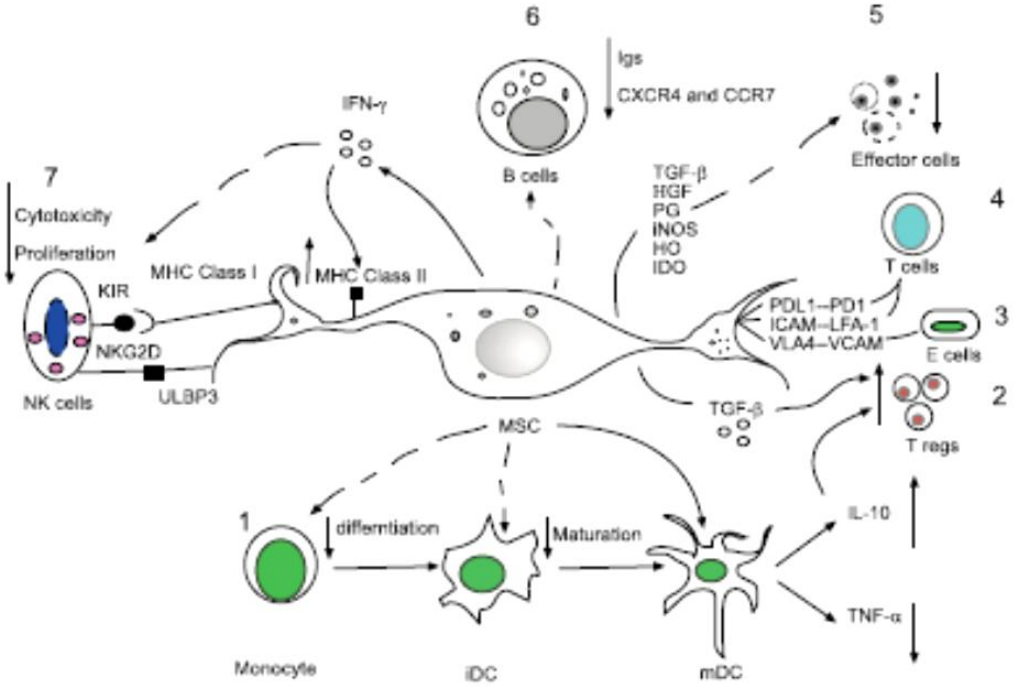


Figure 1.8 - Plausible mechanisms by which MSCs regulate immune responses: reducing the generation and differentiation of DCs (1); increasing the percentage of regulatory T-cells through production of cytokines (2); engaging in cell-to-cell contact with T-cells and ECs (3,4); suppressing effector T-cells through various growth factors (5); acting through down-regulation of immunoglobulin production by B-cells (6); up-regulating MHC II on MSCs thus leading to down-regulation of NK cell cytotoxicity and proliferation (7) (*Abdi et al., 2008*).

It has been reported that MSCs can modulate T-cell proliferation thanks to their low expression of costimulatory molecules including B7-1 (CD80), B7-2 (CD86), or CD40, and the absence of MHC-II. This mechanism may necessitate an initial contact phase as well as specific mediators produced by MSCs, such as transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), interleukin-10 (IL-10), and indoleamine 2,3-deoxygenase (IDO).

MSCs may also determine T-cell arrest in G0-G1 phase by inhibition of cyclin D2 expression resulting in T-cell anergy, and stimulate the production of $CD8^+$ regulatory T-cells (T_{regs}), that inhibit allogeneic lymphocyte proliferation (*Vija et al., 2009*).

MSCs are able to interact with dendritic cells, not only inhibiting differentiation of monocytes into DCs but also inhibiting DC maturation, giving rise to immature DCs that could subsequently render T-cells anergic. MSCs have also been shown to alter the cytokine secretion profile of DCs toward up-regulation of regulatory cytokines such as IL-10 and down-regulation of inflammatory cytokines such as interferon- γ (IFN-γ), IL-12, and tumor necrosis factor TNF-α, inducing a more anti-inflammatory or tolerant DC phenotype (*Abdi et al., 2008*).

Concerning B lymphocytes, MSCs can inhibit their proliferation and activation in a dose-dependent manner and modulate their differentiation, antibody production and chemotactic abilities (*Corcione et al., 2006*).

Lastly, MSCs could suppress proliferation of NK cells, decrease the secretion of IFN-γ by IL-2 stimulated NK cells, and affect cytotoxicity against HLA class I-expressing targets.

1.5.3.2 Tolerance induction

Tolerance to self-antigens is a process of fundamental importance for the correct development of the human immune system. Peripheral tolerance mechanisms act together with central tolerance, based on the role of T_{reg} cells. A key molecule in which a tolerance-induction role has been demonstrated is HLA-G (a non-classical MHC I), which was first characterized in trophoblast cells, where it mediates tolerance towards the semi-allogeneic embryo. HLA-G has been found to be expressed in different MSC populations, such as BM-MSCs and WJ-MSCs. This molecule has been directly linked to the tolerogenic ability of MSCs, e.g. inducing the expansion of $CD4^+CD25^+FoxP3^+$ T_{regs} which would contribute to the suppression of effector responses to alloantigens.

HLA-G would act both in its membrane-bound isoform, implicating direct cell-cell contact, or by its shedding from the cellular surface (the soluble isoform is defined as HLA-G5).

In a recent paper, Gonzalez and colleagues demonstrated that the beneficial action of ASCs on experimental rheumatoid arthritis was due to the generation and activation of T_{reg} cells.

Taken together, these data indicate that one of the great expectations linked to the use of MSCs may reside in their ability to successfully engraft, evading an immune response and inducing peripheral host tolerance (*Anzalone et al., 2011*).

1.5.4 Vasculogenesis and MSCs

1.5.4.1 Vasculogenic differentiation

There are some evidences of the existence of an heterogeneous population in MSCs, that means the presence of undefined subfractions of stem cell populations with a great differentiation potential. There is a subpopulation positive for Flk-1/KDR (kinase insert domain receptor, a major receptors for vascular endothelial growth factor, VEGF) and negative for CD31 and CD34, which can differentiate into endothelial cells.

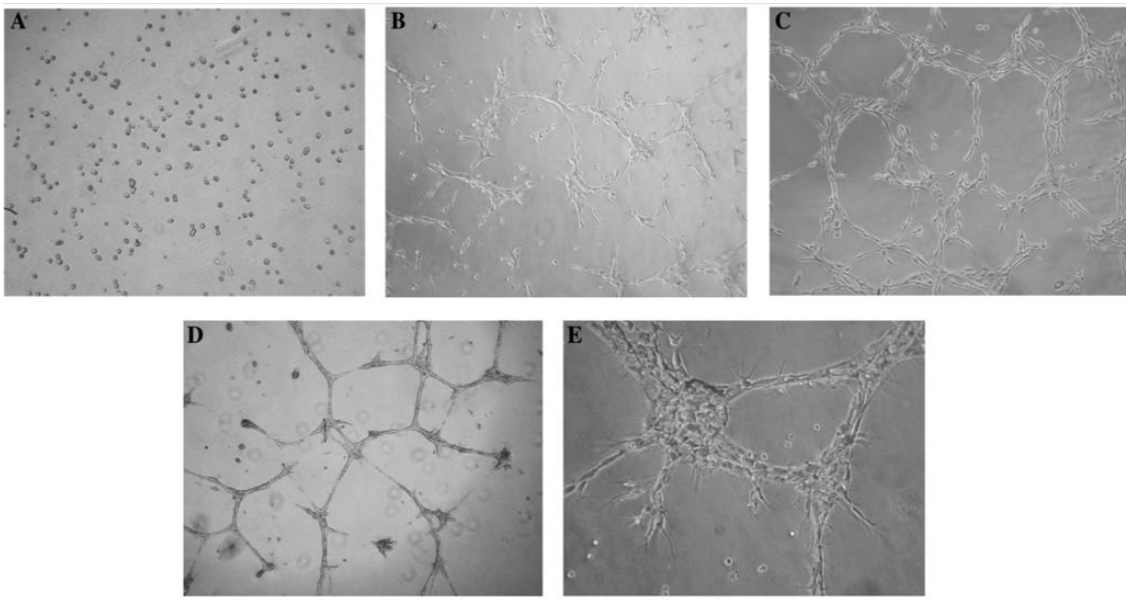


Figure 1.9 - Morphological changes during endothelial differentiation of ASCs. (A) 0 h; (B) 12 h; (C) 24 h; (D,E) 48 h (*Cao et al., 2005*).

Cao and colleagues exploited a method which allows to isolate and expand an ASC subset Flk-1^{+}CD31^{-}CD34^{-} able to differentiate into ECs. The cells were cultured into functional ECs *in vitro*, with 50 ng/ml VEGF, 10 ng/ml bFGF (basic fibroblast growth factor) on Matrigel coated coverslips. Light microscopy observation over a period of 48 hours showed that vascular network was formed by ASCs after incubation with endothelial differentiation medium (fig. 1.9). PCR and immunocitochemistry confirmed the presence of EC markers including CD31 (PECAM-1), CD34, CD144 (VE-cadherin), and endothelial nitric oxide synthase (eNOS) (*Cao et al., 2005*). Positive results were obtained in *in vivo* mouse limb ischaemia model, where injected human ASCs were detected in the injury site.

1.5.4.2 Secretion of paracrine factors

As MSCs enter and progress toward an end-stage phenotype, the quantity and array of secreted bioactive factors changes as the descendants of MSCs enter new lineage stages. The pattern and quantity of such secreted factors is well known to feedback on the cell itself and govern both its functional status and physiology. Of course functional paracrine and autocrine secretion of factors can have profound impact on local cellular dynamics.

The effects of MSC-secreted bioactive molecules can be either direct or indirect or even both: direct by causing intracellular signaling, or indirect by causing another cell in the vicinity to secrete the functionally active agent. As shown in figure 1.10, MSCs can have two distinct functions: they can provide replacement units for expired cells in mesenchymal tissues, and have trophic effects on cells in their vicinity without generating newly differentiated mesenchymal phenotypes and, thus, also influence the regeneration of cells or tissues by purely a bioactive factor effect.

An important characteristic that makes ASCs a good alternative in cell therapy is the participation in endocrine processes by secreting cytokines and growth factors (*Kilroy et al., 2007*). ASCs secrete high levels of epidermal growth factor (EGF), VEGF, bFGF, keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), HGF, TGF-β, insulin-like growth factor (IGF), and brain-derived neurotrophic factor (BDNF). They also secrete cytokines such as Flt-3 ligand, G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF, IL-6, IL-7, IL-8, IL-11, IL-12, LIF, and TNF-α. This secretion of paracrine factors by the adipose tissue likely contributes to the

elevated levels of these cytokines in cases of obesity. Worthy to note, these angiogenic and anti-apoptotic growth factors are secreted in bioactive levels by ASCs and their secretion increases significantly under hypoxic conditions (*Rehman et al., 2004; Lee et al., 2009*).

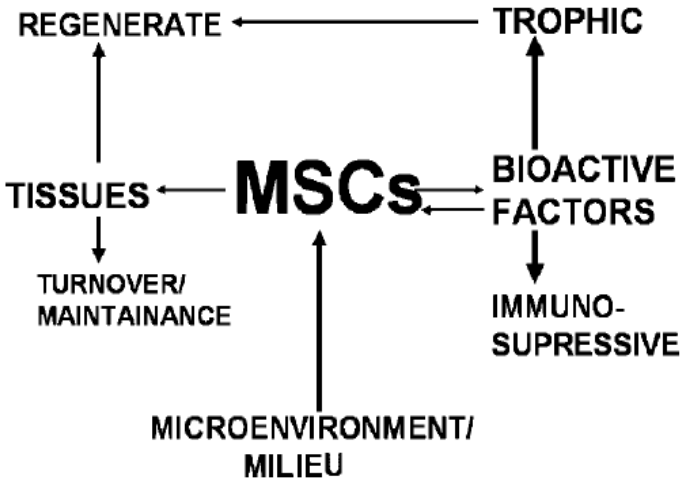


Figure 1.10 - Schema representing the dual role of MSCs: abilities in tissue regeneration and repair, and trophic secretion of bioactive factors that mediate the functional tissue outcomes (*Caplan et al., 2006*).

HGF is possibly the main angiogenic factor secreted by ASCs, playing a central role in the paracrine effects on ASCs. Its suppression has been shown to impair the angiogenic and regenerative effects of ASCs in ischemic tissues. Silencing HGF reduces the ability of ASCs to promote EC proliferation and inhibits the pro-angiogenic effects of HGF *in vitro* (*Cai et al., 2007*).

1.5.5 Stem cells as a tool to address limitations of islet transplants

Recent advances in the field of stem cell research have stimulated significant interest in the potential role that both multipotent (adult), pluripotent (embryonic), and induced pluripotent stem (iPS) cells could play in the replacement of islets in patients with T1DM. The unique properties of different postnatal or adult stem cell populations offer valuable supportive functions that appear to enhance graft function and survival. In addition to a potential role as companion cells during transplantation, pluripotent or embryonic stem cells (ESCs) present a potential alternative for the generation of insulin producing cells (IPCs). The use of adult stem cell populations has also emerged as a

potential source of IPCs, and these cells can be directed down a pancreatic and endocrine lineage of development.

1.5.5.1 Generation of insulin-producing cells

Exploration into the possibility of reprogramming human somatic cells into embryonic-like cells has also resulted in promising outcomes. Although autoimmunity would still present a challenge, this prospect is especially enticing given that patient-specific β-cells could be generated, circumventing the need for immunosuppression to prevent rejection. Social and ethical objections to use of human ESCs would also be avoided. These iPS cells could ideally be directed down a pancreatic endocrine developmental program and then be used to obtain insulin producing cells (IPCs). While this field has not yet advanced as far as the hESC field, several studies utilizing these protocols show the generation of IPCs that express insulin and some other markers of mature β-cells (*Zhang et al., 2009; Tateishi et al., 2008*). Despite some promising results of investigations using hESCs and iPS cells to generate IPCs, several other limitations exist that are unique to these cell populations. In addition to the incomplete differentiation, the recombinant proteins required for the process are extremely expensive. Current research is exploring chemical compounds that could replace these proteins in protocols, while providing more easily regulated and efficient processes of guiding differentiation (*Lin et al., 2009*). Safety concerns have also arisen regarding teratoma formation from undifferentiated cells (*Fujikawa et al., 2005*). Some have explored the possibility of forcing adult stem cells that could otherwise be used as supportive cells in transplantation strategies towards a β-cell lineage *in vitro*. These cells may then be able to directly contribute to islet graft success through differentiation into IPCs (*Dor et al., 2004*).

Conflicting *in vivo* evidence exists for IPC development from MSCs. In mice transplanted with islets and BM-MSCs, an increase in pancreatic and duodenal homeobox gene (PDX-1) positive cells was noted in bone marrow cells. Other *in vivo* studies have had negative results, with no evidence of MSC-derived β-cells observed in murine pancreatic injury or transplantation models, despite improved islet graft function (*Sordi et al., 2010*). These studies reflect that the majority of MSC effect on β-cell regeneration likely occurs through augmentation of endogenous cell survival or regeneration.

Similar to BM-MSCs, ASCs are capable of differentiation into primitive IPCs *in vitro* under certain culture conditions (*Timper et al., 2006*). Analogous to MSCs, much of the evidence regarding ASC effects on islet replacement points to their role as supportive cells.

1.5.5.2 Stem cells as companion cells

1.5.5.2.1 Mesenchymal stem cells

Among the most studied of adult stem cells as companion or supportive cells for islet transplantation are MSCs (fig. 1.11). The multipotent, immunomodulatory, and regenerative properties of these cells have inspired applications in models of tissue injury and immune diseases, ranging from increased neurogenesis in rats to inhibition of pro-inflammatory cytokines in murine acute lung injury models (*Uccelli et al., 2008*).

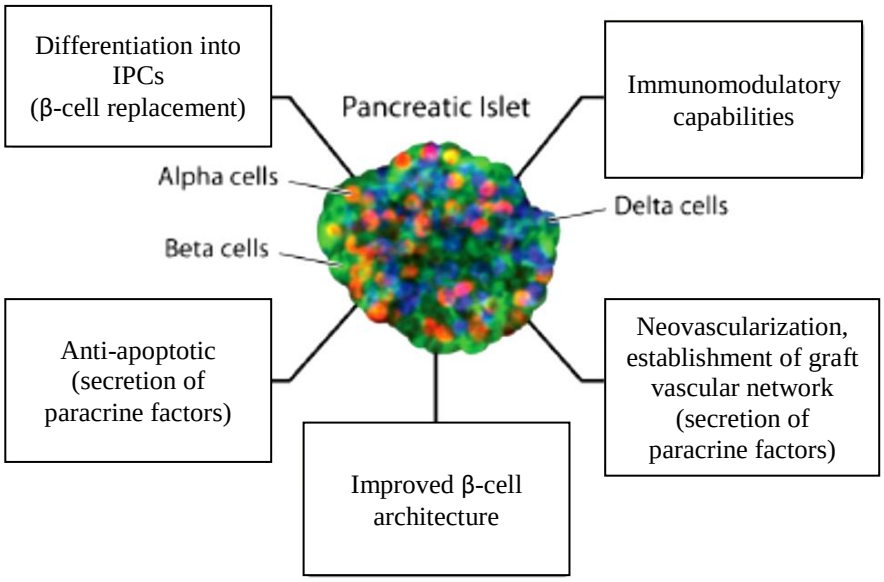


Figure 1.11 - Role of mesenchymal stem cells in islet transplantation (modified from *Harlan et al., 2009*).

In preclinical studies, cotransplantation of islets and MSCs has emerged as a promising tool to improve graft survival. Early studies focused on the effects of BM-MSCs and the benefits of this cell population on transplanted islet function have been demonstrated repeatedly through *in vivo* experiments in rodents and primates. Cotransplantation with syngeneic MSCs results in a lower β-cell requirement for

normoglycemia, with observed improvements in glucose tolerance and prolonged viability of allogeneic islet transplants in mice (*Ito et al., 2010; Figliuzzi et al., 2009*). In diabetic cynomolgus monkeys at 1 month after transplantation, the combination of MSCs with islets confers prolonged graft function with significantly increased C-peptide levels compared to islets transplanted with nonspecific bone marrow cells (*Berman et al., 2010*). Because of their adhesive properties, MSCs have also been shown to coat islets in a coculture system. This characteristic provides a potential model for transplantation that may improve interactions between the cell types after engraftment (*Duprez et al., 2011*).

Numerous studies have been undertaken to investigate the mechanisms behind the beneficial effects of BM-MSCs. One important contribution of MSCs appears to be related to their immunomodulatory capabilities. MSC administration in mice with allogeneic islet grafts was associated with decreased delayed-type hypersensitivity. Additional MSC dosing was associated with reversal of acute rejection of allogeneic transplants in monkeys (*Berman et al., 2010*). These properties are mediated via production of multiple factors that collectively act to suppress T-cell proliferation and function, DC maturation, and NK-cell proliferation. MSC-derived factors act on these immune cells to decrease secretion of pro-inflammatory cytokines including INF-γ, GM-CSF, TNF-α, and monocyte chemoattractant protein-1 (*Ding et al., 2009; Longoni et al., 2010*). MSCs also act to induce regulatory T-cells and the generation of anti-inflammatory cytokines like IL-10, modulate neutrophil function, and B-cell function and differentiation (*Aggarwal et al., 2005*). Collectively, these effects create a shift away from antigen-specific cytotoxicity and inflammation.

Another component to the advantageous effects of MSCs is their contribution to establishing a vascular network for new islet grafts. Compared to islets transplanted alone, multiple studies have demonstrated that mice transplanted with BM-MSCs combined with islets had a significant increase in periislet vessel number, with a higher capillary to β-cell ratio observed post-operatively. In cotransplantation models, the development of new vessels in hybrid grafts was observed earlier (*Sakata et al., 2010*). This earlier and more pronounced increase in capillary density seems to occur secondary to secretion of multiple pro-angiogenic factors, including VEGF, IL-6, IL-8, HGF, TGF-β, and PDGF (*Golocheikine et al., 2010*). MSC secretion of matrix metalloproteinases is also thought to initiate degradation of the preexisting extracellular matrix (ECM), allowing endothelial cells to migrate into islets.

In addition to pro-angiogenic effects, MSCs also have potent anti-apoptotic effects that protect islets from the hypoxia and inflammatory destruction which occurs as a result of the isolation and transplantation process. In an *in vitro* model of islet hypoxia and reoxygenation, rat islets cocultured with BM-MSCs had increased expression of protective hypoxia-induced genes, along with decreased apoptotic rates, and improved glucose-stimulated insulin secretion when compared to islets cultured alone (*Lu et al., 2010*). Cocultured islets also had an increased ATP/ADP ratio leading to improved glucose-stimulated insulin release (*Park et al., 2010; Xu et al., 2008*). *In vivo* benefits of these cells on early islet death from the isolation process are demonstrated by improved blood glucose values in diabetic mice receiving a marginal mass of human islets that were cultured in MSC media for 48 hours before transplantation. This improvement was noted when compared to results obtained from transplantation of islets that had undergone more typical isolation procedures (*Park et al., 2010*).

Many of the effects of MSCs appear to be mediated via secretion of paracrine factors, including HGF, TGF-β, IL-6, VEGF, and PDGF. The importance of this influence is supported by a decrease in benefits on islet survival and vessel development observed when human islets are cocultured with bone marrow cells and antibodies that selectively deplete these paracrine factors. Direct cell contact between the MSCs and islets may also play a role as immunomodulatory effects and IL-10 production are decreased *in vitro* when islets are separated from MSCs by a permeable membrane.

Concurrent transplantation of islets with MSCs also has advantageous effects on islet remodeling and structure that may lead to improved insulin secretion as well as improved intraislet paracrine communication between β-cells and other islet endocrine cells (*Kelly et al., 2011*). Immunostaining reveals in mice that islets transplanted with MSCs develop graft morphology characteristic of native islet architecture, versus a more diffuse distribution of α-cells and δ-cells in grafts containing only islets (*Rackham et al., 2011*).

1.5.5.2.2 Adipose-derived stem cells

While ASCs are functionally similar to BM-MSCs, they are more easily accessible with minimal risk to the patient. Adipose also yields a greater number of stem cells per gram of tissue than bone marrow (*Nakao et al., 2010*). This accessibility is especially attractive as patients could easily provide their own populations of cells.

ASCs exhibit a number of potential characteristics and effects that are similar to MSCs and benefit islet grafts comparably. Combined transplantation of ASCs with a marginal islet mass resulted in prolonged graft survival and glucose tolerance similar to that observed when using significantly higher numbers of islets. Hybrid grafts had a well-preserved islet structure compared to those transplanted with islets alone. These hybrid islets also had decreased presence of $CD4^+$ and $CD8^+$ cells, reflecting an anti-inflammatory effect (*Ohmura et al., 2010*).

While research into the application of ASCs to diabetic models is ongoing, ASCs have been studied in several other injury and disease models. Studies in mice with proximal femoral artery ligation and subsequent hind limb ischemia have demonstrated the pro-angiogenic influence of ASC administration (*Mi et al., 2006*). *In vitro* studies suggest the etiology is a combination of differentiation and direct incorporation of ASCs into vascular structures combined with secretion of angiogenic and anti-apoptotic growth factors (*Traktuev et al., 2008*). These specifically include VEGF, HGF, bFGF, GM-CSF, and TGF-β (*Rehman et al., 2004*). ASC-hybrid grafts demonstrate an increased presence of ECs, which appear to be differentiated from ASCs.

1.5.5.2.3 Endothelial progenitor cells

Possible benefits on vasculogenesis have generated interest in endothelial progenitor cells, which promote angiogenesis at sites of hypoxia or injury and can be obtained from bone marrow, cord blood, vessel walls, or peripheral blood. The use of EPCs in ischemic injury models has previously been undertaken (*Botta et al., 2004*).

Concerning the use of EPCs for therapeutic neovascularization, we have to consider that EPCs in T1DM patients are dysfunctional, and their dysfunction may contribute to the pathogenesis of vascular complications (*Loomans et al., 2004*).

Generation of a more stable vascular network has been achieved by cotransplantation of endothelial cells with ASCs. In this context, ASCs are able to function similarly to pericytes, which are cells that line vessel walls and support vasculature. This role is supported by frequent ASC expression of pericyte surface markers and the periendothelial location of ASCs in adipose tissue *in vivo*. Through paracrine interaction, ECs promote mitosis and chemoattraction of ASCs, while ASCs promote EC survival and migration (*Traktuev et al., 2008*).

1.5.5.3 Human clinical trials

Although no clinical human trials have been published that employ stem cells in islet transplantation strategies, they are beginning to be employed in other ways.

Recently, patients with T1DM whose serum lymphocytes were "educated" by multipotent human cord blood stem cells demonstrated a progressive improvement in fasting and stimulated C-peptide levels up to 40 weeks after treatment. "Education" was performed by removing the cells from peripheral blood and returning them to the circulation after stem cell exposure. Patients receiving this novel treatment exhibited a significant increase in regulatory T-cells and TGF-β1, reflecting immune modulation as an explanation for the improved β-cell function (*Zhao et al., 2012*). Even with clear differences in the treatment approach, these results provide promise for a future role of stem cells in islet transplants for T1DM in humans.

Despite the lack of published human clinical trials, a search of registered clinical trials (http://clinicaltrials.gov/) revealed about fifteen active studies involving stem cell treatments for T1DM. Thirteen studies involved infusion of stem cells (mostly autologous MSCs), while one study used a stem cell "educator" as outlined above. Only one study planned to evaluate cotransplantation of islets with MSCs. The trials listed appear to be of varying quality, and many factors regarding the administration of these cells will need to be carefully and rigorously studied.

1.6 Molecules with differentiating logic

In eukaryotes, many fundamental biological phenomena, such as transcription, replication and DNA repair, involve the interaction of factors with DNA sequences. Most of these interactions occur in the context of chromatin environment. Transcription factors bind DNA and recruit cofactors that introduce covalent changes in chromatin, including DNA methylation and histone modifications (acetylation, methylation, phosphorylation). These so-called epigenetic changes have profound effects on gene expression, both in physiological and pathological processes (e.g. differentiation and cancer).

The possibility of chemically modifying a gene program, without the aid of gene transfer technology, has become more and more interesting as well as real. Targeting stem cell fate at the level of gene expression could represent a potentially powerful

therapeutic approach to afford a high-throughput of vascular lineage commitment and paracrine secretion of trophic factors.

The current logics in chemical manipulation of stem cell fate for tissue repair are to apply single powerful natural or synthetic factors to trigger and/or enhance the onset of phenotypic processes. However, stem cell fate is controlled by a complex interplay between cell signaling, the establishment of multifaceted transcriptional motifs, the temporal and spatial organization of chromatin in loops and domains, and the ability to "perceive" the fine architecture and the composition of the extracellular matrix (*Ventura et al., 2008*).

Based on the above, it's interesting to design strategies which utilize natural occurring molecules (alone or in combinatorial treatment) to induce specific commitments and the secretion of trophic mediators of tissue repair.

1.6.1 Hyaluronic acid (HA)

Hyaluronic acid (or Hyaluronan, HA), an important constituent of the ECM, is a glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine (fig. 1.12). In the cell, hyaluronan synthase enzymes (Has1, Has2 and Has3), localized in the plasmatic membrane, synthesize this linear, high molecular weight polymer in which the number of repeating disaccharide units can reach 10,000 or more and a molecular mass of about 4 million of Daltons (each disaccharide unit is about 400 Da).

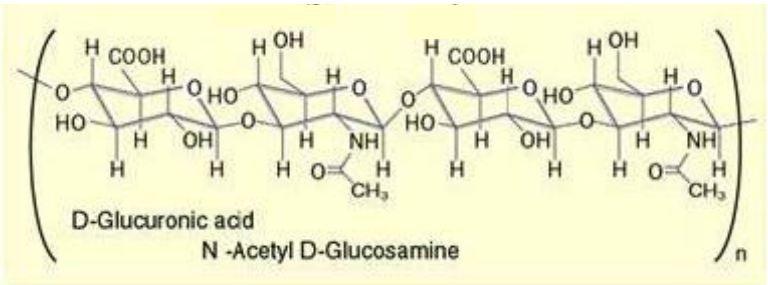


Figure 1.12 - Hyaluronan basic structure: the repeating disaccharide unit.

This polysaccharide, ubiquitously distributed in the ECM of almost all animal tissues, regulates cellular events such as cell proliferation and locomotion that are required for a variety of biological processes.

HA has an extraordinarily high rate of turnover, and at the cellular level it is considered to be degraded progressively by a series of enzymatic reactions mediated by hyaladherins that generate polymers of decreasing sizes.

Hyaluronic acid functions differently depending on its molecular size and concentration. High molecular weight HA or native HA (about 10^7 Da) plays a structural role and inhibits inflammation, immune response and angiogenesis (inhibits EC proliferation and disrupts confluent endothelial monolayers). Consistent with these findings are the observations in chick embryo limb buds that avascular regions are rich in high molecular weight HA, and that expression of this form of hyaluronic acid in normally vascular areas results in decreased vascularity. On the other hand, low molecular weight HA (HA fragments in the 6-20 kDa size range) stimulates inflammation and immune response, and promote angiogenesis *in vivo*. HA fragments have also been demonstrated to induce proliferation, migration and capillary tube formation in cultured ECs *in vitro* (*Wang et al., 2011*).

HA appears to exert its biological effects through binding interactions with specific cell-associated receptors. A number of hyaluronan-binding proteins have been identified, and two molecularly distinct cell-surface receptors for hyaluronan have been characterized, namely CD44 and RHAMM (receptor for HA-mediated motility). Although several other binding interactions for CD44 and RHAMM have been reported, currently the interaction with HA appears to be the one most likely to directly activate intracellular signals required to stimulate processes relevant to angiogenesis (*Savani et al., 2001*). Indeed intracellular hyaluronan-binding molecules (hyaladherins) translocate to the nucleus, serving as substrates or activators for crucial signaling kinases or acting as vertebrate homologs of proteins involved in cell growth and differentiation, as well as in modulation of ECM and rearrangement of the cytoskeleton (*Takahashi et al., 2005*).

In particular, CD44 is a type 1 transmembrane glycoprotein that is expressed by most cell types, including ECs, and is considered the major receptor for HA. It’s expressed also on bone marrow-derived cells such as recruited leukocytes, tissue macrophages, and circulating EPCs that participate in angiogenesis (*Cao et al., 2006*). It is worthy of note that CD44 is highly expressed by human MSCs.

1.6.2 Butyric acid (BU)

Butyric acid (sodium salt), a volatile short-chain fatty acid (fig. 1.13), is the most common and physiologic member of histone deacetylase (HDAC) inhibitors because it is formed in the colon by fermentation of dietary fibers. This small molecular weight molecule belongs to class I HDAC inhibitors, and exerts its action by altering chromatin structure and consequently increasing transcription factor accessibility to target *cis*-acting regulatory sites.

In detail, butyric acid inhibits the function of histone deacetylase enzymes, thereby favoring an acetylated state of histones in the cell. Acetylated histones have a lower affinity for DNA compared with non-acetylated histones, due to the neutralization of electrostatic charge interactions. In general, it is thought that transcription factors will be unable to access regions where histones are tightly associated with DNA (i.e. non-acetylated, e.g. heterochromatin). Therefore, butyric acid is thought to enhance the transcriptional activity at promoters, which are typically silenced or down-regulated due to HDAC activity.

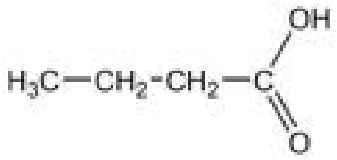


Figure 1.13 - Butyric acid structure.

Anyway, given the known function of histone acetylation in transcription, it seems logical to postulate that inhibition of HDACs alone is unlikely to lead to a generalized increase in the transcription of all known genes. In fact, acetylation works together with other post-translational modifications, and blocking deacetylation might have very different outcomes depending on the previous chromatin state. Up to 20% of all known genes are affected by HDAC inhibitors (*Van Lint et al., 1996*). Not all of these genes are necessarily up-regulated by treatment (the ratio of up-regulated to down-regulated genes is close to 1:1).

1.6.3 Retinoic acid (RA)

Retinoic acid (RA) is a natural occurring retinoid derivative of Vitamin A (retinol) that exerts a wide variety of profound effects on vertebrate development (fig. 1.14).

Vitamin A is an essential nutrient, required for crucial biological functions in quantities that far exceed what can be metabolically generated. Thus, survival of mammalian and avian species is dependent upon acquisition of adequate dietary vitamin A, which must be enzymatically converted to active RA. The terminal step in RA synthesis is carried out by members of the class I aldehyde dehydrogenase (ALDH) family. Embryos lacking this enzyme are RA deficient and die in utero at E10.5.

The RA signal is transduced through two families of ligand dependent transcriptional regulators, RA receptors (RARs) and retinoid X receptors (RXR), which bind as heterodimers to DNA motifs (RA response elements, RAREs), thus regulating the transcriptional activity of target genes controlling differentiation of a variety of cell types (*Lai et al., 2003*).

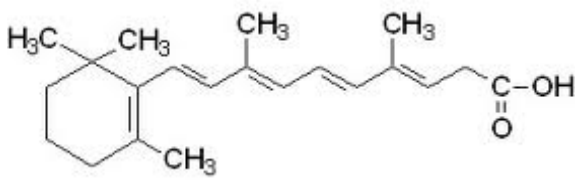


Figure 1.14 - Structure of all-trans retinoic acid (atRA), one of the more active forms of RA.

Lack of retinaldehyde dehydrogenase 2 (*Raldh2*) expression in the yolk sac is correlated with disrupted formation of extra-embryonic vessels in *Raldh2*$^{-/-}$ mutants. However, *Raldh2*$^{-/-}$ embryos also exhibit defects in heart looping morphogenesis, and severely hypoplastic atria and sinus venosus development. Vascular defects can be the indirect result of cardiac malformations. Anyway, histological examination revealed vascular malformations in RA-deficient yolk sacs and embryos prior to the onset of cardiac function and blood circulation. Cellular and molecular analyses indicated that RA was not required for EC differentiation, but was required for EC maturation and the control of cell cycle progression. Continuous RA supplementation of maternal diet restored cell cycle control and rescued the observed vascular defects in *Raldh2*$^{-/-}$ embryos and yolk sacs.

Hence, RA plays a crucial role in mammalian vascular development, and is required throughout development to control EC growth and vascular remodeling.

Therapeutic applications of RA and its derivatives against various malignancies have been increasing. Among other things, RA has been reported to be beneficial for

atherosclerotic vascular disorders by inducing differentiation and inhibiting proliferation in vascular smooth muscle cells. Moreover, Uruno and colleagues have recently shown that RA increases nitric oxide production by eNOS phosphorylation through RAR-mediated PI3-kinase/Akt pathway activation in vascular ECs. Because NO is a potent vasodilator and signal modulator molecule and plays important roles in controlling vascular function, RA may be a candidate for novel therapeutic agents against vascular disorders with endothelial damage (*Saito et al., 2007*).

EXPERIMENTAL DESIGN

2 - AIM OF THE THESIS

Mesenchymal stem cells have already made their mark in the young field of regenerative medicine. Easily derived from many adult tissues, their therapeutic worth has already been validated for a number of conditions. Unlike embryonic stem cells, neither their procurement nor their use is deemed controversial (*Domìnguez-Bendala et al., 2012*). Here we suggest the potential use of MSCs for the treatment of type 1 diabetes mellitus, a devastating chronic disease in which the insulin-producing β-cells of the pancreas are the target of an autoimmune process.

Islet transplantation is a form of cell therapy that has already proven effective at clinically restoring normoglycemia. The publication of the promising results of the Edmonton protocol in 2000 generated optimism for islet transplantation as a potential cure for T1DM. Unfortunately, follow-up data revealed that less than 10% of patients achieved long-term insulin independence. More recent data from other large trials show incremental improvement with 44% of islet transplant recipients maintaining insulin independence at three years of follow-up. Multiple underlying issues have been identified that contribute to islet graft failure, first of all the instant blood-mediated inflammatory reaction. Even hypoxia plays an important role in limiting the engraftment, survival and function of transplanted islets. MSCs were recently used in animal models of islet transplantation not only to reduce allograft rejection by immune modulation mechanisms (*Berman et al., 2010*), but also to promote islet revascularization (*Ito et al., 2010*).

Beside bone marrow and human cord blood, adipose tissue represents a novel and high-throughput source for human MSCs (*Zuk et al., 2002*). Moreover, the capability of adipose-derived stem cells to improve islet graft revascularization was recently reported after hybrid transplantation in mice (*Ohmura et al., 2010*). Within this context, we have previously shown that hyaluronan esters of butyric and retinoic acids can significantly enhance the rescuing potential of human MSCs in both heart and kidney rat models of tissue damage (*La Manna et al. 2010, Ventura et al. 2007*), and that these beneficial effects were mediated by the transcriptional action of hyaluronan grafted moieties intracellularly released by the mixed ester. Based on these underpinnings, the aim of the

current thesis was to define a sort of chemical preconditioning able to increase the angiogenic potential of human ASCs *in vitro*. In particular we tried to find the best combination of naturally occurring molecules capable to enhance and optimize both vascular and perivascular cell commitment as well as paracrine secretion of angiogenic and anti-apoptotic factors.

Then we investigated whether *ex vivo* preconditioning of human ASCs with a mixture of hyaluronic, butyric, and retinoic acids may result in optimization of graft revascularization and function after islet/stem cell intrahepatic cotransplantation in syngeneic diabetic rats.

3 - MATERIALS AND METHODS

3.1 Adipose-derived stem cell isolation, culture and characterization

3.1.1 Cell isolation

According to the policy approved by the local ethical committee, all tissue samples were obtained after informed consent from healthy donors. Human subcutaneous adipose tissue samples were obtained from lipoaspiration/liposuction procedures from several regions of the body including hip, thigh and abdominal regions, and collected into a sterile container. After washing, lipoaspirates were digested with 0.2% collagenase A type I solution (Sigma-Aldrich), under gentle agitation for 45 min at 37°C, and centrifuged at 650 xg for 10 min to separate the SVF from adipocytes. If necessary, the MSC fraction was treated with red blood cell lysis buffer for 5 min, and then centrifuged again. The supernatant was discarded and the cell pellet was resuspended and seeded in culture flasks in Dulbecco's Modified Eagle Medium (DMEM)-low glucose (Lonza) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, 2mM L-glutamine, and incubated at 37°C in a humidified atmosphere with 5% CO_2. When the cultures were near confluence, the cells were detached by treatment with trypsin-EDTA solution (Sigma-Aldrich), characterized, subcultured and used at passage 3-5 (fig. 3.1).

3.1.2 ASC expansion in culture

The cultures at passage 0 were checked for several days under an optical microscope, the medium was changed twice a week, and when the flasks reached 90% confluence, the cells were collected and expanded.

Cells were detached with trypsin-EDTA solution and replated at the desired density in culture medium supplemented with 20% (then 10%) FBS, subcultured for expansion and *in vitro* analysis (including characterization). Human ASCs maintain their characteristics at least until passage 8; for experiments cells from 3-6 passage were used.

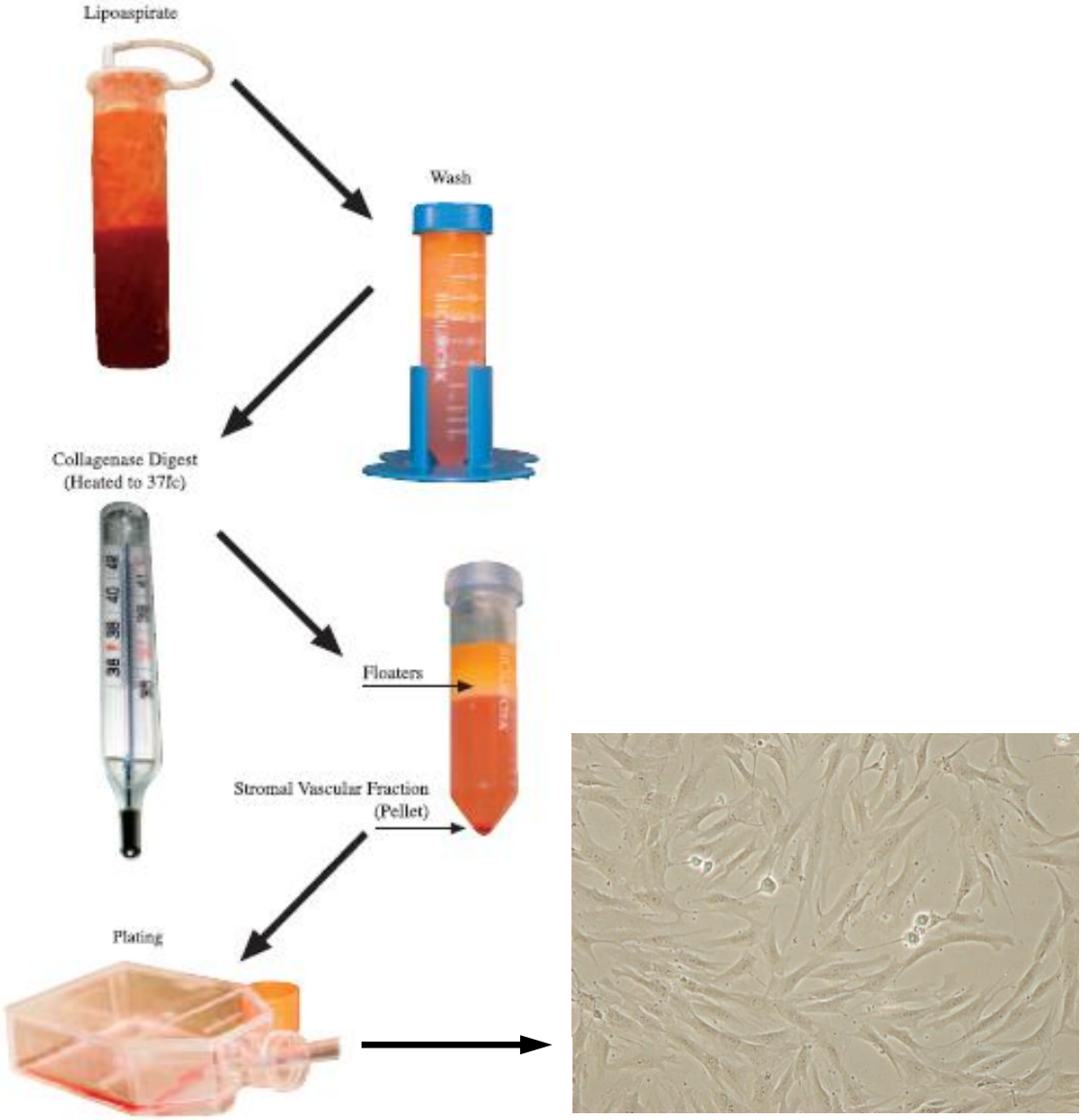


Figure 3.1 - Processing of lipoaspirate and isolation of adipose-derived stem cells (*right image*: hASCs in culture, 100x original magnification) (modified from *Gimble et al., 2007*).

3.2 Characterization

3.2.1 Differentiation assays

To evaluate the cell plasticity we tested the ability to differentiate into multiple tissue type lineages, by using commercial available kits. In particular cells derived from lipoaspirate should be able to undergo adipogenic, osteogenic, and chondrogenic differentiation (the classical 3 mesenchymal lineages).

3.2.1.1 Adipogenic differentiation

ASCs were seeded at a density of 2 x 10^4 cells/cm^2 in a 24-well microplate, cultured until 100% confluence, and treated with DMEM-low glucose supplemented with 10% FBS, 0.1 µM dexamethasone, 0.5 mM isobutyl-methyl xanthine (IBMX), 100 µM indomethacin, 10 µg/ml insulin, 1% penicillin-streptomycin (Mesenchymal Stem Cell Adipogenesis Kit, Chemicon International). The induction medium was replaced every 2-3 days, interchanging with cycles of adipogenesis maintenance medium containing DMEM-low glucose supplemented with 10% FBS, 10 µg/ml insulin, 1% penicillin-streptomycin.

Control cells were cultured in basal medium (DMEM/10% FBS) and finally assayed in the same manner.

After 2-3 weeks of culture, induced cells, observed with optical microscope, should show storages of neutral lipids in cytoplasmic vacuoles; the cells were fixed with 10% formalin and stained with Oil Red-O that specifically highlights lipids in the mature adipocytes.

3.2.1.2 Osteogenic differentiation

ASCs were seeded at a density of 2 x 10^4 cells/cm^2 in a 24-well microplate, cultured until 100% confluence, and treated with DMEM-low glucose supplemented with 10% FBS, 10 mM glycerol-2-phosphate, 0.2 mM ascorbic acid 2-phosphate, 0.1 µM dexamethasone, 1% L-glutamine, 1% penicillin-streptomycin (Mesenchymal Stem Cell Osteogenesis Kit, Chemicon International). Control cells were cultured in basal medium (DMEM/10% FBS).

The day before seeding the cells, the wells were coated with 0.5 ml of a vitronectin/collagen mixture prepared in PBS at a final concentration of 12 µg/ml for each ECM molecule. This solution was removed and washed just before plating.

Cells were cultured for 3-4 weeks and the medium was changed every 2-3 days. To assess the occurred osteogenic differentiation, cells are fixed with 10% formaldehyde, and coloured with Alizarin Red Solution. Samples were observed under an inverted optical microscope (Nikon ECLIPSE TS100), equipped with a digital sight camera and the imaging software NIS-Elements (Nikon).

3.2.1.3 Chondrogenic differentiation

Aliquots of 2.5 × 10^5 ASCs were pelleted in polypropylene conical tubes, by centrifugation at 150 xg for 5 min. To induce chondrogenic differentiation, cell pellets were fed every 2-3 days by completely replacing the medium with 0.5 ml of freshly prepared complete chondrogenic medium: Chondrogenic Basal Medium supplemented with Supplements and Growth Factors (ITS + supplement, dexamethasone, ascorbate, sodium pyruvate, proline, penicillin-streptomycin, L-glutamine), and 10 ng/ml Transforming Growth Factor-β3 (all the reagents were purchased from Lonza).

Chondrogenic pellets were harvested after 21 to 28 days in culture, formalin-fixed, then embedded in paraffin for histological analysis or to be freezed for the sectioning. The thin slices were mounted on slides, examined morphologically and immunostained for the research of GAG with Alcian blue or type II collagen.

3.3 Preconditioning of hASCs

The use of a cell preconditioning strategy has the aim to evaluate how natural molecules like HA, BU, and RA can affect the cellular pattern, and address a possible vascular/perivascular commitment.

Human ASCs were seeded at 8 x 10^3 cells/cm^2 in DMEM/20% FBS, and after 24 hours the medium was changed to DMEM/10% FBS containing low molecular weight HA (2 mg/ml), BU (5 mM), and RA (1 μM). Chemicals were purchased from Sigma-Aldrich, except for 16.9 kDa hyaluronic acid which comes from Lifecore Biomedicals. Control cells were cultured in DMEM/10% FBS.

3.4 Gene expression

Total RNA from control or preconditioned hASCs was extracted at 1-3-6 days using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and quantified using Nanodrop. One μg of RNA from each sample was reverse-transcribed into cDNA in a 20-μl reaction volume with SuperScriptTM III reverse transcriptase (Invitrogen). To assess gene expression, 2 μl of cDNA were used for real-time PCR performed with a Lightcycler system (Roche Applied Science) and with the SYBR Green I FastStart kit (Lightcycler® FastStart DNA MasterPLUS SYBR Green I), following the manufacturer's instructions.

Primers for real-time PCR were designed by using Primer3 program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), checked by BLAST (http://www.ncbi.nlm.nih.gov/BLAST), and ordered from Sigma-Aldrich. Primer sequences are shown in table 3.1; each forward/reverse primer was used at a final concentration of 0.25 μM. Amplification program: denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 8 s, annealing at 55-65°C (depending on the melting temperature of each couple of primers) for 8 s, extension at 72°C for 5 s. Fluorescence is measured at the end of every cycle. The melting curve program is performed starting at 55°C and ending at 95°C, with increments of 0.1°C/second and a continuous fluorescence measurement. The final step is cooling to 4°C.

Table 3.1 - PCR primer sequences (5'-3').

GENES	**Forward (5'-3')**	**Reverse (5'-3')**
ACTB	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA
HPRT-1	CATTATGCTGAGGATTTGGAAAGG	CTTGAGCACACAGAGGGCTACA
VEGF	AGAAGGAGGAGGGCAGAATC	ACACAGGATGGCTTGAAGATG
KDR	CTGCAAATTTGGAAACCTGTC	GAGCTCTGGCTACTGGTGATG
HGF	ATTTGGCCATGAATTTGACCT	ACTCCAGGGCTGACATTTGAT
ISL1	GAGGGTTTCTCCGGATTTGG	TCCCATCCCTAACAAAGCATGT

Data were normalized using three housekeeping genes (beta-actin ACTB, glyceraldehyde 3-phosphate dehydrogenase GAPDH, hypoxanthine phosphoribosyltransferase 1 HPRT1) as an index of cDNA content after reverse transcription. Relative quantification of mRNA expression was calculated with the comparative Ct method using the "delta-delta method" for comparing relative expression results between treatments in real-time PCR (*Pfaffl, 2001*).

3.5 Secretome analysis

To investigate whether the mixture of HA, BU, and RA may affect the production of angiogenic and anti-apoptotic factors, we performed some enzyme immunoassays (Human HGF ELISA Kit and Human VEGF ELISA Kit, from Boster Biosciences Co., LTD).

Samples were prepared starting from cells, cultured in absence (control) or presence (preconditioned) of HA, BU, and RA in DMEM supplemented with a reduced concentration of FBS (2%) to limit the possible interference of factors normally contained in serum.

At defined time points (1-2-3-6 days) cell media were collected, and centrifuged to remove particulates/debris. Cell supernatants were dispensed into ELISA plate wells, and assayed immediately according to the manufacturer's instructions or aliquoted and stored at -20°C.

Plates were read on an Opsys MR Microplate Reader (Dynex Technologies) at an optical density (O.D.) of 450 nm. For each well the relative O.D. was calculated according to the formula: (relative O.D.) = ($O.D._{450}$) - ($O.D._{450}$ of Zero well). The standard curve was plotted as the relative $O.D._{450}$ of each standard solution (Y) vs. the respective concentration of the standard solution (X). The unknown cytokine concentrations in the samples (pg/ml) were interpolated from the standard curve. For each sample, cell proteins were extracted by trichloroacetic acid (TCA) precipitation/NaOH lysis, and the total protein amount was quantified by Bradford assay, and used for normalization. Data are expressed as pg of secreted factor per μg of protein at the time of harvest.

3.6 Flow cytometry analysis: hASC characterization and expression of perivascular/vascular markers

For characterizing hASCs as mesenchymal stem cells, as well as assessing if treatment with HA, BU, and RA could change protein expression, we harvested cells by trypsinization, washed with phosphate buffered saline (PBS), and centrifuged at 650 xg for 5 min at room temperature. After removing the supernatant, the cell pellet was resuspended with a solution of 1% BSA (Bovine Serum Albumin) in PBS, and divided into aliquots (each of at least 1 x 10^5 cells). One aliquot was used as negative to acquire cell parameters, the others were marked adding fluorescent specific anti-human antibodies (1 μg/10^6 cells) for 40 min at 4°C in the dark.

In case of NG2, two different incubations were necessary: the first one with the primary antibody (mouse anti-human NG2, Millipore) and, after a washing step, the second one in presence of the secondary antibody (goat anti-mouse IgG Alexa Fluor 555, Invitrogen).

Finally cells were washed with BSA/PBS and centrifuged. The pellet was resuspended with 300 µl of BSA/PBS, and cells analyzed on a flow cytometer (FACSAria, BD Biosciences) by collecting 10,000 events. Data were processed using the FACSDiva Software.

To characterize ASCs we used a MSC specific panel of antibodies conjugated with different fluorophores including anti-CD90-R-PE and anti-CD105-FITC (Biolegend), anti-CD14-APC, anti-CD29-PE-Cy5, anti-CD34-FITC, anti-CD44-FITC, anti-CD45-PerCP, anti-CD73-PE, anti-CD166-R-PE (BD Biosciences). Other investigated markers were CD146-Alexa647, PDGF-Rβ-PE, KDR-PerCP/Cy5.5 (Biolegend).

Flow cytometry analysis was also exploited to assess the percentage of cells expressing alpha smooth muscle actin (α-SMA), an intracellular antigen requiring a different preparation protocol. After 10-min fixation with 0.25% formaldehyde on ice, followed by 15-min permeabilization with 1% Tween20, ASCs were incubated with an anti-human antibody directed against α-SMA (FITC-conjugated, AbCam) for 30 min at 4°C in the dark.

3.7 Immunofluorescent analysis

Immunofluorescence allows the identification of cell or tissue antigens through the employment of specific antibodies conjugated with fluorescent dyes (fluorophores). The samples are observed with a fluorescence microscope that excites the fluorophore, detecting its signal and showing the potential presence and localization/distribution within the cell. By means of this technique, we researched the presence of some vascular protein, as well as the expression of endothelial markers (CD31 and von Willebrand Factor, vWF) in cells cultured up to 14 days in absence (control) or presence of HA, BU, and RA (preconditioned). After 14 days cells, which had been grown in 24-well microplate on sterile glass coverslips, were fixed with ice cold methanol for 10 min on ice. Only for intracellular antigen detection, cells were also permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Non-specific antibody binding sites were blocked by incubating the cells with 4% BSA/PBS for at least 30 min at room temperature (or overnight at 4°C). The blocking solution was then carefully removed and the cells were incubated for 45 min at 37°C with the primary antibodies (or conjugated antibodies). Following several washing in PBS/Tween 20 (0.25 %), the samples were incubated with the secondary antibody (where needed) for 45 min at 37°C

in the dark. After this incubation time, the cells were extensively washed, then the nuclei were counterstained with DAPI solution (0.1 μg/ml final concentration, Invitrogen). The samples were mounted with antifade reagent (ProLong® Gold, Invitrogen). Negative controls were done by omitting the primary antibodies. Samples were observed under a fluorescence microscope (Nikon), and images acquired and merged with a digital camera through the imaging software NIS-Elements.

Used antibodies:

- Mouse anti-human PECAM-1 (CD31) (1:50 dilution, Santa Cruz);
- Monoclonal mouse anti-human von Willebrand Factor (vWF, 1:30 dilution, DakoCytomation);
- Mouse anti-human chondroitin sulfate proteoglycan (NG2, 1:100 dilution, Millipore);
- Goat anti-mouse IgG Alexa Fluor 555 (1:1000 dilution, Invitrogen);
- Mouse monoclonal anti-alpha smooth muscle Actin (FITC) (α-SMA, 1:100 dilution, AbCam).

3.8 Vasculogenesis (*in vitro* study of angiogenic potential)

ASCs express markers and functional properties of pericytes *in vitro* and, in combination with endothelial cells, are able to establish multilayer functional vessels *in vivo* (*Traktuev et al., 2009*). However, the factors that coordinate EC-ASC communications to promote migration of these cells toward one another, and their heterotypic assembly into vascular structures are not well defined. To understand the mechanisms of EC-ASC interaction, we drew inspiration from the *in vitro* model published by Merfeld-Clauss and colleagues, which consists in coculturing ECs with ASCs, without additional exogenous cytokines or extracellular matrix proteins.

The culture medium we used was a 1:1 mixture of Endothelial Basal Medium EBM-2/DMEM-low glucose containing 5% FBS. At first ASCs were seeded in a 24-well microplate on sterile glass cover slips with a density of 4 x 10^4 cells/cm^2; after 4 hours in the same wells HUVECs (Human Umbilical Vein Endothelial Cells) were seeded (1 x 10^4 cells/cm^2). For negative control with the same procedure we plated Human Foreskin Fibroblasts (HFFs) in place of hASCs, and HUVECs after 4 hours.

In the aftermath we observed the wells with an optical microscope, and fixed cells for fluorescence immunostaining between 2-6 days from coculture beginning.

Following the protocol described in the previous paragraph, we stained cocultures with endothelial markers (CD31 and vWF), to analyze how HUVECs reorganized themselves to eventually form cord structures, and with α-SMA antibody to indagate how its expression in ASCs eventually changed.

3.9 *In vivo* study

3.9.1 Experimental animals

Male Lewis rats were purchased from Charles River Laboratories, Inc. Rats weighing approximately 200 g were used as recipients, and those weighing 300-350 g were used as donors. The use of animals and the animal procedures of this study were approved by the Bioethics Committee of the University of Bologna.

3.9.2 Isolation and culture of rat pancreatic islets

Rats were anesthetized with Isoflurane (Abbott Laboratories), and subsequently sacrificed by neck dislocation. Islet isolation and purification were performed according to a modified procedure described earlier (*Gotoh et al., 1985*). Briefly, after cannulation of the common bile duct, 10 ml of enzyme solution of collagenase NB8 (SERVA Electrophoresis), at a concentration of 1.3 mg/ml in Hank's Balanced Salt Solution (HBSS), were injected by retrograde perfusion of the pancreas. After organ procurement, enzymatic digestion took place for 12-15 min at 37°C in a water bath with two intermitted 1-min periods of vigorous shaking. Following a wash step with 10% FBS/HBSS and filtration through cheesecloth, islets were obtained by centrifugation in a discontinuous density gradient of Histopaque 1077 (Sigma-Aldrich) and HBSS, with subsequent hand-picking and microscope examination. The total number of islets in each diameter class was counted using an optical graticule. The number was then converted to the standard number of islet equivalents. Islet viability was assessed using fluorescent staining with acridine orange and propidium iodide (Sigma-Aldrich). Islets with viability >90% were used for transplantation purposes.

Immediately after counting, isolated rat islets (fig. 3.2-A) were incubated with hASCs (fig. 3.2-B) that had been previously preconditioned for 5 days (as already described above). To facilitate the adhesion of hASCs to the islet surface, we used the procedure described by Johansson and colleagues in 2008. In brief, the total volume of

isolated islets was mixed with control or preconditioned hASCs at a cell/islet ratio of 5,000/100 in islet medium RPMI-1640 (Lonza), supplemented with 10% FBS, 2 mM L-glutamine, 1% antibiotic-antimycotic solution. The cell suspensions were incubated at 37°C for 2 hours in culture tubes, and mixed gently twice per hour. Thereafter, the mixture of islets and hASCs was seeded in ultra-low attachment plates (Corning), and cultured for up to 3 days in a humidified incubator at 37°C, 5% CO_2 (fig. 3.2-C).

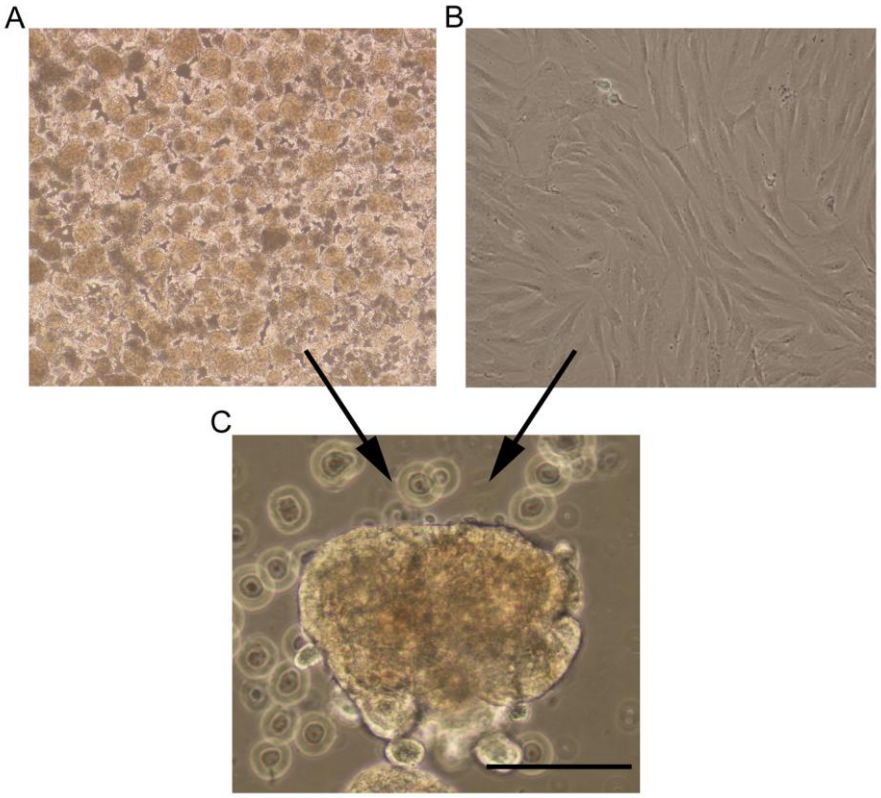


Figure 3.2 - Islets cocultured with hASCs. Isolated rat islets (A, magnification 40x), and hASCs (B, magnification 100x) were cocultured up to 3 days (C). Scale bar = 50 μm.

To detect the capability of hASCs to adhere to the islet surface, in a pilot test we cultured islets with hASCs transfected with green fluorescent protein (GFP). At the end of a 3-day coculture period, the eventual formation of composite hASCs-islets was evaluated by immunofluorescence microscopy.

For transplant purpose, at the end of the coculture period, islets and hASCs were hand-picked, washed 3 times with 10 ml of HBSS containing 2% Lewis rat serum, suspended in transplant medium (HBSS/10% rat serum), and kept on ice into a sterile Eppendorf tube until the time of transplantation.

3.9.3 Cotransplantation of islets and hASCs, and monitoring of islet graft function

Diabetes was induced in rats by a single 70 mg/kg intraperitoneal injection of streptozotocin (Sigma-Aldrich). Only rats with blood glucose levels of more than 400 mg/dl for 3 consecutive days were used as recipients of islet grafts. The animals were anesthetized with xylazine-tiletamine-zolazepam (Zoletil®, Virbac), and placed on a deltaphase heating pad (2Biol). A suboptimal volume of 500 cultured islets (2,500 islet/Kg), that in our experience allowed the reduction of blood glucose levels of transplanted rats without achieving reversal of diabetes, and the cocultured control or preconditioned hASCs were transplanted in diabetic rats weighing approximately 200 g (n=7 per group, control or preconditioned group, respectively). With the aim to favor graft detection after intraportal injection and to permit a comparative analysis between the two study groups, we transplanted islets only in the caudate lobe of the rat liver, as already described (*Kugelmeier et al., 2008*). Briefly, the cavity was accessed by midline incision. The portal branches to the left, middle and right liver lobes were temporarily closed with microvascular clamps, after which islets of both study groups were slowly injected into the portal vein by a 26-gauge needle, in a volume of 300 µl of transplant medium, directing the islets to the caudate liver lobe (fig. 3.3). The clamps were released and the injection needle was removed. Bleeding was stopped with a cotton swap by gentle compression at the site of injection. After closing the abdomen with sutures, the recipient was allowed to recover.

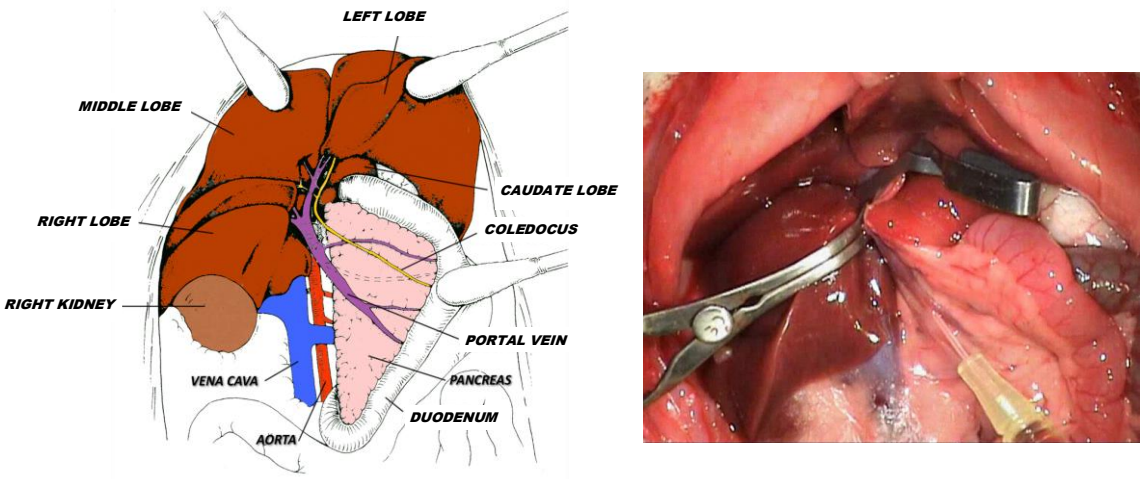


Figure 3.3 - Pancreatic islets were injected in the portal vein and selectively directed in the caudate lobe of the rat liver.

On the 15th post-transplantation day, intraperitoneal glucose tolerance test (IPGTT) was performed. After overnight fasting, rats were intraperitoneally injected with normal saline containing 2 g glucose/kg of body weight. Glucose disposal was analyzed by measuring blood glucose levels at 30, 60, 90, and 120 min after injection by tail snipping (fig. 3.4).

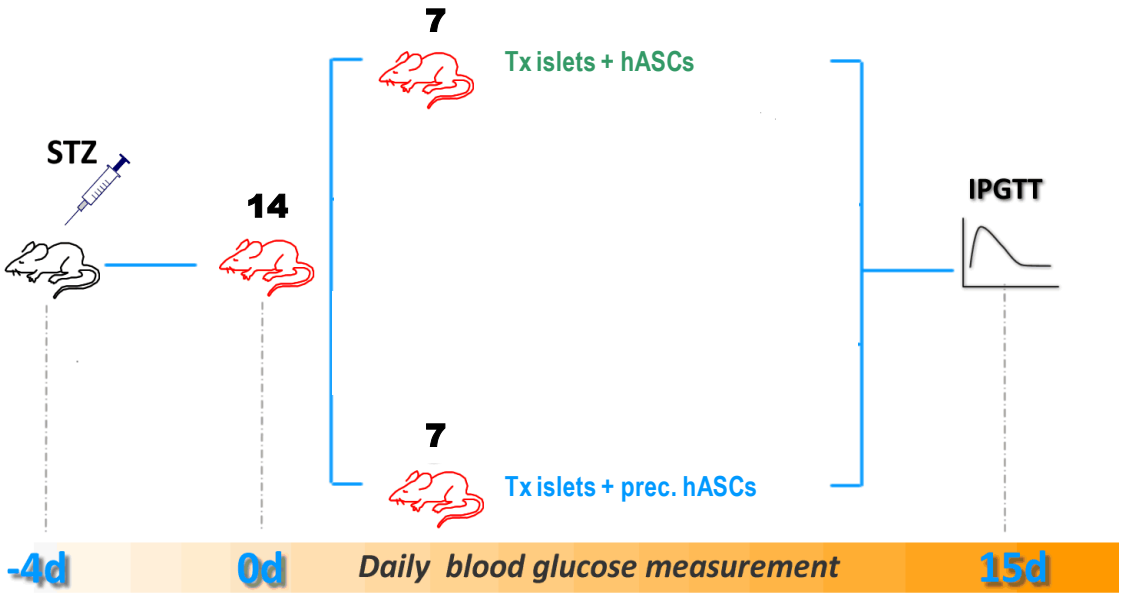


Figure 3.4 - Experimental design for islet/stem cell intrahepatic cotransplantation in syngeneic diabetic rats (Tx: transplant, STZ: streptozotocin).

3.9.4 Morphological and immunohistochemical examination

At the end of a 3-day coculture period, islet morphology was evaluated by direct microscopic observation, then prepared for histological examination (*ex vivo* analysis). Islets were fixed in Bouin solution, embedded in 2% agarose in PBS, and then allowed to polymerize on ice. The enrobed islets were processed for paraffin embedding and 4-µm sections were cut on a microtome (Leica RM2125RT). Sections were deparaffinized in xylene, rehydrated through decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E). Deparaffinized sections were placed in TRIS/EDTA Buffer (pH 9.0), and heated for 1 min in a pressure cooker (110°C) for antigen retrieval. Subsequently, retrieved sections were blocked with 2% normal goat serum (NGS) in 1% BSA in PBS for 30 min at room temperature. The slides were then incubated with the anti-human mitochondria monoclonal antibody, clone 113-1 (1:50, Millipore) for 60 min at room temperature to evaluate the adhesion of the human MSCs to the islets. The

islet sections were then stained for immunofluorescence by the mouse anti-insulin monoclonal antibody clone K36AC10 (1:500, Sigma-Aldrich) used at 4°C for overnight, followed by the secondary antibodies anti-mouse IgG (Fc specific) FITC-conjugated (1:250, Sigma-Aldrich) or Alexa Fluor® 555 (1:1000, Invitrogen) in 1% BSA in PBS for 1 hour at 37°C to evaluate β-cell viability. To detect intraislet endothelial cells, lectin Bandeiraea simplicifolia (BS-1) TRITC-conjugated (1:100, Sigma-Aldrich) was employed in 1% BSA in PBS for 45 min at 37°C, after a second blocking with normal mouse serum (NMS) for 30 min at room temperature.

To evaluate islet graft morphology and in particular their revascularization process *in vivo*, immediately after sacrifice the caudate liver lobes were harvested from the transplanted rats, fixed in formalin solution for 24 hours, embedded in paraffin, and cut into 4-µm sections. Paraffin sections were stained for insulin and lectin BS-1 using the same products reported above. To evaluate the level of the islet graft revascularization, the number of insulin-positive cells and lectin BS-1-positive capillary segments were counted in 7 pictures randomly selected for both study groups, and the obtained capillary/β-cell ratios were compared.

Furthermore, to evaluate the presence of hASCs after transplantation, sections were stained with the anti-human mitochondria monoclonal antibody plus the secondary goat anti-mouse Alexa Fluor® 488 (1:250, Invitrogen) in 1% BSA in PBS for 1 hour at 37°C. Samples were coverslipped with ProLong antifade reagent with DAPI (Molecular Probes).

Slides were studied with a Leica DMI4000 B inverted fluorescence microscope and the image acquisition LAS AF software (Leica Microsystems).

3.10 Statistical analysis

Data are presented as mean ± SEM (standard error of the mean), or mean ± SD (standard deviation). Statistical comparison of two groups was carried out by two-sided unpaired Student's t test and log-rank test. Comparison of more than two groups was performed by one-way ANOVA, followed by Bonferroni post hoc test (GraphPad Prism ver.5). A p value less than 0.05 was assumed as the limit of significance. * denotes significance level of $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

4 - RESULTS

4.1 Immunophenotypic characterization of hASCs

Human mesenchymal stem cells isolated from adipose tissue (hASCs) grew adherent to plastic flasks, with a fibroblast-like morphology (fig. 4.1).

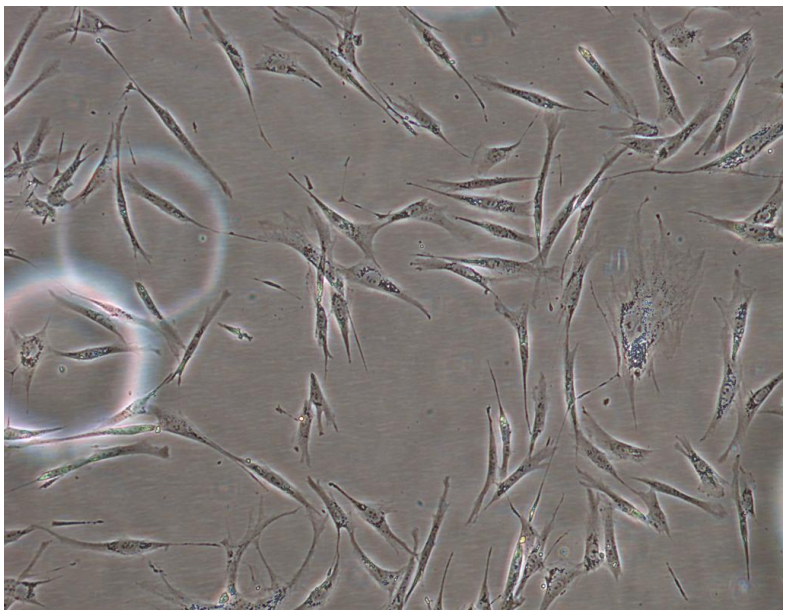


Figure 4.1 - Human ASCs in culture at passage 0, 5 days after the first seeding (original magnification, 100x).

4.1.1 Cytofluorometric analysis of mesenchymal markers

Immunophenotypic characterization was assessed by flow cytometry analysis, measuring the fluorescence expression of the following conjugated monoclonal antibody: CD14-APC, CD29-PECy5, CD34-FITC, CD44-FITC, CD45-PerCP, CD73-PE, CD90-R-PE, CD105-FITC, CD166-R-PE.

ASCs were positively stained (>90%) with an epitope of endoglin (CD105 or SH2), the TGF-β receptor III present on endothelial cells, erythroblasts, monocytes, and connective tissue stromal cells. They were positive for CD73 (SH3/SH4), a molecule involved in B-cell activation, and for CD29, the β-subunit of an integrin family behaving as the major receptor for ECM molecules. Cells also expressed CD166, an hMSC marker not found in hematopoietic precursors, and were uniformly positive for

the CD44 hyaluronate receptor (fig. 4.2). Conversely, antigen profiles were negative for the hematopoietic markers CD14 and CD34, and the leukocyte common antigen CD45. This analysis of surface antigen expression indicates that hASCs can be regarded as alternative sources for hMSC-like elements.

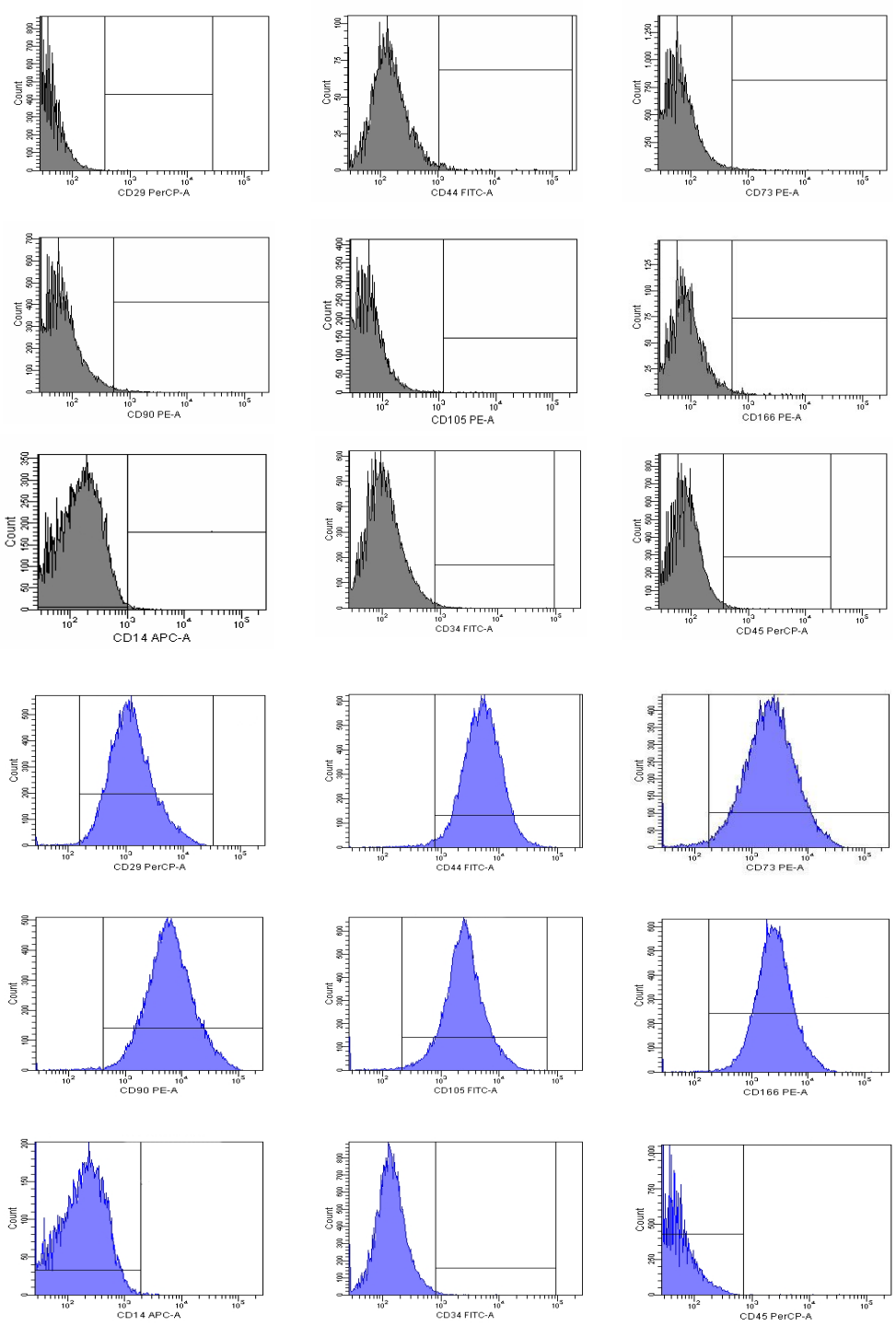


Figure 4.2 - Flow cytometry histograms show the immunophenotypic profile of hASCs: unstained hASCs (negative control, *grey*); stained hASCs (*violet*).

4.1.2 Differentiation tests

Differentiation assays *in vitro* further confirmed that the cell population isolated from adipose tissue was mesenchymal. Classical differentiations toward an adipogenic, osteogenic or chondrogenic fate were induced by using lineage-specific induction media. As showed in figure 4.3 (*left panels*), Oil Red-O staining, which highlights the accumulation of lipid vacuoles within the cytoplasm, confirmed the ability of hASCs to become mature adipocytes.

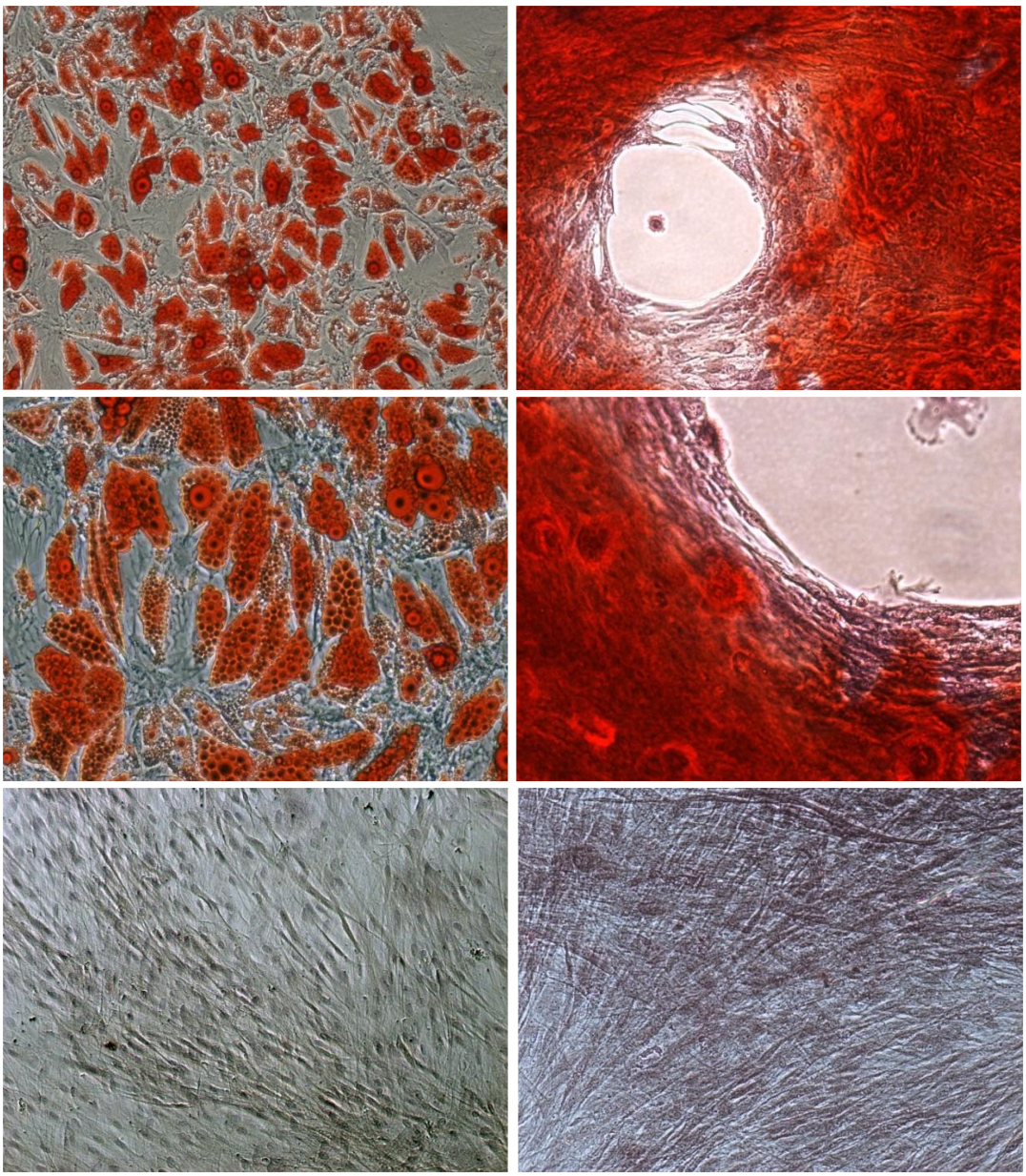


Figure 4.3 - Induction of classical mesenchymal differentiations in hASCs: adipogenic differentiation (*left panels*), and osteogenic differentiation (*right panels*). From top to bottom: positive samples (original magnification 100x and 200x respectively), control negative (original magnification 40x).

Human ASCs could also differentiate into cells functionally able to mineralize the extracellular matrix with calcium salts, as revealed by histochemical staining with Alizarin Red (fig. 4.3, *right panels*).

At the end of the chondrogenic differentiation assay, cells gave origin to a pellet which stained positive for Alcian blue (which colours sulphated glycosaminoglycans, GAGs), indicating that a chondrogenic matrix has formed (fig. 4.4).

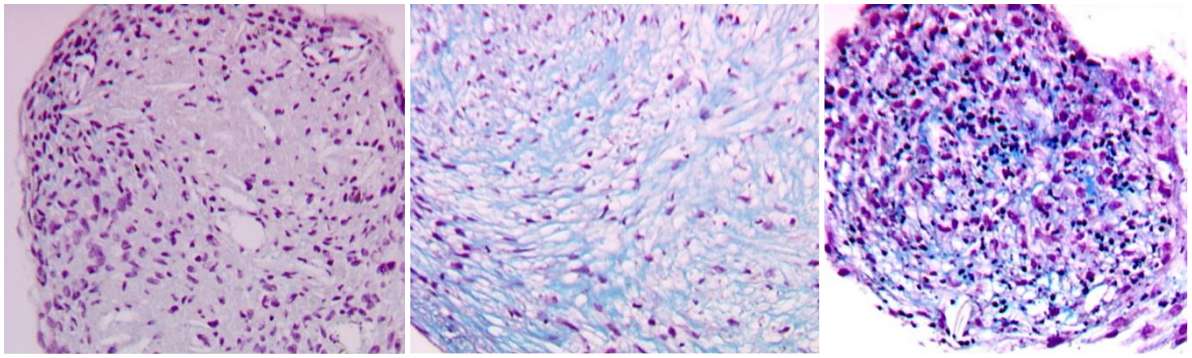


Figure 4.4 - Induction of chondrogenesis in hASCs: from left to right, control negative, positive samples stained respectively with Alcian blue pH 1 and pH 2.5.

4.2 Gene expression analysis

Real-time PCR provided evidence that stem cell preconditioning increased the transcription of genes that have been shown to play a crucial role in the orchestration of angiogenic signals, including VEGF, KDR, encoding a major VEGF receptor, HGF and ISL1 (Islet-1).

Data were normalized using three housekeeping genes (ACTB, GAPDH, HPRT1) as an index of cDNA content after reverse transcription. Relative quantification of mRNA expression was calculated with the comparative Ct method using the "delta-delta method" for comparing relative expression results between treatments in real-time PCR (*Pfaffl, 2001*).

In detail, VEGF expression (fig. 4.5-A) peaked at 24 hours after treatment with BU+RA, then it decreased in spite of maintaining its level higher than control cells. The transcriptional response to HA+BU+RA was even more remarkable compared to BU+RA, reaching a nearly 3-fold increase at 1 day (compared to 2.1-fold of BU+RA, at the same time point). Down-regulation of VEGF gene (concomitant to the increased VEGF secretion showed in figure 4.6-A) suggested that an autocrine regulation was likely to occur.

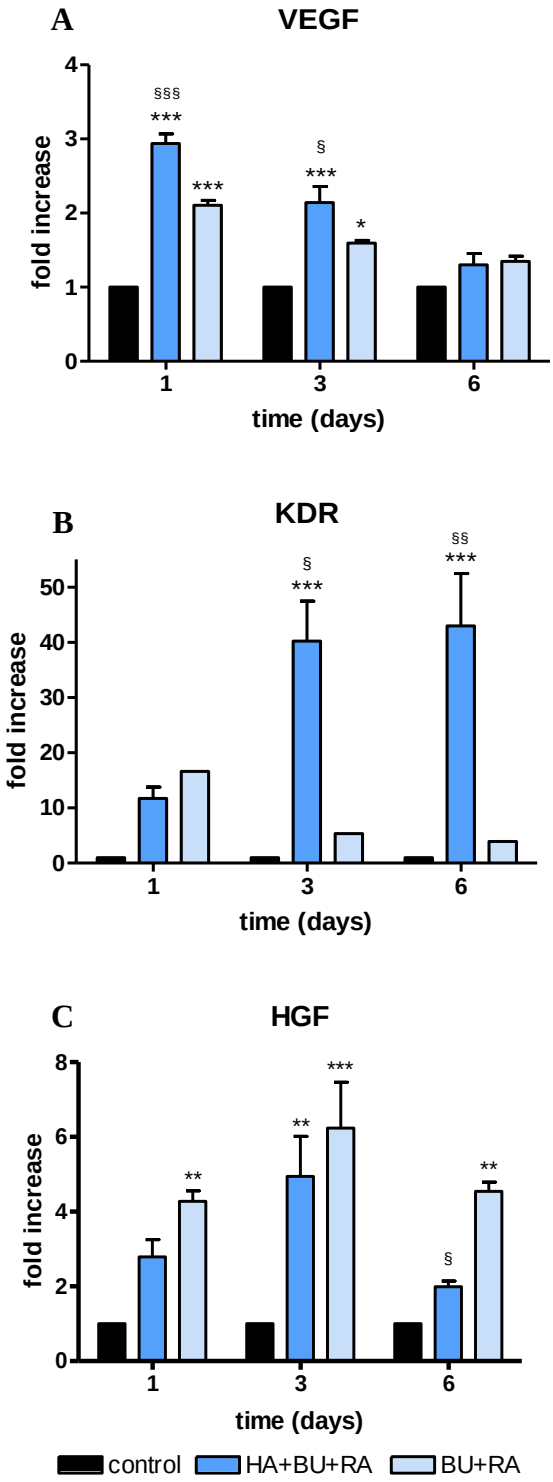


Figure 4.5 - Enhanced angiogenic gene expression in treated hASCs. The abundance of each mRNA in control cells was defined as 1, and the amounts of VEGF (A), KDR (B), or HGF (C) mRNA from treated cells are plotted relative to that value. Values are mean ± SEM (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control; § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ vs. BU+RA.

Treatment with HA+BU+RA dramatically enhanced KDR expression (fig. 4.5-B) starting as early as 1 day and peaking at 6 days, reaching more than a 40-fold increase, as compared with control cells, while BU+RA peaks at 1 day decreasing with time.

Concerning HGF (fig. 4.5-C), chemical preconditioning with BU+RA increased its expression significantly at all the time points, peaking at 3 days. HA+BU+RA treatment obtained the same trend but at a lower extent compared with BU+RA (fold increase: 2.79 vs. 4.28 at 1 day, 4.94 vs. 6.94 at 3 days, 1.99 vs. 4.54 at 6 days).

In further experiments, when the combination HA+BU+RA had already been chosen for the preconditioning, we analyzed the expression of ISL1 gene. During embryonic development, ISL1 has been identified as a marker for cardiovascular progenitor cells that form the three major cell types in the heart (cardiac, smooth muscle, and endothelial cells). In addition, it is a marker for endocrine progenitor cells in the pancreas, playing a critical role in the development of mature islet β-cells. ISL1, together with PDX-1 and Sonic hedgehog (Shh), affects the early events of pancreas development (*Sordi et al., 2008*).

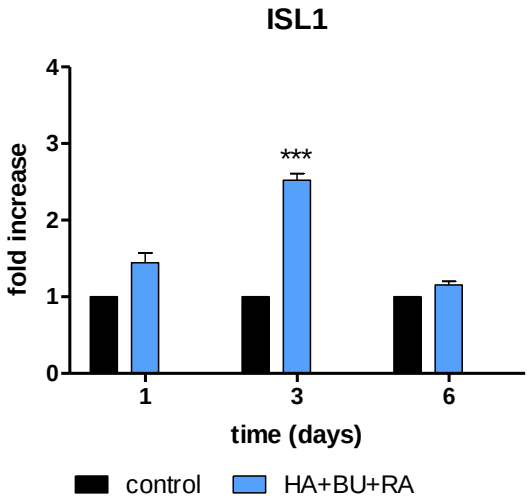


Figure 4.6 - Enhanced ISL1 gene expression in treated hASCs. The amounts of mRNA from treated cells are plotted relative to abundance of mRNA in control cells. Values are mean ± SEM (n=3). *** $p<0.001$.

The treatment with HA+BU+RA increased the ISL1 gene expression significantly at 3 days (2.52-fold increase, fig. 4.6), showing a trend similar to HGF gene. Worthy to note, ISL1 is a master transcription factor which may influence pro-angiogenic and pro-vasculogenic programs in adult MSCs. Barzelay et al. showed the potential of ISL1 in

promoting postnatal angiogenesis and vasculogenesis by triggering the intrinsic pro-angiogenic functional properties of these cells, as well as by endowing paracrine amplification on angiogenesis.

4.3 Cytokine secretion

Cytokine secretion analysis was performed over a 6-day period in basal medium with 2% FBS. ELISA revealed that control hASCs secreted consistent amounts of VEGF throughout the investigated time course. Noteworthy, culturing of hASCs in the presence of the preconditioning mixture resulted in a time-dependent increase in VEGF secretion, starting as early as 24 hours, and peaking after 6-day exposure (fig. 4.7-A).

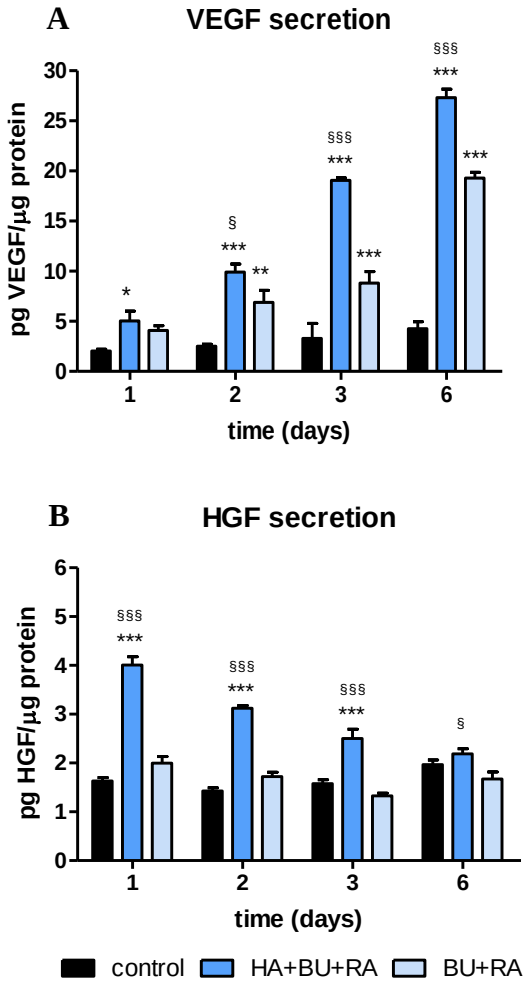


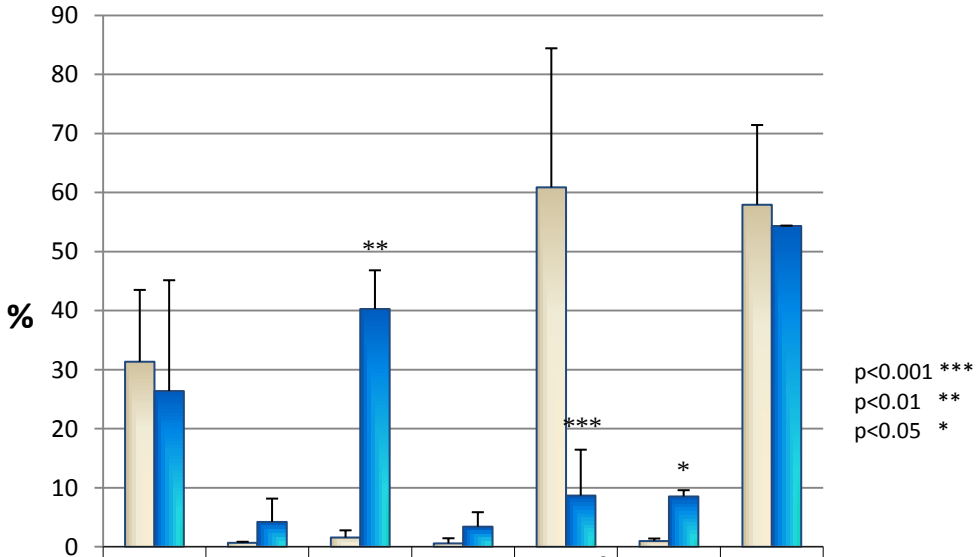
Figure 4.7 - Enhanced cytokine secretion in treated hASCs. Time-course analysis of VEGF (A), and HGF (B) released by hASCs cultured for up to 6 days in the absence (*black bar*) or presence of natural molecules (*light blue bars*). Values are mean ± SEM (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control; § $p<0.05$, §§§ $p<0.001$ vs. BU+RA.

The increase was more remarkable in case of HA+BU+RA treatment, which resulted not only significantly different (higher) than control at all the time points, but also higher than BU+RA treated-cells (significantly from 2 to 6 days, $p<0.05$).

Concerning HGF, only the combination HA+BU+RA was able to significantly increase its secretion from hASCs. This treatment caused a peak of HGF release at 24 hours (the first investigated time point), followed by a decrease, probably due to a saturation effect or negative feedback (fig. 4.7-B).

4.4 Flow cytometry analysis of perivascular and vascular markers

Based on previously obtained data, we chose the combination of molecules that in our opinion could be able to best force a vascular/perivascular fate: HA+BU+RA. Flow cytometry analysis, performed after 14 days of treatment (only one induction), showed that combined exposure to 2 mg/ml HA, 5 mM BU and 1µM RA remarkably augmented the percentage of cells expressing perivascular markers, like CD146 and NG2 ($p<0.01$ and $p<0.05$ respectively), compared to control (fig. 4.8).



	α-SMA	KDR	CD146	CD34	PDGF-Rβ	NG2	CD105
Control	31,37	0,7	1,6	0,6	60,9	1	57,95
HA+BU+RA	26,40	4,23	40,30	3,43	8,70	8,55	54,35

Figure 4.8 - Histogram of flow cytometry analysis for vascular/perivascular markers in hASCs 14 days after treatment. Below a table with percentage values. Data are mean ± SD (n=3).

Expression of PDGF-Rβ, that can be involved in proliferation of undifferentiated cells, is dramatically reduced in preconditioned cells, suggesting a possible cellular commitment. Alpha-SMA, KDR, CD34, and CD105 didn't change significantly.

4.5 Immunofluorescence

The protein expression profile investigated by flow cytometry was confirmed by means of immunofluorescent analysis (fig. 4.9), that highlighted the presence of the pericytic protein NG2 (panel A) as well as the marker for endothelial specification vWF (panel B), not detectable in untreated cells. The amount of α-SMA-positive cells was not different in the two groups, but the fluorescence intensity was higher with HA+BU+RA preconditioning (panels C,D).

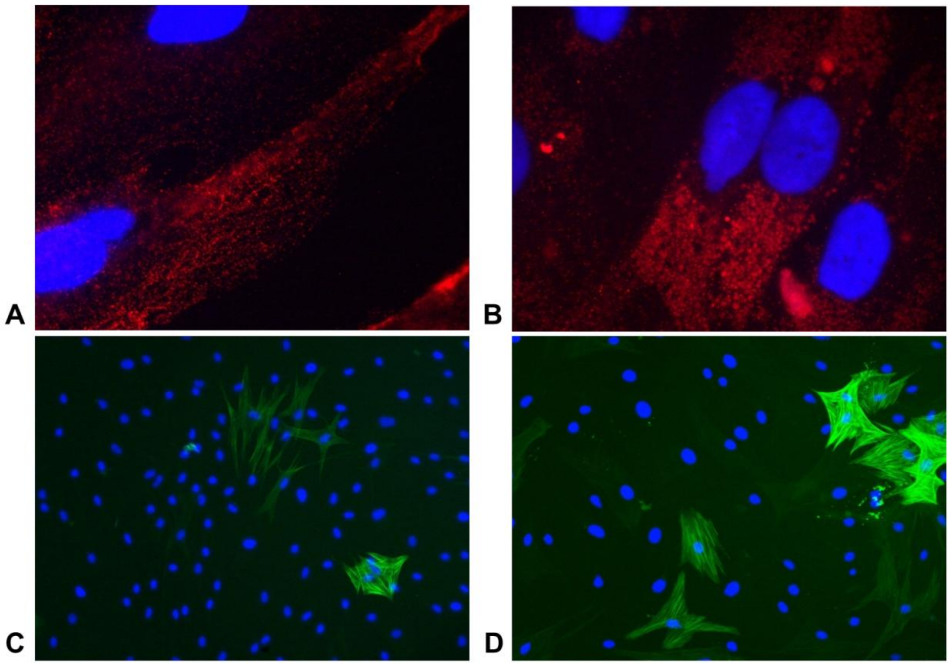


Figure 4.9 - Immunofluorescent staining of hASCs after 14-day culturing in presence of HA+BU+RA. NG2-positive (*red*, A) and vWF-positive cells (*red*, B); original magnification, 1000x. Alpha-SMA-positive cells cultured in absence (*green*, C) or presence (*green*, D) of the three molecules. Nuclei are counterstained with DAPI (*blue*).

4.6 Vasculogenesis

We performed cocultures of ASCs and HUVECs to estimate *in vitro* the relevance of the contact between the two different cells types and to assess whether cell-to-cell interaction may induce a differentiation of ASCs toward a vasculogenic lineage. We

analyzed whether the treatment with HA+BU+RA can increase or induce this kind of differentiation, forcing vascular network formation, by immunofluorescence analysis.

Endothelial cells (all vWF-positive), seeded over ASC monolayer, were able to organize into vascular network in both cases (control or preconditioned cells, fig. 4.10), but not when seeded over HFF monolayer (data not shown).

Anyway in the preconditioned samples costained with α-SMA, we observed an increased α-SMA expression in ASCs (recognizable also for the larger nuclei) and its organization into fibers, quite selectively in those ASCs that were in direct contact or in proximity with HUVECs. We can hypothesize that preconditioned ASCs could be more inclined to receive signals from HUVECs and differentiate toward a mural/perivascular cell type.

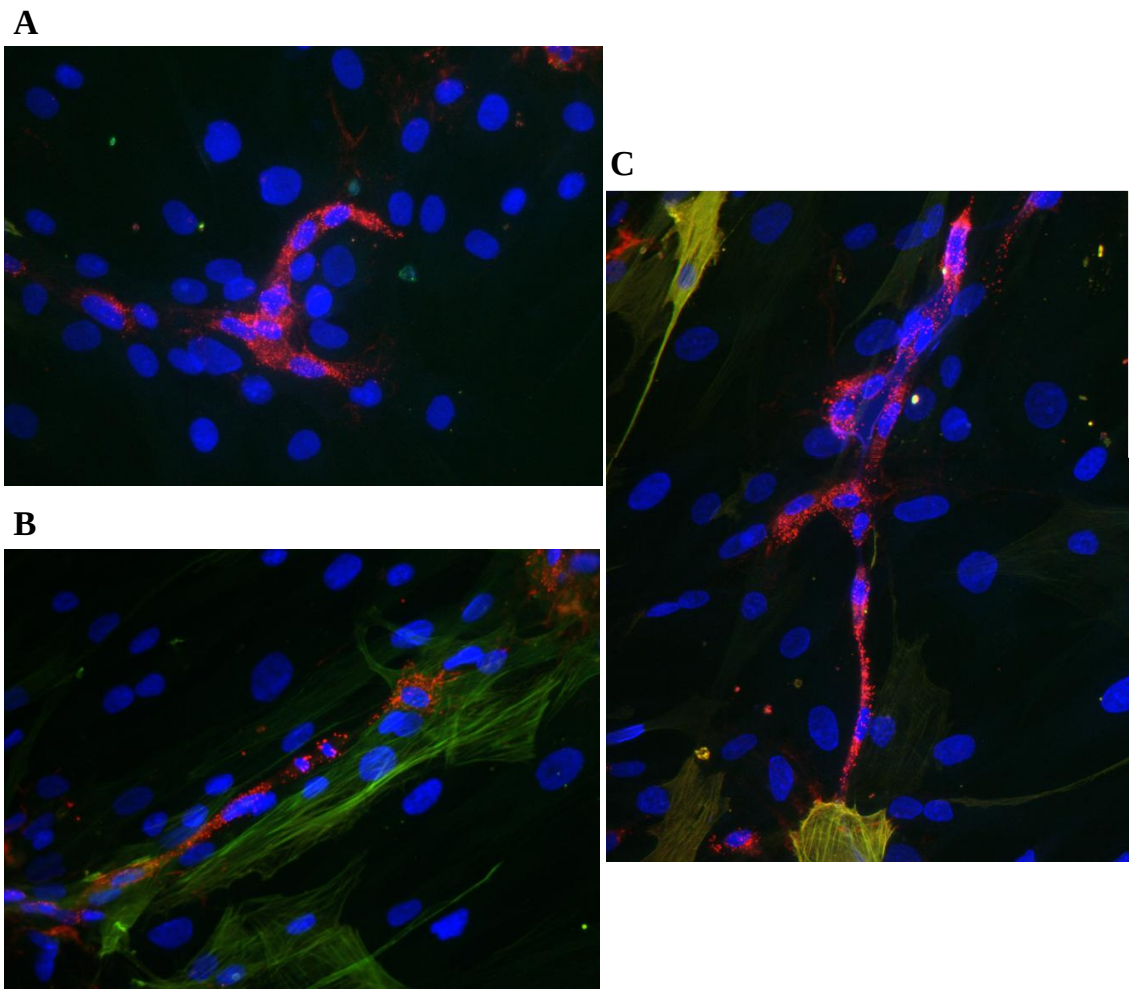


Figure 4.10 - Immunofluorescent staining of HUVECs cocultured for 6 days over a monolayer of ASCs, previously cultured for 5 days in absence (A) or presence of HA+BU+RA (B,C). Merged images: HUVECs are stained with anti-vWF antibody (*red*), ASCs (larger nuclei) are stained with anti α-SMA (*green*); nuclei are counterstained with DAPI (*blue*). Original magnification, 400x.

4.7 Formation of composite hASCs-islets

Stem cell adhesion to the surface of cocultured islets was clearly detectable at microscopic examination using GFP-expressing hASCs. Approximately 50% of the islets presented hASCs on their surface at the end of the coculture period (fig. 4.11-A). The half part of the islets, however, appeared without hASCs coating their surface (fig. 4.11-B).

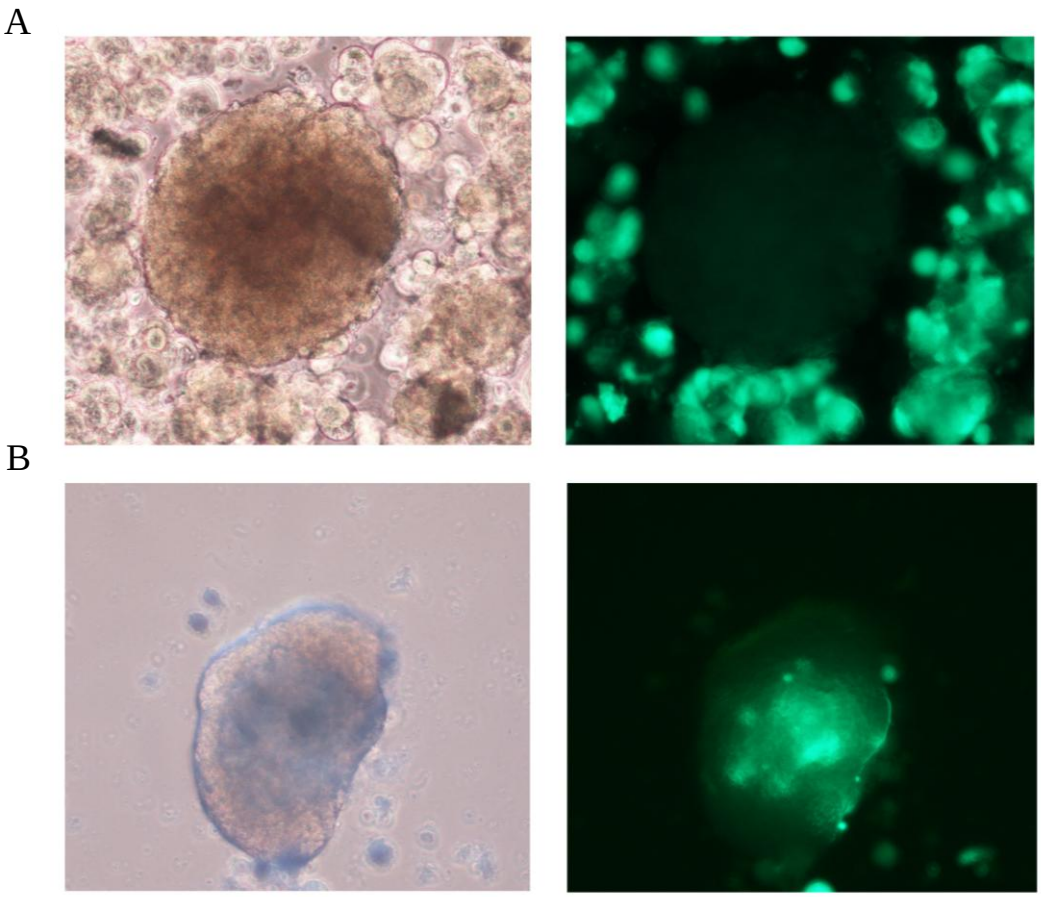


Figure 4.11 - Formation of composite hASCs-islets. The half part of islets remained uncovered (A), while approximately 50% of islets appeared covered by GFP-expressing hASCs (B), without difference between the two study groups.

The formation of composite hASCs-islets was confirmed by immunohistochemistry using the anti-human mitochondria monoclonal antibody (fig. 4.12). No difference in the formation frequency of composite hASCs-islets was evidenced between the two study groups. Concerning the low number of hASCs linked to the islets, we have to consider that the formation of composite hASCs-islets is an accessory, although

favourable event, that is not mandatory to determine the revascularization of the transplanted islets.

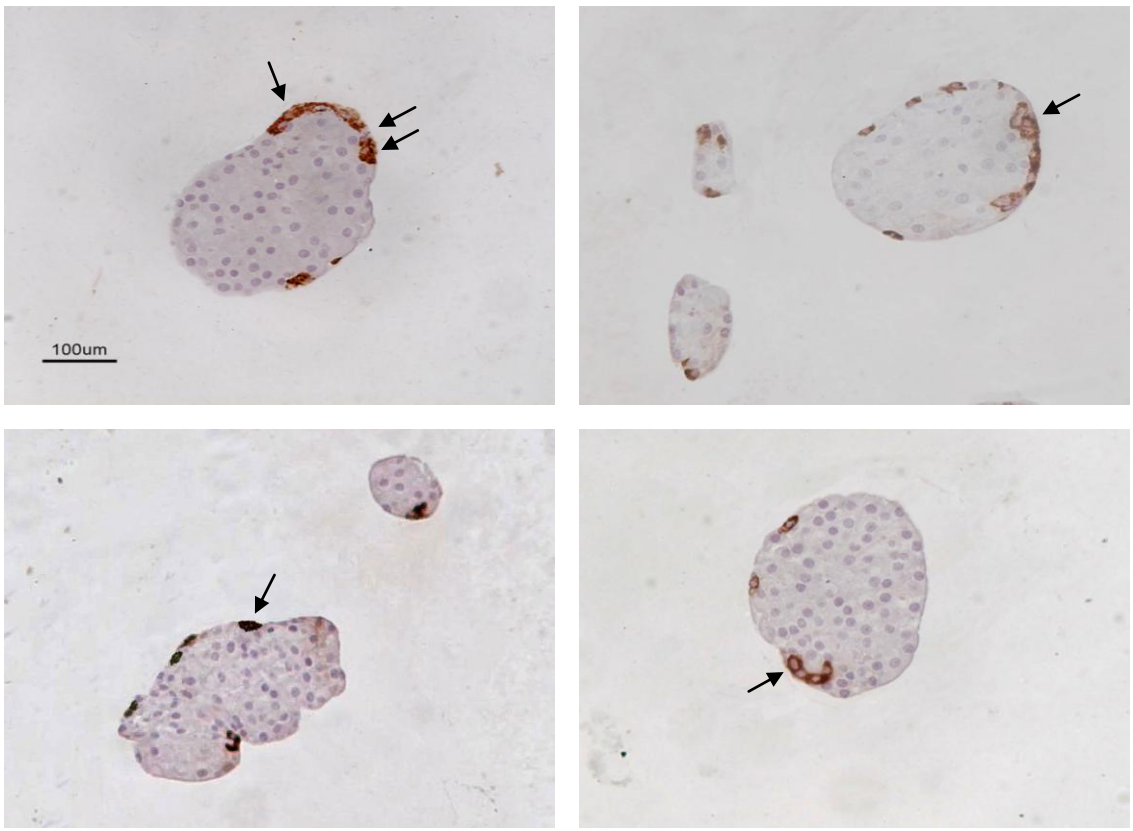


Figure 4.12 - Immunohistochemical analysis of composite hASCs-islets: detection of anti-human mitochondria-positive cells (*arrows*) in cultured rat pancreatic islets. Scale bar: 100 μm.

4.8 High presence of intraislet endothelial cells after coculture with preconditioned hASCs

The majority of islets from both study groups, except for the occasional presence of central necrosis in the core region of the larger ones, showed an intact morphology at immunohistochemical examination at the end of the 3-day coculture period. Interestingly, by means of immunofluorescent analysis we were able to show that islets cultured with preconditioned hASCs revealed a higher presence of intraislet endothelial cells, as compared to islets from control group (fig. 4.13-A,B). Most part of the islets are marked green by antibodies anti-insulin, which specifically stain viable insulin-producing β-cells (nuclei are blue). Red spots identify endothelial cells inside the islet.

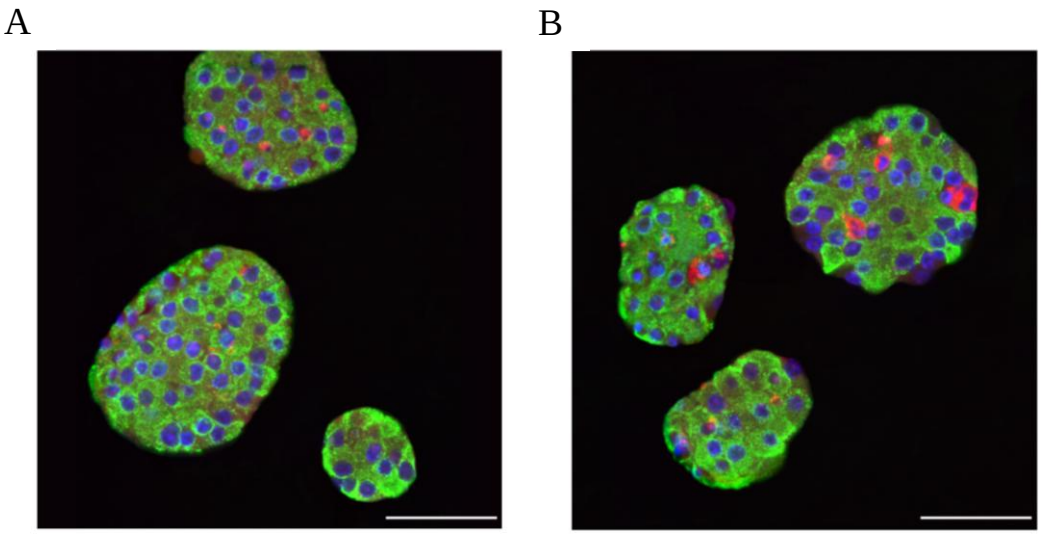


Figure 4.13 - Immunofluorescent staining of 3-day cultured islets in presence of hASCs (A) or preconditioned hASCs (B). β-cells are stained with antibody anti-insulin (*green*), intraislet ECs are stained with lectin BS-1 (*red*), and nuclei with DAPI (*blue*). Scale bar: 50 μm.

4.9 Cotransplantation of preconditioned hASCs enhances islet graft function

With the aim of evaluating differences between the two study groups, for transplantation we used a marginal islet mass which was sufficient to improve glycemic control, but not to obtain a complete reversion of diabetes. Diabetic rats transplanted with 500 syngeneic islets of preconditioned group demonstrated enhanced glycemic control, compared with rats of control group. In particular, significant differences ($p<0.05$, $p<0.01$, $p<0.001$) in non-fasting glucose levels were registered between the rats of the two study groups at all the time points one week after transplantation (fig. 4.14-A). The percentage of recipient rats with achievement of improved blood glucose control (non-fasting blood glucose <300 mg/dl for at least 3 consecutive days) is reported in fig. 4.14-B. Approximately 500 islets were sufficient to achieve a remarkable improvement in blood glucose control in 6 of 7 (85.7%) rats of preconditioned group, compared with 3 of 7 (42.8%) rats of control group ($p<0.05$ by log-rank test). Moreover, two of the recipients of preconditioned group (28.5%) became normoglycemic (non-fasting glucose <200mg/dl), while none of the rats of the control group achieved normal glucose levels. The IPGTT performed in overnight fasting rats 15 days after transplantation detected significantly lower levels of blood glucose at 60

and 90 min ($p<0.01$) in the rats transplanted with islets of the preconditioned group (fig. 4.14-C), confirming that preconditioning improved islet functionality. These results became more important considering some experiments previously performed in a group of rats using islet alone cultured for 3 days before transplant (data not shown). Here we observed a lower graft function compared to rats transplanted with an equal volume of islets cocultured with stem cells.

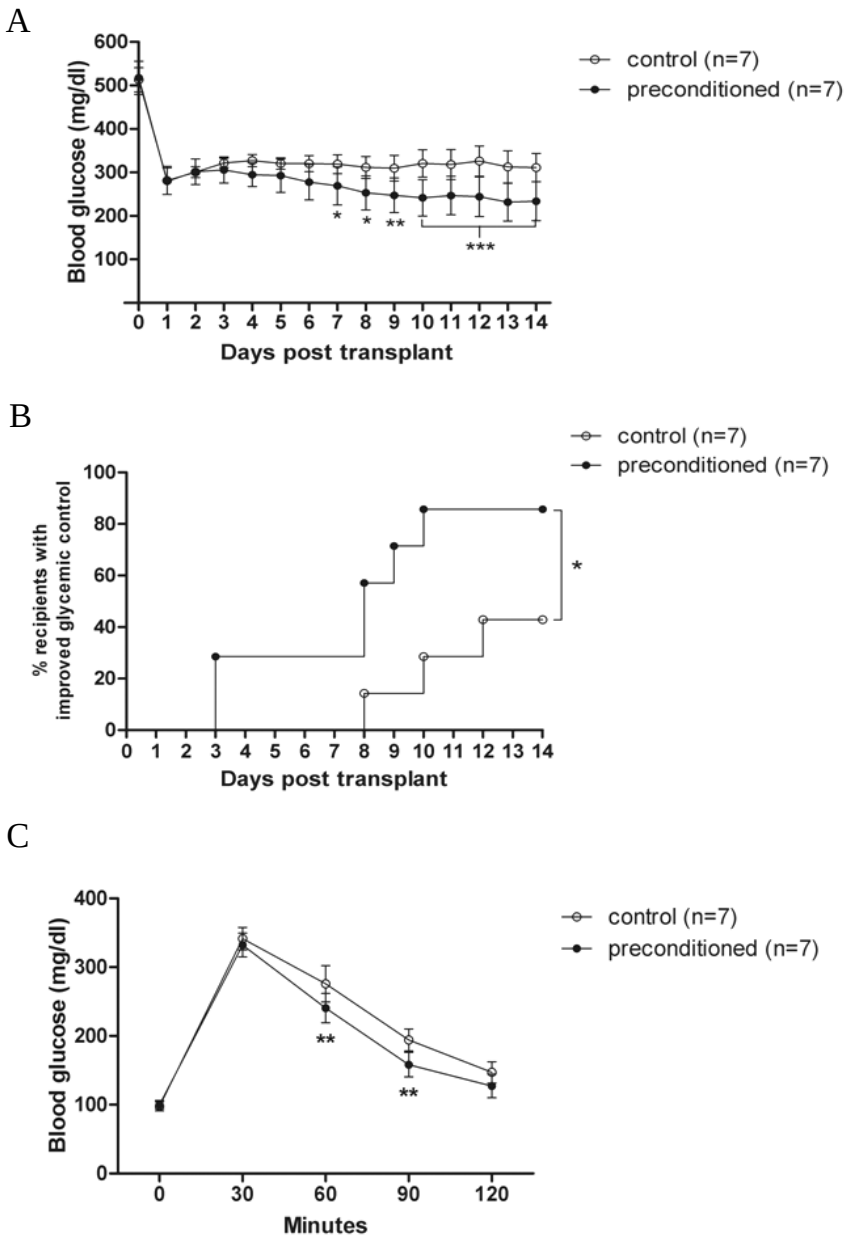


Figure 4.14 - Improved islet graft function after cotransplantation with preconditioned hASCs. Daily measures of non-fasting glucose levels in diabetic rats after transplant (A). Percentage of recipient rats with improved glycemic control in the two study groups (B). IPGTT performed on the 15th post-transplantation day in overnight fasting rats (C). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control group at respective time points.

4.10 Cotransplantation of preconditioned hASCs increases the revascularization process of transplanted islets

Islet transplantation only in the caudate liver lobe of the recipient rats allowed the detection of a high number of islet grafts at the histological examination, and consequently permitted an accurate comparative immunohistochemical analysis of the graft vascularization between the two study groups (fig. 4.15). Two weeks after transplant, islets of preconditioned group showed intact β-cells detected by antibodies anti-insulin (fig. 4.15-A), and a dense capillary network stained with BS-1 (fig. 4.15-B). Graft revascularization appeared preferable in islets of preconditioned group (fig. 4.15-C) compared to control group (fig. 4.15-D).

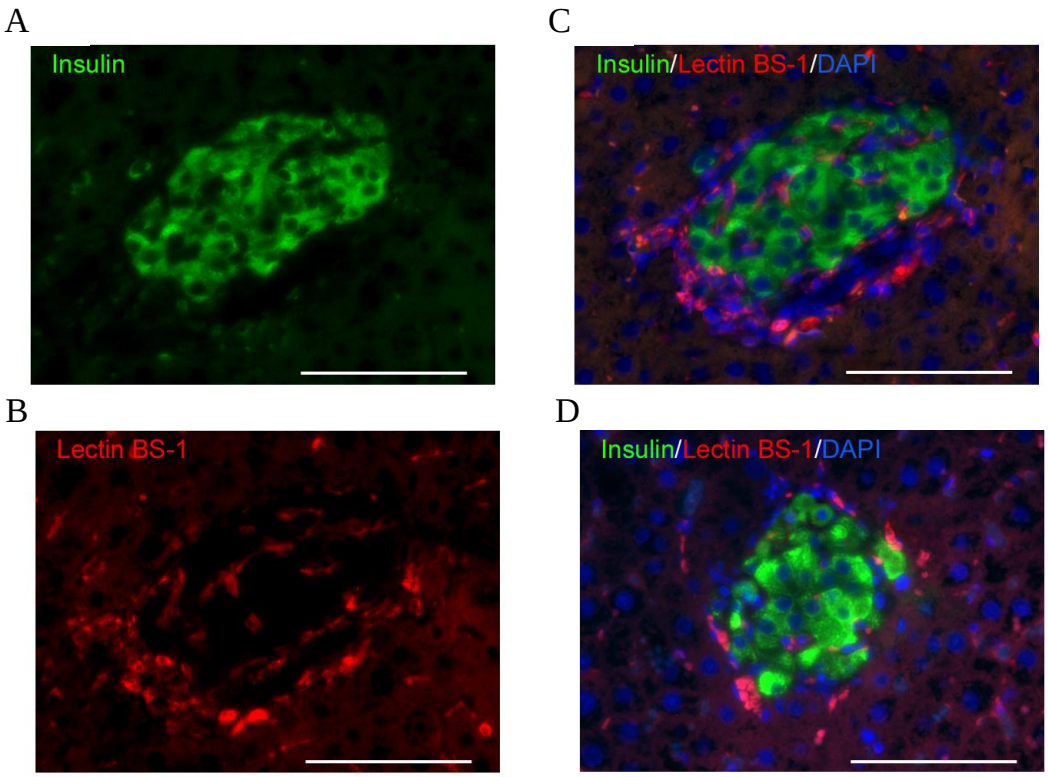


Figure 4.15 - Enhanced islet graft revascularization by preconditioned hASCs cotransplantation two weeks after transplant. Islets of preconditioned group stained with antibody anti-insulin (A, *green*), and anti-lectin BS-1 (B, *red*). Merged photograph of A and B (C). Merged photograph of control group islet (D). Scale bar: 100 μm.

As shown in fig. 4.16, the capillary number per β-cell in the islet graft of preconditioned group was sensibly superior to that of control group (n=7 sections examined per group) (0.117±0.02 vs. 0.062±0.02 capillary fragments per β-cell, $p<0.01$). The presence of anti-human mitochondria-positive cells, located in proximity

of the engrafted β-cells, indicated the viability of hASCs for up to two weeks after transplantation (fig. 4.17).

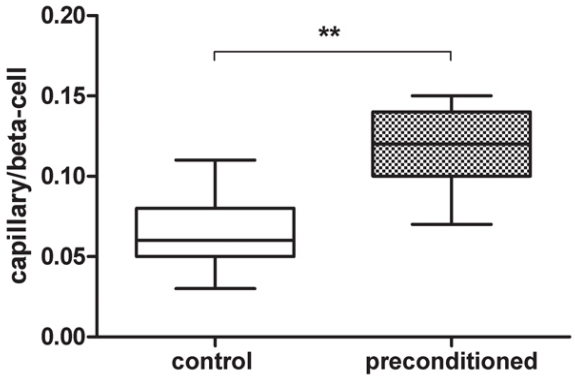


Figure 4.16 - Analysing 7 islet graft pictures for group, a higher capillary segments/β-cell ratio was detected in preconditioned vs. control group (** $p<0.01$).

Thus, as reported by other authors in literature, hASCs are able to protect β-cell viability during pre-transplant coculture, preserving at least partially islet function and improving graft revascularization process after transplantation in an animal model. Together these *in vivo* results clearly show that our preconditioning strategy with natural molecules fulfilled to strengthen the aforementioned ASC ability.

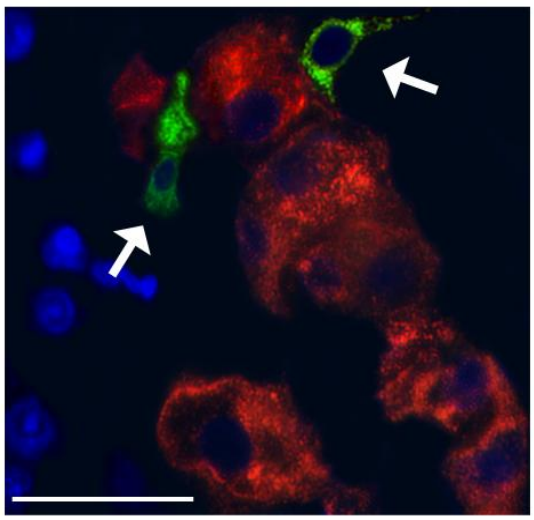


Figure 4.17 – Anti-human mitochondria-positive cells (*green, arrows*) revealed hASCs surrounding engrafted β-cells stained for insulin (*red*); scale bar: 25 μm.

5 - DISCUSSION

The employment of MSCs to improve the efficacy of pancreatic islet transplantation represents a new promising research approach (*Berman et al., 2010; Ito et al., 2010; Johansson et al., 2008; Park et al., 2010*). Recently the ability of ASCs to enhance pancreatic islet engraftment and functionality by inhibition of inflammatory host response and improvement of graft revascularization was demonstrated in an animal model of diabetes (*Ohmura et al., 2010*). An important element involved in the maintenance of a good function of the graft is the secretion of anti-apoptotic and vasculogenic factors by MSCs (*Zuk et al., 2002*), that exert a protective effect on islet cells during the culture period, and increase the revascularization of transplanted islets (*Brissova et al., 2006; Ito et al., 2010; Park et al., 2010*). One of the most important cytokines produced by both mesenchymal stem cells and endothelial cells is VEGF, which has an anti-apoptotic effect and exerts a fundamental role in vessel development. During the procedure of islet isolation and culture, not only a significant portion of β-cell mass but even intraislet endothelial cells, which are essential for the process of revascularization after transplant, get lost (*Brissova et al., 2004; Nyqvist et al., 2005*). Therefore, the reduced production of VEGF, caused by necrosis of endocrine cells, impairs the already poor process of post-transplant revascularization (*Kampf et al., 2006; Lau et al., 2009*).

In this study, we showed that the regenerative action exerted by hASCs could be increased by preconditioning these cells with a mixture of natural molecules as hyaluronic, butyric, and retinoic acids. Specifically, the intrahepatic transplantation of a subtherapeutic volume of islets cultured for 3 days in the presence of preconditioned hASCs showed an enhanced revascularization and function, compared to islets cultured with control cells. The beneficial effects of this new strategy may result from the ability of the preconditioning mixture to increase the gene and protein expression of VEGF, as well as the transcription of KDR, HGF, and ISL1 which have been shown to be involved in autocrine/paracrine circuitries of angiogenic signaling amplification (*Ancelin et al., 2004; Millauer et al., 1993; Barzelay et al., 2010*).

The higher production of vasculogenic and anti-apoptotic cytokines by preconditioned cells, together with the increased expression of perivascular markers (CD146 and NG2), could also explain the higher presence of intraislet endothelial cells

in islets evaluated after a 3-day coculture with preconditioned hASCs, and the reduction of necrosis, that is only occasionally observed in the core region of larger islets.

The *in vivo* studies indicate that the favorable pro-survival effect on intraislet endothelial cells, provided *in vitro* by stem cell preconditioning, leads to an improved vascular network after islet/preconditioned hASC cotransplantation in syngeneic diabetic rats, and that the animals subjected to this protocol could benefit from a remarkably enhanced glycemic control. This finding indicates that the currently described procedure not only preserves structural features of the islets and their inherent vascular network, but can also impact on the molecular plight that controls the attainment of a proper islet function.

The use of adipose tissue as an alternative stem cell source is one of the most intriguing fields of recent interest. Fat can be used in autologous fashion, and exhibits ideal characteristics, being readily available, inexpensive, and host compatible. It is also abundant, can be repeatedly harvested with a minimally invasive procedure, and cryopreserved for banking and subsequent use in one of the several therapeutic applications that may be envisioned from hMSCs. Moreover, hASCs have already been used in clinical settings in the treatment of several pathologies (*García-Olmo et al., 2005; Rigotti et al., 2007; Valina et al., 2007*), and their efficacy and safety allow their clinical use in the field of islet transplantation.

It has been recently shown that lipoaspirates can also be processed with mild mechanical forces in the absence of any enzymatic digestion or growth factor additives, leading to a significant simplification and cost decrease in fat processing (*Tremolada et al., 2010*). Our preconditioning strategy may also be applied in principle to hASCs isolated from these non-enzymatic fat products, and studies are on the way to assess such a perspective.

Although the rescuing ability of MSCs has been a driving force behind initial studies examining their therapeutic effectiveness, their immunomodulatory properties are equally exciting in terms of exploring their potential implications in various disease models, including autoimmune diseases, as type 1 diabetes (*Anzalone et al., 2011*). An important immunological feature of MSCs is the inhibition of T-cell proliferation and dendritic cell differentiation, due to their low expression of costimulatory molecules and the absence of class II HLA. In addition MSCs are able to synthesize trophic mediators involved in immunomodulation, and to induce T-cell anergy and regulatory T-cells (*Zhou et al., 2011*). One of the great expectations linked to the use of MSCs may

therefore reside in their ability to successfully engraft, evading an immune response and inducing peripheral host tolerance, allowing hMSC xenotransplantation (*La Manna et al., 2011; Rossignol et al., 2009; Ventura et al., 2007; Zhou et al., 2011*).

The results from our study show that preconditioning with natural molecules increases the protective effect of hMSCs on cultured islets, improving their post-transplant functionality and efficiency. These results may pave the way to an optimization in the employment of hASCs for islet transplantation, and may also lead to a consistent reduction in the total volume of islets necessary for the transplant.

Nowadays several centers have adopted the strategy to perform two or more islet infusions in the same recipient using different pancreas donors, in order to extend the period of insulin independence after transplant. However, this strategy could increase not only the costs related to the procedure, but also the risk of sensitization of the patients. A period of islet culture, optimized with the employment of preconditioned hASCs, may provide several advantages in the clinical setting of islet transplantation, optimizing a number of critical features in this biomedical area of enquiry, including the quality of islet preparations, the availability of additional time for the selection and treatment of the recipient with suitable pre-transplant strategies (*Shapiro et al., 2011*), and the possibility to ship processed islets to remote transplant centers.

The results of the Edmonton study showed that the outcome of the islet transplantation is strictly dependent on the experience of the isolation center (*Shapiro et al., 2006*): the strategy of creating regional centers isolating cells and sending them to other transplant centers has been successful so far (*Goss et al., 2004; Kempf et al., 2005).* In particular, this type of collaboration allowed a decrease in the total procedure costs and an increase in the experience of the isolation center with a positive rebound on the percentage of successful isolations, and consequently on the number of the transplants performed. In this type of collaboration, the use of cultured islets is frequently required and a pre-transplant islet culture within 72 hours is considered as safe by most transplant centers. However, a short culture period could also be detrimental in the post-transplantation islet function, further reducing the already poor process of graft revascularization (*Olsson et al., 2005*).

In order to move this field forward, human data will be necessary to provide confirmation of preclinical studies and provide further characterization of the therapeutic benefits offered by stem cells.

Concerning the possibility to apply preconditioning strategies in cell therapies to treat other diseases, studies are in progress to evaluate the effects of the injection of preconditioned hASCs as well as Lipogems in a rat model of hind limb ischemia, with special interest toward revascularization and perfusion recovery. Should future studies confirm our promising results, then the employment of hASCs preconditioned with the currently described mixture may be considered as a useful strategy even in a clinical setting.

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