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EVALUATION OF THE THERAPEUTIC POTENTIAL OF AMNIOTIC FLUID STEM CELLS FOR THE TREATMENT OF A MODEL OF TYPE I DIABETES

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Alla mia famiglia ed Andrea,

CONTENTS

ABSTRACT

Type I diabetes (T1D), also known as diabetes mellitus or juvenile diabetes, is a metabolic disorder characterized by the selective destruction of pancreatic β cells of the islets of Langerhans. This loss, caused by an autoimmune attack of the endocrine cells, leads to hypoinsulinemia and hyperglycemia. T1D has nowadays reached epidemic proportions and the number of patients is rapidly increasing. To date, treatments are limited to administration of exogenous insulin and islets transplantation therapy, whose widespread applicability is though limited by scarcity of donors and risks related to immunosuppressive regimens. Stem cell therapy could represent a reliable treatment in the near future. In the context of regenerative medicine, stem cells have garnered much attention as a new potent source for the treatment of severe pathologies in which a specific tissue is lost or damaged, as in diabetes. Both embryonic and mesenchymal stem cells have been frequently investigated and represent an alternative source of *in vitro*-differentiated implantable β cells or can be directly used in stem cell therapy treatments. Different studies have already reported the application of mesenchymal stem cell (MSC) therapy for the treatment of autoimmune disease as T1D, showing that tissue protection and repair are obtained primarily via paracrine immunomodulatory functions.

Amniotic fluid represents a new source of pluripotent stem cells. It contains a heterogeneous population of cells originating from the developing fetus, including c-kit positive cells, which exhibit both embryonic and mesenchymal stem cell characteristics. Amniotic fluid stem

cells (AFSC) can be easily isolated without causing any harm to the fetus, thus there is no ethical concern in their use, can be extensively expanded *in vitro* and have the advantage of not forming tumors *in vivo*. Our laboratory has already demonstrated the successful therapeutic application of AFSC in a murine model of acute and chronic kidney injury as well as in model of acute and chronic lung injury.

The aim of the project was to test the therapeutic potential of AFSC for the treatment of an acute mouse model of T1D. We hypothesize that intracardiac injections of AFSC on diabetic mice have the potential to restore pancreatic functional loss and to protect the pancreatic tissue from further damage.

Our approach was based on the *in vivo* injection of human AFSC into immunodeficient NOD/SCID mice. Mice were rendered diabetic by selective drug treatment with streptozotocin. Injected mice were divided into two distinct groups, the first one receiving a single cell injection and the second group receiving a double dose of cells. This approach was chosen in order to evaluate whether the effect of stem cells could depend from the frequency of injections. Mice treated with AFSC were compared to not-treated diabetic mice and wild type mice. Mice were constantly monitored for blood glucose levels and in all experimental groups that received AFSC some of the mice maintained normoglycemic values during the time frame of investigation.

Immunohistochemical assays reveal that diabetic mice treated with AFSC had significantly higher levels of insulin when compared to diabetic mice. Moreover, islets from AFSC-treated mice showed morphology similar to that of wild type islets. Integration of AFSC into the target organ was as well evaluated by immunofluorescence. However, despite the presence of

some AFSC into the host tissue, the percentage of integrated cells resulted very low. Our hypothesis is that AFSC have the ability to restore pancreas functionality by mainly protecting endogenous beta cells, thus modifying the local microenvironment and/or by stimulating regeneration from endogenous progenitors rather than differentiate themselves into functional beta cells.

In summary, we were able to show that AFSC can hold the potential to treat a severe condition such as diabetes by mainly protecting endogenous cells from damage. However the response to cell treatment is not equal among mice. We speculate that differences in outcomes among the mice can be due mainly to the type of injury that we performed, creating variations between different animals in the early phase of disease development.

ABSTRACT IN ITALIANO

Il diabete di tipo I, noto anche come diabete mellito o diabete giovanile, è una malattia metabolica caratterizzata dalla distruzione selettiva delle cellule β che risiedono nelle isole del Langherans del pancreas. Tale perdita, causata da un attacco autoimmune da parte di linfociti T e macrofagi che infiltrano il tessuto endocrino del pancreas, ha come immediata conseguenza lo sviluppo delle condizioni di ipoinsulinemia e relativa iperglicemia.

Ad oggi, il diabete mellito ha raggiunto proporzioni epidemiche ed il numero di persone affette è in rapida espansione. Le attuali terapie per il trattamento del diabete mellito si limitano alla somministrazione d' insulina esogena e, in alcuni casi, al trapianto di insule pancreatiche da donatore. Entrambe tuttavia presentano alcuni aspetti negativi tra cui la difficoltà nel mantenere sotto stretto controllo costante il livello di glucosio nel sangue, nel caso della terapia insulinica, ed il numero ridotto di donatori disponibili, nonché i rischi legati alle terapie immunosoppressive, per quanto riguarda il trapianto.

Negli anni più recenti, la ricerca nel campo della medicina rigenerativa si è concentrata sulla possibilità di utilizzare le cellule staminali come fonte alternativa per la rigenerazione *in vivo* o *in vitro* di cellule che producano insulina e possano rimpiazzarne la perdita negli individui affetti. Sia le cellule staminali embrionali che mesenchimali sono state già analizzate e studiate come fonte alternativa di cellule da cui derivare *in vitro* beta cellule impiantabili o per un loro utilizzo diretto in trattamenti basati sulla terapia cellulare. Diversi studi hanno già riportato l'applicazione di terapie

basate sull'uso di staminali mesenchimali per il trattamento di malattie autoimmuni quali il diabete mellito, dimostrando come la protezione e la riparazione del tessuto si ottengano principalmente mediante funzioni paracrine immunomodulatorie.

Il liquido amniotico rappresenta una fonte nuova e alternativa di cellule staminali pluripotenti in quanto contenente una popolazione eterogenea di cellule che derivano dal feto in via di sviluppo, incluse le cellule positive per il fattore c-kit, le quali rappresentano circa lo 0.8-1 % dell'intera popolazione. Le cellule staminali da liquido amniotico (AFSC), positive per c-kit, possiedono caratteristiche delle cellule staminali embrionali e mesenchimali. L'uso delle AFSC presenta alcuni vantaggi tra cui la facilità di isolamento da campioni di liquido amniotico senza danno per il feto, il che permette di aggirare i problemi etici legati all'uso delle staminali embrionali. Inoltre, le AFSC possono essere espanse *in vitro* per numerosi passaggi ed è stato dimostrato, mediante la loro applicazione *in vivo,* che le AFSC non hanno proprietà tumorigeniche. Il potenziale terapeutico delle AFSC nel campo della medicina rigenerativa è già stato dimostrato con successo in modelli murini di danno, acuto e cronico, in rene e polmone.

Lo scopo del progetto è la valutazione del potenziale terapeutico delle AFSC per il trattamento di un modello murino acuto di diabete mellito. L'ipotesi è che le AFSC, iniettate per via intracardiaca in topi diabetici, abbiano il potenziale di proteggere il tessuto pancreatico dal danno e favorire la rigenerazione.

Il nostro approccio si è basato essenzialmente sull'applicazione *in vivo* di AFSC umane mediante iniezione intracardiaca in topi immunodeficienti NOD/SCID. Il diabete è stato indotto mediante trattamento chimico selettivo con streptozotocina ed i topi sono stati successivamente divisi in

due gruppi al fine di valutare l'effetto di una singola iniezione di AFSC o di una doppia iniezione. I topi trattati con AFSC sono stati confrontati con topi diabetici e topi wild type. Gli animali sono stati monitorati ad intervalli di tempo regolari per quanto riguarda il parametro fisiologico del glucosio sanguigno e, al termine dell'esperimento, sacrificati. Solo alcuni dei topi trattati hanno risposto al trattamento con le staminali da liquido amniotico, mantenendo valori normoglicemici pressoché per tutta la durata dell'esperimento. Saggi immunoistochimici hanno permesso di evidenziare che i livelli di insulina di topi trattati con streptozotocina e iniettati con AFSC sono risultati significativamente superiori a quelli dei topi diabetici di controllo. Inoltre, le isole pancreatiche dei topi trattati con AFSC mostrano una morfologia molto simile a quella dei topi wild type. La percentuale di integrazione delle AFSC nel tessuto dell'ospite è risultata piuttosto scarsa, come già emerso in precedenti studi, supportando l'ipotesi secondo cui le AFSC abbiano la capacità di ripristinare la funzionalità pancreatica principalmente mediante protezione delle cellule beta endogene ed eventualmente stimolando la rigenerazione a partire da precursori endogeni piuttosto che per differenziamento diretto.

Con l'approccio *in vivo* è stato possibile ottenere risultati preliminari promettenti per una futura applicazione di terapia cellulare possibilmente in ambito clinico. Tuttavia, il responso al trattamento non è uguale per tutti gli animali. Ipotizziamo che differenze nella risposta alla terapia cellulare possano dipendere dal tipo di danno acuto indotto, creando variazioni tra i topi nella prima fase di sviluppo della malattia.

INTRODUCTION

1. Pancreas: anatomy, physiology

The pancreas is a compound gland of the body, which consists of two different tissues, respectively the exocrine and the endocrine, responsible of distinct physiological functions. The exocrine pancreas is composed of various lobules, each of them consisting in one of the ultimate ramifications of the main duct, which ends in a number of cecal alveoli or acini. The alveoli are almost completely filled with secreting cells. These cells produce the digestive enzymes, such as proteases, lipases and amylases, which are collected in the pancreatic juice and passed through the main duct to the small intestine where it is needed for the further digestion of complex carbohydrates, proteins and lipids still present in the chyme.

The endocrine tissue is instead mainly responsible for the glucose homeostasis in the body. It has a peculiar structure since it is composed of cell clusters known as Islets of Langerhans that are dispersed among the exocrine tissue. The islet clusters represent 1-2 % of the total pancreatic mass and contain specific cells that are in contact with a dense network of capillaries surrounding the islet structure, which facilitate the release of hormones directly in the blood stream. Five different types of cell are present within the islet of Langerhans and are classified by the specific hormone they secret for:

- β cells producing insulin and amylin
- α cells producing glucagon

- δ cells producing somatostatin
- PP cells producing the pancreatic polypeptide
- ε cells producing ghrelin

β cells represent around 65-80 % of the islet mass and their main product insulin, together with glucagon, plays a fundamental role in glucose metabolism and the maintenance of euglycemic state. The two hormones act as antagonists responding to opposite stimuli. Insulin is released in response to the increase of glucose level in the blood. β cells are able to sense this difference by the presence of a specific glucose transporter GLUT2 on their membrane. Insulin release immediately promotes conversion of glucose in glycogen, which is then stored in the liver. When the physiological blood glucose needs to be re-established from low level, glucagon is secreted by the pancreas promoting the degradation of glycogen stocks and release of glucose in the blood.

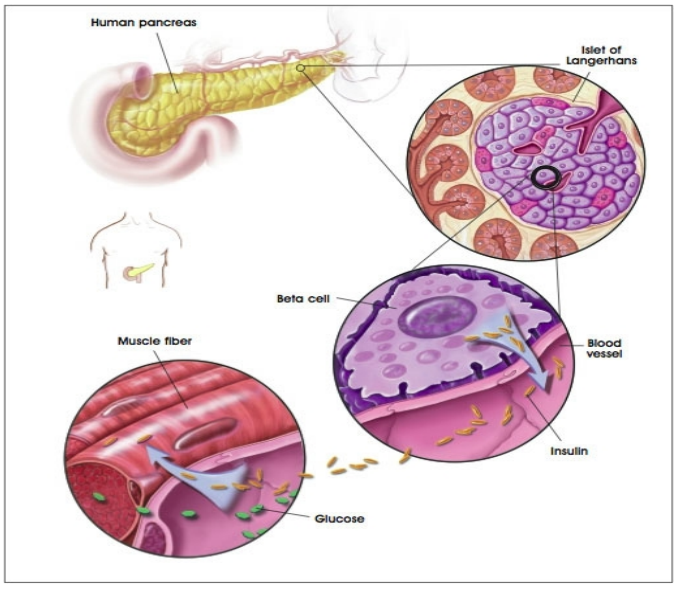


Fig. 1 Schematic representation of pancreas and the microscopic structure of endocrine tissue composed of islets of Langerhans, where insulin-expressing β cells release their product directly into the blood capillaries.

2. Type I diabetes

2.1 Incidence, causes, outcomes

Type I diabetes (T1D), also known as juvenile diabetes or diabetes mellitus, is a metabolic disorder characterized by the immune-mediated destruction of pancreatic β cells of the islets of Langerhans. This selective loss is caused by an autoimmune mononuclear infiltration of the endocrine cells leading to cytokine mediated β cell apoptosis[1]. Type I diabetes is a life-long disease which can occur at any age. However, it is most often diagnosed in children, adolescents and young adults.

Diabetes mellitus is recognized as a major health problem and nowadays has reached epidemic proportions. According to the American Diabetes Association, almost 26 million people, among children and adults, are affected by this chronic disorder only in the United States and its incidence is rapidly increasing. It is estimated that the number of patients is going to double by 2030.

Diabetes is a complex pathology with multifactorial etiology. Even though the actual trigger of the disease hasn't been discovered yet, evidence so far suggests that the development of the pathology has to be attributed to a combination of different factors, including genetic susceptibility, exposure to a driving antigen which could be of viral origin and environmental factors that could expose genetically predisposed individuals to the risk of developing diabetes[2].

Environmental factors have been implicated in the pathogenesis of diabetes mellitus both as triggers and potentiators of β cell destruction[3], even if the contribution of any individual exogenous factor has not yet been

definitely proven.

As far as concern genetic susceptibility, the HLA (human leukocyte antigen) class II locus on the short arm of chromosome 6 was the first to be identified and associated with the disease[4,5]. Despite an increasing evidence that genetic predisposition might have an important role in determining development of diabetes, only a relative small proportion of genetically susceptible individuals progress to clinical disease. This implies that additional external factors are required to trigger and drive β cell destruction in genetically predisposed subjects[6].

Patients with type I diabetes cannot live without a constant source of exogenous insulin. The elevated blood sugar levels developed cause several acute and chronic complications that affect other major organs of the body. Among the main complications that T1D patients present in long-term there are chronic renal failure, known as diabetic nephropathy, diabetic ketoacidosis, proliferative retinopathy that can lead to blindness, peripheral neuropathy and vascular disease[7].

The discovery of insulin in 1922[8] and later the progresses made by recombinant DNA techniques, allowing the production of the enzyme on large scale, have prevented death from acute diabetes. However, despite the improvement in the treatment with exogenous insulin, tight glucose level control is difficult to achieve, making the prevention of the related systemic complications hard.

2.2 Prevention and clinical treatments to type I diabetes

2.2.1 Prevention

As previously mentioned, the pathologic hallmark of T1D is the selective autoimmune attack of pancreatic β cells by CD4+ and CD8+ T cells and macrophages infiltrating the islets[9]. In the past years many efforts have been spent in studying a way to prevent or predict the possible development of the disease. More than 30 years ago, it was recognized that antibodies found in sera of patients with T1D could bind to sections of pancreatic islets. These antibodies have been called “islet cell antibodies” and the three main autoantigens identified were glutamic acid decarboxylase (GAD 65), a protein tyrosine phosphatase-like molecule (IA-2) and insulin[10,11].

The discovery of these autoantibodies gave insight on the possibility of using such antibodies as a tool for the prediction of future disease. It is now clear that about 90% of people with newly diagnosed T1D have autoantibodies to at least one of the three antigens[2]. However, there is a great variability in the pattern of humoral immunity among patients and it is in the pre-diabetic phase that islet autoantibodies have been most useful, because they appear to be already present in most cases of future diabetes by the age of 5 years. Thus, the appearance of these markers is a major risk for the future development of T1D and the presence of 2 or more autoantibodies in people in the general population is highly predictive of future disease[12]. This is why one of the main targets of diabetes research is to find appropriate agents to reduce or delay the autoimmune process in people found to have these antibodies. Although relatively good

predictions of T1D can be obtained by measuring autoantibodies, a wide-spread successful method of prevention has not yet been discovered and no treatment so far has been shown to prevent T1D in humans. Two major trials have been conducted and completed to try to prevent T1D. The European Nicotinamide Diabetes Intervention trial (ENDIT) was a randomized double-blind placebo-controlled trial of high dose nicotinamide therapy whose participants were first-degree relative of T1D patients. Although nicotinamide showed to be protective in animal models, no difference was observed among participants in terms of protection from diabetes during the 5-year test period[13].

In the United States, the Diabetes Prevention Trial (DPT-1) was started in 1994 with the aim of determining the efficacy of low-dose insulin injections in high risk (>50%) first-degree relatives of T1D patients to prevent or delay appearance of diabetes. The trial didn't overall show a slow progression of diabetes. Insulin treatment had no effect except for a subset of participants (those with high levels of autoantibodies), in which a delay and a reduction in the incidence of the disease was observed[14,15].

2.2.2 Current therapies

To date, therapeutic treatments to T1D are limited to the administration of exogenous insulin and, to a less extent, islet transplantation therapy. Both therapies though present some disadvantage, strengthening the need to find new, wide-spreadable and more reliable therapeutic approaches.

Administration of exogenous insulin

Considering the difficulties in finding a way to prevent and reduce the incidence of appearance of T1D, nowadays the only widely available treatment to this disease is the administration of exogenous insulin once the symptoms have already developed. The use of genetically engineered analogs of insulin that are rapidly absorbed has been widely investigated and demonstrated to reduce the variability of insulin absorption[16]. Another recent introduction in the treatment of T1D patients is the use of insulin glargine, which functions as very long acting insulin. Conventional NPH (neutral protamin Hagedorn) insulin, an intermediate acting insulin normally given to patient to help control blood sugar level, is not efficient for the treatment of patients who need continuous basal coverage. Premixed insulin preparations do not meet the individual needs of patients to adequately control postprandial glucose excursion. This is why combinations of engineered very long acting insulin and rapid acting insulin can provide a more efficient control of blood glucose levels, comparable to that obtained with insulin pumps[17]. However, the risk of hypoglycemia still represents a major limiting factor in achieving euglycemia with insulin treatment.

Islet transplantation

An alternative therapy to exogenous insulin administration has been islet transplantation, even though different factors put limits on the wide applicability of such approach. In clinic, two types of islet cell transplant protocols are recognized which are respectively the allogeneic transplant

and the autologous transplant. In the latter case, islet transplantation is performed for the prevention of diabetes occurring after total or semi-total pancreatectomy using tissue of the same patient. Allogeneic transplantation takes place when a T1D patient receives purified islets from a donor[18].

The basis for clinical application of allogeneic islet cell transplant was laid with the completion of the Edmonton protocol[19]. The group published the result of a study conducted on seven diabetic patient enrolled for allogeneic islet transplant therapy. All patients were able to become insulin independent after the treatment for a long period of time, making the outcome of the study quite sensational for the period since a minimally invasive procedure seemed to be able to reverse diabetes. One of the main goals of the study was to test islet cell transplantation in a glucocorticoid-free immunosuppressive regimen, which included sirolimus, low-dose tacrolimus and a monoclonal antibody against the IL-2 receptor (daclizumab). Sirolimus and tacrolimus are the main immunosuppressant drugs used to prevent organ rejection in transplantation therapy. This new protocol was intended to provide a greater immunologic protection without the diabetogenic side effects related to immunosuppression therapy, which is one of the main drawbacks in this kind of intervention. On the basis of that study many efforts have been spent over the past decade in order to render islet transplantation the standard therapy to treat diabetes mellitus. However, there are still disadvantages in its application. One of the main negative issues is the scarcity of donors for islets isolation. The Edmonton protocol itself showed that more than one transplant is often required to achieve insulin independence. Usually, more than one cadaver donor would be necessary to get the proper amount of cells for a single patient.

The small number of donors is not the only limiting factor though. The rate of isolation and the good quality of purified islets are as well factors that can affect the outcome of islet transplantation.

In order to confirm the results of the Edmonton protocol, a multi-center, international clinical trial was conducted[20], demonstrating that the success of such therapy depends on different factors including the efficiency of isolation and purification protocols, the amelioration of immunosuppressive regimens to be less toxic, the enhancing of donor islet engraftment and the necessity of multiple transplants. In 2005, the long-term clinical outcome of the Edmonton protocol was published by the same group[21], indicating that approximately 80% of the patients maintained viable transplanted islets. However, less than 10% of patients could maintain insulin independence 5 years after the beginning of the transplantation trial, despite the fact that multiple transplants were initially performed.

Islet cell transplantation is still considered a therapy for highly selected patients with severe hypoglycemia caused by normal insulin therapy. Whole pancreas transplantation seems to offer greater metabolic reserve compared to the yield of islet engraftment in islet transplantation. Still, donor shortage represents the stronger limiting factor to its application. The demand for transplantation therapy, either with whole pancreas or purified islets, cannot be met also considering the increasing number of people affected by T1D. Other options are being investigated such as the possibility of transplanting islets from living donors[22,23,24].

Given the clinical burdens that belong to islet cell transplantation therapy, the search for alternative ways to treat diabetes mellitus must be pursued. In recent years, research has in particular focused on finding new sources of functional insulin secreting cells. In the context of regenerative

medicine, the possible use of stem cells to replace damaged β cells holds great promise for the future treatment of type I diabetes.

3. Regenerative medicine and stem cells: new strategies for the treatment of type I diabetes

3.1 Regenerative medicine

Regenerative medicine is a discipline that has been widely growing in the last years in order to overcome limitations of current therapies in many degenerative diseases, diabetes included. Regenerative medicine can be defined as a whole of research and clinical approaches that aim to functionally restore or replace damaged organs or tissues through the *in vivo* regeneration or the transplantation of biological substitutes, obtained *in vitro*.

The development of engineered pancreatic substitutes is still far from being achieved. In general, as for any other functional living-tissue replacement, it requires the integration of cell technology, responsible for studying and identifying the proper functional cell candidate and construct technology that provides a three-dimensional scaffold, of natural or synthetic origin, on which the cells are seeded or encapsulated. Lately, one of the most common approaches to address this issue has focused on the design of three-dimensional encapsulated systems where functional insulin producing cells are encapsulated into scaffolds of different sort[25,26]. Such strategy allows immunoisolation of cell graft once implanted *in vivo.* However, before *in vivo* implantation, still many issues must be considered such as the ability of these systems to support the cells for long periods of

time and the capability of the cells to maintain their responsiveness and secretory properties.

Islet cell transplantation, as previously described, has provided critical proof of principle that restoration of functional β cell mass in patients with T1D can restore nearly normal glucose metabolism, letting patients free from the burden of exogenous insulin injections. In such context thus the main goal of regenerative medicine is to find a way to replace the damaged β cells of diabetic patients by finding new sources of insulin expressing cells. These cells must meet some important criteria in order to be considered for possible transplants. The cells need to be easily expandable so as to grow large amounts of cells with minimum expenses, avoid rejection typical of allogeneic transplants and be easily controllable in term of genetic stability. The maintenance of long-term efficacy and functionality of the cells post engraftment is another important issue.

In recent years, many progresses have been documented in this field and stem cell-based therapies now represent an attractive strategy to address this problem. Intensive research is nowadays being conducted to look for alternative sources of β cells and stem cells are regarded as the key target. Regenerative medicine has focused on the potential use of stem cells for the treatment of several compromising degenerative diseases since the discovery of their existence back in the '60s. The use of stem cells is promising for the cure of type I diabetes due to both their regenerative capabilities and immunological properties[27].

3.2 Stem cells

Stem cells are known to be special cells defined by two fundamental

characteristics. First, they are unspecialized cells capable of long-term self-renewal through cell division. Self-renewal is the process by which a stem cell divides into two different daughter cells by asymmetric division. One of the daughter cells will keep the undifferentiated state while the other one starts the differentiation process towards a specific cell lineage. The second fundamental property of stem cells is their plasticity, also defined as pluripotentiality, intended as the ability, under certain physiologic or experimentally-induced conditions, to differentiate into any of the three germ layers (endoderm, mesoderm, ectoderm) and give rise to any fetal or adult cell type with determined functions.

Stem cells can be basically divided into distinct groups based on the degree of their plasticity (totipotent, pluripotent, multipotent) and their origin. Totipotent stem cells can give rise to any embryonic tissue, comprised the extra-embryonic, and adult tissue. They can actually give rise to the whole individual. Pluripotent stem cells are those that can give rise to any cell of the three germ layers while multipotent cells are partially committed progenitors, usually resident in different adult tissues, that can differentiate into multiple but still limited cell types. One main distinction is between embryonic stem cells (ESC) and “somatic” or adult stem cells (ASC).

ESC derive form the inner cell mass of a blastocyst at 5 days post fertilization. They typically express markers of pluripotency such as Oct 4, Nanog-1, Sox-2, which code transcription factors involved in self-renewal and are associated with the maintenance of the undifferentiated state[28]. A landmark breakthrough of pluripotent stem cell research was made in 2006 when Takahashi and Yamanaka developed a protocol to induce adult somatic cells to become pluripotent stem cells, also known as induced

pluripotent stem cells (IPSC), by insertion of pluripotent genes within the DNA of a somatic cell, including Oct 3/4, Sox 2, C-Myc and Klf4[29].

ASC are undifferentiated cells found among differentiated cells in a tissue or an organ of the adult body. They localize in the so-called “stem cell niche” where the surrounding environment protect them from differentiation. ASC are multipotent cells and under certain conditions can differentiate to yield some or all of the major specialized cell types of the specific tissue. Thus, their primary function is to maintain and repair the tissue in which they are found. The first studies involving ASC date back to the early '60s when scientists discovered that bone marrow contains at least two kind of stem cells, hematopoietic stem cells (HSC) which form all the type of blood cells and bone marrow stromal cells, also known as mesenchymal stem cells (MSC).

Another important source of stem cells is represented by cord blood stem cells (CB-SC). They are harvested at birth from the umbilical cord and include undifferentiated progenitors cells capable of self-renewal for many cycles. Cord blood is reported to be source of various stem cells including HSC, MSC and very small embryonic-like stem cells (VSEL)[30].

3.3 Stem cells for β cell regeneration: state of the art

The use of stem cells holds great promise for the future treatment of T1D due to their regenerative capabilities and, in some case, immunological properties. Within β cell replacement therapy, the main goal is to find the proper source of stem cells that can be directed to produce insulin. However, different cell-based approaches might be addressed to achieve solutions to the problem. Such solutions could space from generating new

implantable insulin-producing cells (IPC) that can be easily grown on large scale to protecting endogenous endocrine cells and favoring endogenous *in vivo* regeneration by use of stem cells with immunomodulatory properties, or either a combination of both.

In order to obtain fully functional IPC, many attempts have been made, using different types of stem cell, in which the main steps of pancreas development, occurring during embriogenesis, are followed as we learn from developmental biology. Many studies have focused on deriving β cell progenitors from pancreas and pluripotent stem cells. The success of β cell regeneration depends on manipulation of *in vitro* culture conditions and the induction of key regulatory genes implicated in pancreas development[31].

A successful approach could be either replace damaged β cells with *in vitro*-derived insulin expressing cells or preserve the remaining β cells and possibly restore endogenous β cell function mainly by “activation” of progenitor cells which are known to reside in the pancreas, both within endocrine and ductal tissue[32,33].

However, the challenge of a successful stem cell-based therapy for the treatment of diabetes mellitus is not only to generate *in vitro* or *in vivo* functional β cells but also to overcome the immune response both in terms of autoimmunity and rejection of allogeneic tissue.

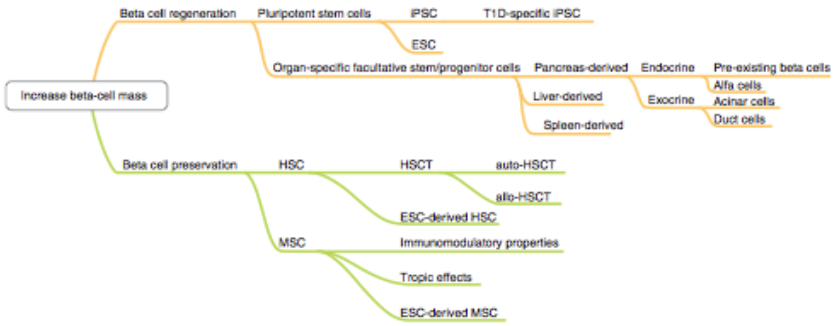


Fig. 2 Schematic representation of research directions for stem cell-based therapies to treat diabetes mellitus. The diagram shows different stem cell sources and their possible application for beta cell regeneration or endogenous preservation [34].

The induction of endocrine stem/progenitor cells in pancreas to differentiate into IPC cells could become a viable therapeutic approach. Even so, any effort to replace β cells in diabetes will require an approach for dealing with recurrent autoimmunity in patients with T1D[34]. Thus, the amelioration of autoimmune stress together with stimulus for regeneration of endogenous β cells would be the key to a successful therapy.

To date, a number of protocols have been designed to generate IPC *in vitro* using pluripotent stem cells from different sources, including human and mouse ESC, IPSC, CB-SC and bone marrow-derived MSC[35]. Despite the results so far achieved, multiple issues regarding the use of different sources of stem cells still need to be addressed, when considering both regenerative and immunological use of stem cells. Main concerns regard the possibility of generating sufficient amounts of IPC that are able to maintain secretory properties and responsiveness to glucose stimulation and the necessity of finding ways to promote long-term survival of the newly generated IPC after transplantation.

3.3.1 Embryonic Stem Cells (ESC) and Induced Pluripotent Stem Cells (IPSC)

One of the first studies describing derivation of IPC from ESC was published in 2000 by Soria *et al*[36]. ESC-derived IPC were selected by means of a neomycin-resistant gene placed under the insulin promoter's transcriptional control. Such obtained IPC were afterwards transplanted under the kidney capsule of streptozotocin (STZ)-induced diabetic mice, leading to a transient correction of the hyperglycemic state. The result was though quite controversial and raised doubts because surgical removal of the grafts didn't cause hyperglycemia. In other studies, where ESC-derived IPC were transplanted under the kidney capsule or into the spleen of diabetic mice, despite a transient correction of glycemia, formation of tumors was observed in the kidney and spleen of some animals[37].

In 2001, Lumelsky *et al* proposed to produce highly enriched nestin-positive cells from embryoid bodies under experimental conditions that minimized the differentiation to neuronal progeny[38]. Implantation of these cells into diabetic mice was associated with a time-dependent improvement of glucose metabolism, even if formation of teratomas was documented[39]. In 2006, D'Amour *et al* described a novel five-step protocol for the differentiation of human ESC into IPC, mimicking as closer as possible the physiological multistep process of pancreas development[40].

Recently, it was demonstrated that ESC-derived pancreatic endoderm-like cells, transplanted into diabetic mice, were able to migrate to the damaged pancreas and initiate *in vivo* a spontaneous differentiation to IPC, resulting in a long-term correction of hyperglycemia up to 40 days post transplantation[41].

It is clear that further investigations are anyway required to better understand the mechanism of *in vitro* maturation of IPC and to improve differentiation protocols in order to increase the yield of functional differentiated cells and promote long-term survival and function of ESC-derived IPC.

The two actual major limitations in the use of ESC for future clinical trials are the risk of tumor development and a lack of glucose responsiveness, which is a characteristic of fetal β cells. It has been shown that ESC can give rise to teratomas and teratocarcinomas in humans[42].

Generation of IPC comparable to functional β cells by differentiation of IPSC is far from being achieved. It was recently reported the possibility of obtaining IPC from dermal fibroblasts of T1D patients[43]. Researchers demonstrated that β cells derived form IPSC were c-peptide positive and able to release insulin in response to stimulation with low and high levels of glucose[44].

The use of IPSC to generate β cells could be advantageous for it would allow to overcome ethical concerns related to the use of human ESC and problems of post-transplant rejection. It would offer the opportunity of generating cells that are specific for the single patient. However, the negative drawback regarding the applicability of IPSC for cell-based therapies is their genetic instability. It was demonstrated that IPSC can acquire genetic abnormalities and carry chromosomal aberrations[45]. Not to mention the fact that their pluripotency is induced by protocols that require viral transfection, adding an additional risk for the translation of such approach to the clinical practice.

3.3.2 Mesenchymal Stem Cells (MSC)

Mesenchymal stem cells (MSC) have been shown to differentiate into various lineages, are readily available and easy to grow and manipulate, making them an attractive source for regenerative cell replacement therapy for T1D.

The ability of both human and mouse MSC to differentiate into IPC has been extensively investigated through development of *in vitro* differentiation protocols and *in vivo* transplantation protocols in murine models.

One of the first studies, published in 2003, showed that c-kit positive murine bone marrow-derived MSC (BM-MSC) were able to initiate endogenous pancreatic tissue regeneration after transplantation into chemically-induced diabetic mice, showing that these cells can contribute to the restoration of organ function[46].

More recently, a method was established to induce differentiation of murine BM-MSC into IPC after 6 days of culturing with conophylline and betacellulin-δ4 to accelerate the differentiation process. When transplanted into diabetic mice, the cells markedly reduced plasma glucose levels up to 4 weeks post transplant[47].

In 2009, Chandra *et al* demonstrated that adipose tissue can also be a potential source of MSC with the generation of pancreatic hormone-expressing islet-like cell aggregates (ICA) from murine adipose tissue-derived stem cells in a three-step protocol[48].

This result led to further investigate the potential role of human adipose tissue as a source of stem cells able to form functional islet-like cell aggregates. Transplantation of mature hormone-expressing ICA into

diabetic mice resulted in restoration of glucose levels near normoglycemia within 3-4 weeks post treatment[49].

Other protocols for the differentiation of MSC to IPC require transfection of cells with sequences of transcription factors involved in pancreas development. IPC were obtained by transfecting human MSC with an adenovirus containing integrated human Pdx1 cDNA, a pivotal factor for pancreatic commitment, followed by specific culturing conditions[50]. These cells showed the ability to produce insulin *in vitro* and when transplanted under the renal capsule of STZ-induced diabetic mice were able to normalize glucose levels by 14 days post transplant. Despite this result, the use of viral transfection to induce cell differentiation still represent a strong limit for the application of such strategy in the clinic. However, IPC were also obtained from human MSC collected from diabetic patients following a three-step protocol without the need for genetic manipulation[51].

Another recent study reports the possibility of differentiating MSC derived from rat bone marrow into pancreatic endocrine and ductal cells with an effective multistep *in vitro* protocol without using viral transfection[52].

Few clinical trials in which MSC are being administered to T1D patients are ongoing. The first one is a US based trial on the use of allogeneic MSC, a commercial formulation produced by Osiris Therapeutics, to determine the safety and efficacy in T1D patients. Other two trials, respectively in China and Europe, have been planned in order to evaluate the efficacy of autologous transplantation of MSC and have already started recruiting T1D candidates[35].

3.3.3 Hematopoietic stem cells (HSC)

Hematopoietic stem cells (HSC) are multipotent stem cells that can be readily harvested from bone marrow, cord blood, peripheral blood and fetal tissue[53].

They are able to differentiate to myeloid lineages (macrophages, erythrocytes, granulocytes) and lymphoid lineages (natural killer, T and B cells) of the immune system[54].

Due to their immunological properties, HSC have already been used for the treatment of autoimmune diseases[55,56] and have been extensively used for leukemia treatment in the clinic. In fact, growing evidence suggests that autologous HSC can induce central and immunological tolerance *per se*[57], thus showing promise for the treatment of T1D, eventually in combination with the administration of other stem cells, either differentiated *in vitro* into IPC or that can promote endogenous regeneration, as previously discussed.

HSC themselves are not considered as a potential source of IPC since *in vivo* transplantation in either human or animal models didn't determine β cell regeneration[58]. It is more likely that these cells might contribute to facilitate endogenous β cell regeneration from endocrine progenitors *in situ.*

In 2003, Voltarelli *et al* initiated a phase I/II trial to study the use of HSC for the treatment of diabetes mellitus. The main goal of the project was to evaluate and determine the safety and metabolic effects of high-dose immunosuppression followed by autologous nonmyeloablative HSC transplantation in newly diagnosed T1D patients[59]. During a mean follow-up of 18 months, 14 patients out of 15 became insulin free for a certain

period of time. In all, except one, patients HSC transplantation induced prolonged exogenous insulin independence. However, still several issues remain to be clarified, especially with regards to the potential contribution of concomitant immunosuppression[56].

3.3.4 Endogenous precursors

As previously mentioned, another line of research might focus on the potential of endogenous pancreatic progenitors. Despite the initial hypothesis that β cells could regenerate by division of pre-existing β cells or differentiate from exocrine progenitors, recent studies proposed an alternative mechanism in which newly formed β cells differentiate primarily from glucagon-expressing α cells present within the islets[60,61]. Protection of remaining functional β cells and/or activation of endogenous progenitors by means of stem cell therapy might represent an alternative to pancreatic regeneration for the treatment of diabetes mellitus.

To conclude, many encouraging results have emerged in the last years about the use of stem cells for the treatment of T1D, rising at the same time concerns about the safety use of different kind of stem cells.
Pluripotent stem cells such as ESC or IPSC bring both ethical and safety concerns. On the other hand, adult stem cell are already partially committed cells, which makes them less reliable for a wide range use in different applications and therapies. Choosing the right source is not an easy task. The main goal is to find a source good enough to provide large amount of cells, which are safe, easily expandable, do not raise ethical controversies and give standardized repeatable results.

4. Amniotic fluid: an alternative source of pluripotent stem cells

In recent years, amniotic fluid (AF) has emerged as a new potential source of pluripotent stem cells. AF mostly derives from fetal urine and lung secretions, and due its contact with the developing fetus it contains a wide variety of cells, most of which still poorly known. The presence of putative pluripotent stem cells within the AF was evidenced in 2003 in a study based on the analysis of the expression of the gene Oct-4, a distinct marker of pluripotency expressed by embryonic stem and germ cells[62]. Oct-4 positive cells were found in human AF specimens and in such samples mRNAs of three genes, known to be expressed in pluripotent human stem cells, were detected: stem cell factor, vimentin and alkaline phosphatase. Moreover it was demonstrated that Oct-4 positive cells were able to actively divide.

In 2007, a population of pluripotent stem cells isolated from AF and expressing the surface antigen c-kit (CD 117) was described and proposed as a new potential source for therapeutic application[63]. c-kit is a surface marker expressed by stem cells of mesenchymal origin and it is specific for the stem cell factor. Amniotic fluid stem cells (AFSC), c-kit positive, represent 1% of all the cells present in amniocentesis specimens. AFSC can be easily isolated by AF specimens through immunomagnetic selection with no harm for the fetus and can be expanded in culture in viable lines for many passages. AFSC grow with a doubling time of 36 hrs and present an *in vitro* fibroblast-like morphology.

AFSC pluripotency has been confirmed by their capability to give rise to cells of the three germ layers. AFSC were demonstrated to be able to

differentiate *in vitro* into adipocytes, myocytes, neurons and bone[63]. AFSC stained positive for a number of markers typical of mesenchymal and neural stem cells such as CD29, CD44, CD73, CD90, CD105.

AFSC present advantages over other sources of stem cells. They can be harvested from amniocentesis specimens with no harm to the developing fetus, thus not raising ethical issues typical of ESC, and can also be easily expanded *in vitro.* Moreover, previous studies involving their use *in vivo* demonstrated that they do not form teratomas, making them a suitable source for future cell-based applications in the clinic.

AFSC have already showed their therapeutic potential in terms of functional protection and damage reduction in models of acute renal injury[64] and chronic kidney disease such as Alport syndrome[65].

Perin *et al* demonstrated that human AFSC injected in the kidney of an immunodeficient model of acute tubular necrosis provide a protective effect, as reflected by a decrease in creatinine and BUN blood levels and reduction of damage, in the acute necrosis phase by modulating the kidney immune milieu[64]. In 2008, Carraro *et al* showed *in vivo* integration and differentiation of AFSC into murine injured lungs[66].

Based on these previous results it is to believe that the use of AFSC also holds the potential for the therapeutic treatment of type I diabetes.

AIM OF THE PROJECT

Type I diabetes is a major health problem whose incidence is rapidly increasing and has already reached epidemic proportions. It is estimated that almost 26 million people, only in the United States, are affected by this severe metabolic disorder. T1D is a complex pathology with a multifactorial etiology which is what has made challenging to find a cure so far. The current therapies are limited to the administration of exogenous insulin and donor islet transplantation. None of such treatments though has met yet the demand for a suitable widespread therapy that could overcome the limits and negative side effects related to conventional treatments, such as scarcity of donors and risks of immunosuppressive regimens in the case of transplant therapy and the difficulty of exogenous insulin therapy to keep blood glucose levels under constant strict control. Not to mention that pancreas functionality is never completely re-established.

There is therefore an urgent need for a tissue replacement therapy that can restore pancreas functionality, thus preventing patients from dependence on exogenous insulin or allowing overcoming the negative burdens that come from islet transplantation.

Stem cell therapy could provide a solution to the problem and has emerged in recent years as a new candidate for future clinical therapy.

In such context, our efforts have focused on the evaluation of the therapeutic potential of a new source of stem cells, recently discovered to reside in the amniotic fluid, for the treatment of a murine model of type I diabetes. The aim of the project was to determine, through an *in vivo* approach, the capacity of amniotic fluid stem cells (AFSC) to functional

restore and protect from damage the pancreatic tissue in an acute model of chemically-induced diabetes, by direct intracardiac injection of the cells into the mice. We investigated the potential of a single or double injection of AFSC to determine whether mice showed amelioration of physiological parameters such as circulating glucose levels.

We looked at AFSC integration into the host target organ in order to understand a possible mechanism of action of the cells and determine whether the cells act by differentiation into functional beta cells or by protecting pre-existing beta cells from damage and/or stimulate endogenous progenitors by modifying the local microenvironment.

MATERIALS AND METHODS

1. Amniotic fluid stem cells

1.1 Expansion of human amniotic total cell population

Under Institutional Review Board approval of Children's Hospital Los Angeles, 28 human amniotic specimens were obtained from discarded amniocentesis fluid between 15 and 20 weeks of gestation. Samples with normal karyotype and normal fetal ultrasound were collected from discarded cultures (Genzyme Pasadena, CA). Cells were expanded in tissue culture dishes (BD Falcon, Franklin Lakes, NJ) with the following culture medium: Chang's medium containing α-MEM supplemented with 20% Chang B, 2% Chang C (Irvine Scientific, Santa Ana, CA), L-glutamine, 20% of ES-FBS and 1% of Pen/Strep antibiotic (Gibco/Invitrogen, Carlsbad, CA). Cells were cultured under humidified conditions at 37°C and 5% CO_2 and passaged up to 50 passages by trypsinizing using trypsin 0.25% EDTA (Gibco/Invitrogen, Carlsbad, CA).

1.2 Selection of c-kit (CD117) positive human amniotic fluid stem cells

Selection of c-kit positive amniotic fluid stem cells is a well established protocol in our laboratory.

Human amniotic fluid stem cells positive for the c-kit receptor (CD117) were selected by standard magnetic cell sorting using the CD117

Microbead Human Kit (Miltenyi Biotech. Inc.), following manufacturer's instructions.

Cells of a total population were expanded under previously described culturing conditions up to 80% confluence. Around $1x10^6$ cells were collected by trypsinization and centrifuged at 1500 rpm for 5 minutes. After discarding supernatant, cells were resuspended in 300 µl of buffer solution, PBS containing 2% ES-FBS (Gibco/Invitrogen, Carlsbad, CA). FCR blocking reagent (100 µl) and CD117 Magnetic beads (100 µl) were then added to the cell suspension, properly mixed and incubated at 4 °C under shaking conditions for 20 minutes. Terminated the incubation time, cells were centrifuged, the supernatant discarded and cells washed with the buffer solution. The passage was repeated twice and cells were finally resuspended in a volume of 500 µl of buffer prior to magnetic selection.

The selection column was washed with buffer and cell suspension added to the column under magnetic conditions in order to elute negative selected cells. The column was afterwards removed from the magnetic apparatus and positive cells were eluted with 1 ml of the same ES-FBS-containing buffer solution.

Clones were then cultured in Petri dishes in medium containing α-MEM supplemented with 20% Chang B and 2% Chang C solutions, 20% Fetal Bovine Serum, 1% L-Glutamine, and 1% antibiotics (Pen-strep, Gibco-Invitrogen).

2. *In vivo* methods

2.1 Immunodeficient murine model

Immunodeficient NOD/SCID (NOD.CB17-Prkdc^{scid}/J) mice were purchased from the Jackson Laboratory. These mice lack of both functional T and B cells and represent an ideal model for cell transfer experiment with allogeneic grafts. All the procedures described and animal protocols were approved by the IACUC at Children's Hospital Los Angeles (CHLA). IACUC is the Institutional Animal Care and Use Committee on charge of overseeing CHLA's animal programs, animal facilities and policies, ensuring appropriate care, ethical use and humane treatment of animals.

2.2 Induction of diabetes

To induce diabetes, 8 week-old male NOD/SCID mice were treated following a protocol of multiple low dose (MLD) injections of streptozotocin (STZ). STZ is a chemical, analogue of glucose, selectively toxic for pancreatic β cells. STZ enters the cells via the GLUT2 glucose transporter and it has been proven that the main reason for the STZ-induced β cell death is alkylation of DNA[67]. STZ is also a nitric oxide donor[68] and generate other reactive oxygen species that also contribute to DNA fragmentation[69]. Such protocol is often used instead of a single high dose injection of STZ, which is known to cause direct necrosis of the tissue rather than apoptosis as in the case of the MLD-STZ, whose effect is more similar to the diabetic condition.

Briefly, mice were given STZ (2-Deoxy-2-(3-methyl 1-3-nitrosoureido)-D-

glucopyranose, STZ; Sigma-Aldrich, Saint Louis, MO) 50 mg/kg body weight by intraperitoneal (IP) injection, for three consecutive days. Mice were fasted 4 hours before each injection and STZ dissolved in 50 mM sodium citrate buffer, pH 4.5, and injected into mice within 5 minutes from preparation. A health control group did not receive any treatment.

2.3 Glucose measurement

Mice were monitored for blood glucose level starting from the first day of experiment on regular intervals of time, every two days for the first week after treatment and once a week later until the end of the experiment. Mice were fasted 4 hrs in the morning before each measurement. Blood glucose was measured from the tail vein using a OneTouch UltraMini Blood Glucose Monitoring System (Lifescan). The sensitivity of the system does not exceed 600 mg/dL, thus in some cases the extent of hyperglycemia resulted over the limit of sensitivity of the instrument.

2.4 Preparation of AFSC for *in vivo* injections

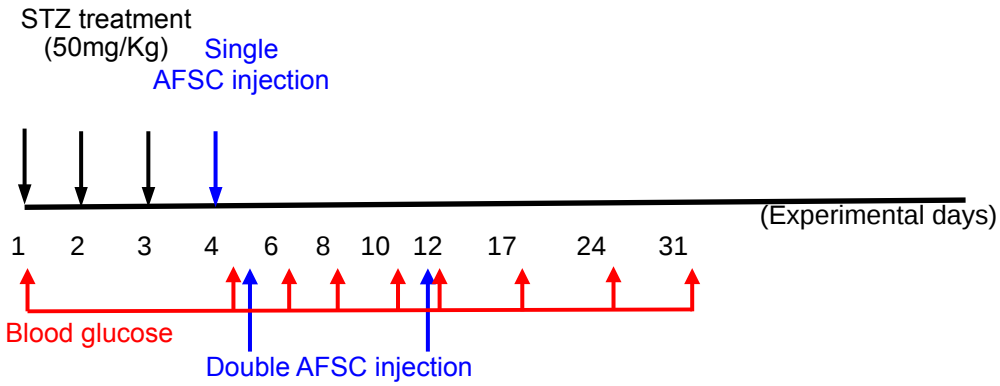
Prior to injection, c-kit positive AFSC were expanded in 150 mm Petri dishes (Corning) using the previously described culturing medium. Cells were passaged when reaching 70% confluence to get the required amount of cells. On the day of injection, cells were trypsinized, centrifuged at 1500 rpm for 5 min and, after counting, labelled with the cell tracker CM-Dil (Molecular Probes), following manufacturer's instructions. CM-Dil is a membrane stain well retained through fixation/permeabilization processes and allows tracking of cells after injection. Briefly, cells were resuspended

in 500 µl PBS and incubated with a working solution of Cm-DiI solution of 1 mg/ml for 5 minutes at 37 °C, followed by an incubation of 15 minutes at 4 °C. Cells were finally rinsed three times with PBS and loaded on sterile U-100 28 G1/2 insulin syringes (Becton Dickinson).

2.5 *In vivo* injection

STZ-treated mice received $1x10^6$ AFSC each. Cells were administered by intracardiac injection through the chest wall into the left ventricle using a 29 gauge needle. Mice were carefully monitored under isofluorane inhalation anesthesia. These procedures and protocols are approved by the IACUC at Children's Hospital Los Angeles. Mice were divided into two groups, one received a single injection of AFSC and the second received a double injection of AFSC. A control group of STZ-treated mice did not receive any cell injection. All animals, STZ-treated and STZ-treated AFSC-injected were compared to wild type.

Scheme 1 shows the experimental time-line. Mice receiving one cell injection were given cells on experimental day 4. Mice receiving the double dose were given cells on experimental days 4 and 11, to give the mice one week to recover from the first intracardiac injection. Mice were regularly controlled for circulating glucose level and sacrificed 4 weeks after AFSC injection.



Scheme 1 Time-line course of mice treatment

3. Histological and immunohistochemical analysis

3.1 Tissue processing

Mice were sacrificed using CO_2 inhalation as recommended by IACUC. Pancreata were removed and fixed in formalin 10%, neutral, buffer phosphate (Polysciences Inc.) for 45 min at 4°C and stored in 70% ethanol at 4°C until processing. Specimens were routinely processed as following described:

90 min in 95% ethanol

60 min in 100% ethanol, twice

40 min in toluene, twice

60 min toluene/paraffin (50:50)

120 min paraffin

Overnight paraffin

The following morning samples were included in paraffin in embedding cassette (Tissue-Tek) and prepared for sectioning. Pancreata were cut in 5

µm sections with a Leica RM2235 microtome and let to dry on a slide warmer (Lab-Line) at 37°C. Once dried, sections were ready to use for histological and immunostaining protocols.

3.2 Histological and immunofluorescence staining

For both histological analysis and immunostaining, sections were deparaffinized by immersion in HistoChoice Clearing Agent (Sigma) for 20 min, rehydrated in ethanol 100%, 90%, 70%, 50%, 30%, 5 min each, and rinsed in distilled water.

For morphological analysis, sections were stained in Hematoxylin solution (Fisher Scientific) for 90 sec, washed in distilled water and stained in Eosin Y solution (Harleco) for 30 sec. Slides were dehydrated in 90% and 100% ethanol, 5 min each, and mounted with xylene mounting medium (Fisher Scientific). Pictures were acquired under a Leica DM1000 light microscope.

For immunofluorescence staining, monoclonal anti-insulin+proinsulin (Abcam) and monoclonal anti-glucagon (Sigma-Aldrich) primary antibodies were used. No antigen retrieval was required for both antibodies and sections were treated as following described. Briefly, pancreatic sections were blocked in a solution of 2% BSA in PBS for 10 min at room temperature (RT) and incubated for 60 min with primary antibody (dil 1:500) at RT. After washing three times with PBS, 5 min each, bound primary antibodies were detected by a RT 30 min incubation with anti-mouse Alexa Fluor 555 (Invitrogen-Molecular Probes) secondary antibody (dil 1:200). After rinsing in PBS slides were mounted with Vectashield (Vector) mounting medium for fluorescence containing DAPI.

Images were captured by digital camera connected to a Leica AF6000 fluorescent microscope.

3.3 Insulin positive β cells count and islet mass quantification

Pancreata from wild type, STZ-treated and STZ-treated AFSC-injected mice were processed and cut as previously described. Different sections were cut at a distance of 100 μm and at least three slides were processed in order to pick 10 different islets/mouse for the counting. Slides were immunostained with anti-insulin antibody as previously described and images captured by fluorescence microscope. The number of hormone-expressing β cells was counted for each islet and normalized over the total number of cells within the islet.

For quantification of islet mass, again 10 different islets/mouse were considered. The total number of cells within the islet was counted. Values of STZ-treated, STZ-treated AFSC-injected mice were expressed as percentage in function of WT number of cell.

RESULTS

1. Selection of c-kit positive human amniotic fluid stem cells

C-kit positive amniotic fluid stem cells (AFSC) were selected from human total amniotic fluid cell population by use of immunoseparation technique. After selection, cells were expanded in Chang medium for several passages, maintaining their characteristic fibroblastoid shape, as shown in Fig.1. Cells were passaged with a dilution rate 1:2 when reaching a maximum confluence of 60-70 % in order to avoid cell-cell contact and possible cell differentiation.

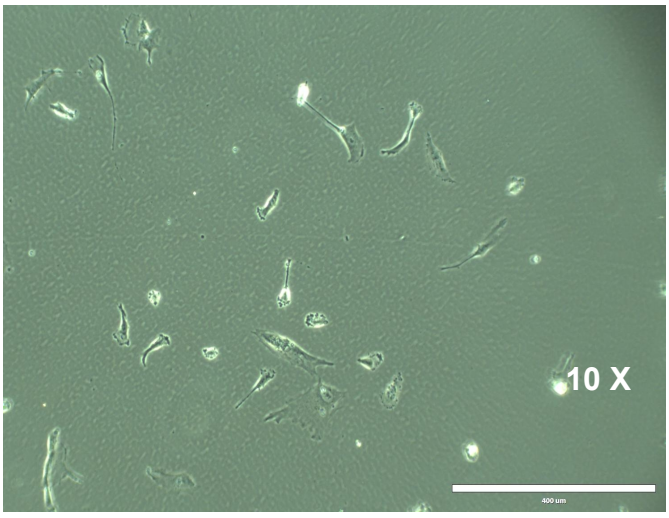


Fig. 1 Morphology of c-kit positive AFSC at light inverted microscope. The sample shown here refers to cells at passage 15, after selection. Cells still present their typical fibroblastoid shape.

2. Establishment of a chemically-induced murine model of T1D

For our study, we adopted a model of multiple low doses MLD-STZ induced diabetes in which mice were given 50 mg/kg body weight for 3 consecutive days. We found that such protocol allows having a more rapid and generalized response of the mice to the chemical treatment.

Mice (n=10) were divided into a control group and a group that received the STZ treatment. Mice were monitored starting on experimental day 1 for blood glucose concentration. Blood glucose was measured every 2 days for the first 10 days and once a week for the rest of experimental time up to 4 weeks post STZ treatment when mice were sacrificed.

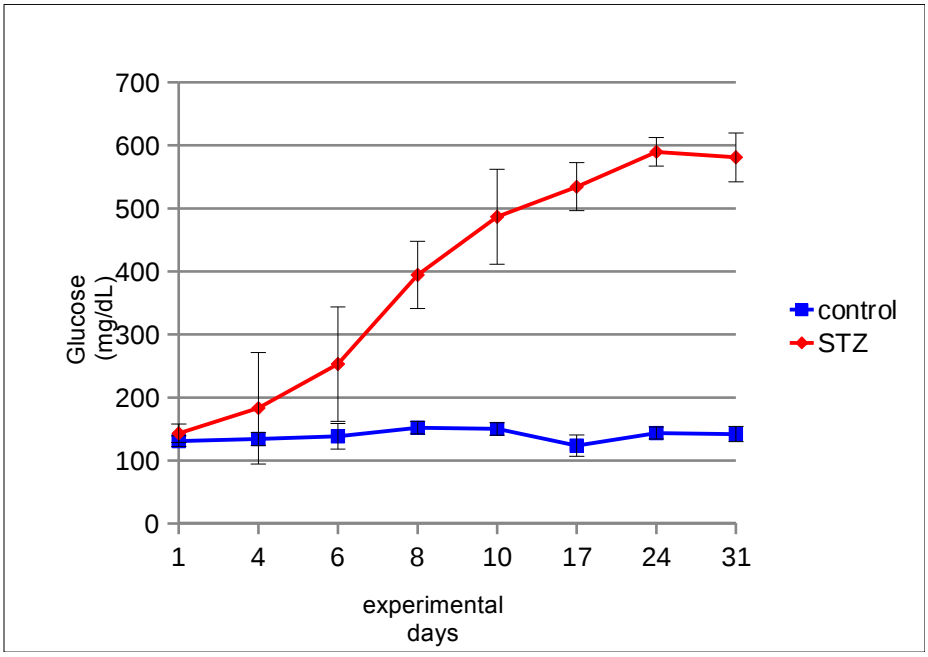


Fig. 2 Time-course progression of the disease. Blood glucose concentration of STZ-treated mice is compared to control animals over time, starting from the first day of STZ treatment.

All mice receiving STZ responded to the treatment developing an average hyperglycemic state by experimental day 8. Highest levels of blood glucose concentration were measured on day 24. Diabetic mice showed phenotypic alterations characteristics of hyperglycemic condition such as weight loss, polydipsia and polyuria. None of the mice though died from the treatment before the experimental deadline.

In Fig. 2, the time-course progression of the disease, as indicated by increase of blood glucose values of treated mice in comparison to control animals, is reported.

We established experimental day 4 as the onset of hyperglycemia, a time at which the drug has already started its action and blood glucose levels have started to increase but are still below the threshold value (250 mg/dL) that defines the hyperglycemic state when mice can be considered as diabetic.

3. Injection of AFSC

Mice were treated with STZ according to the protocol previously described and on day 4^{th} received a dose of $1x10^6$ AFSC/mouse by intracardiac injection into the left ventricle.

STZ-treated mice were divided into two groups. The first group (n=10) received one single AFSC injection on day 4. The second group (n=5) received a double AFSC injection on experimental days 4 and 12.

The aim was to evaluate whether the number of cells that are given to the mice might influence the therapeutic outcome and let mice a better recover. Mice were constantly monitored for circulating glucose levels during the course of the experiment at defined time points.

3.1 Single injection of AFSC

STZ-treated NOD/SCID mice received $1x10^6$ AFSC by intracardiac injection on experimental day 4, as previously specified. Cells were prepared and labelled with Cm-DiI immediately before the injection. As reported in Fig. 3, mice were monitored at defined time points for blood glucose level values up to 4 weeks after the injection, when mice were sacrificed.

Fig. 3 shows the physiological response of AFSC-injected mice compared to STZ treated mice that didn't receive cells. AFSC-injected mice showed average blood glucose levels slightly lower compared to STZ-treated control mice all over the experimental time.

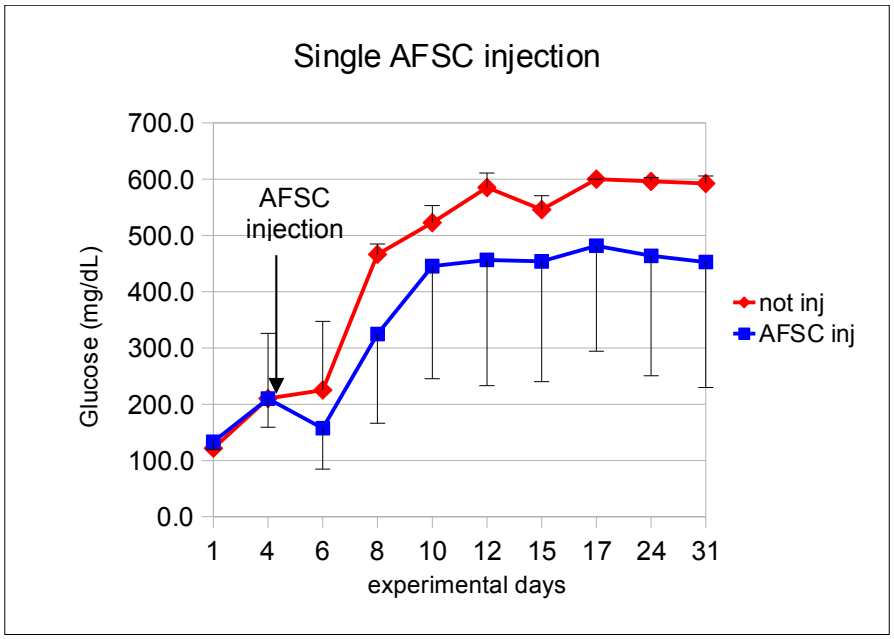


Fig. 3 Mice response to cell treatment by measuring circulating glucose levels. Treatment with a single AFSC injection shows slight improvement when comparing STZ-treated AFSC-injected mice to STZ-treated only mice

Looking into detail, Fig. 4, where only AFSC-injected mice are considered, only a few mice (n=2) were rescued and seemed to properly respond to the cell treatment showing average blood glucose levels, for the duration of the experiment, never exceeding the value of 250 mg/dL, threshold for the mice to be considered diabetic. Rescued mice also presented better phenotypic conditions when compared to diabetic mice that didn't receive cell injection. Unrescued mice developed a high hyperglycemia as observed in untreated diabetic mice. Injection of AFSC was able to prevent development of hyperglycemic state in onset recipient mice.

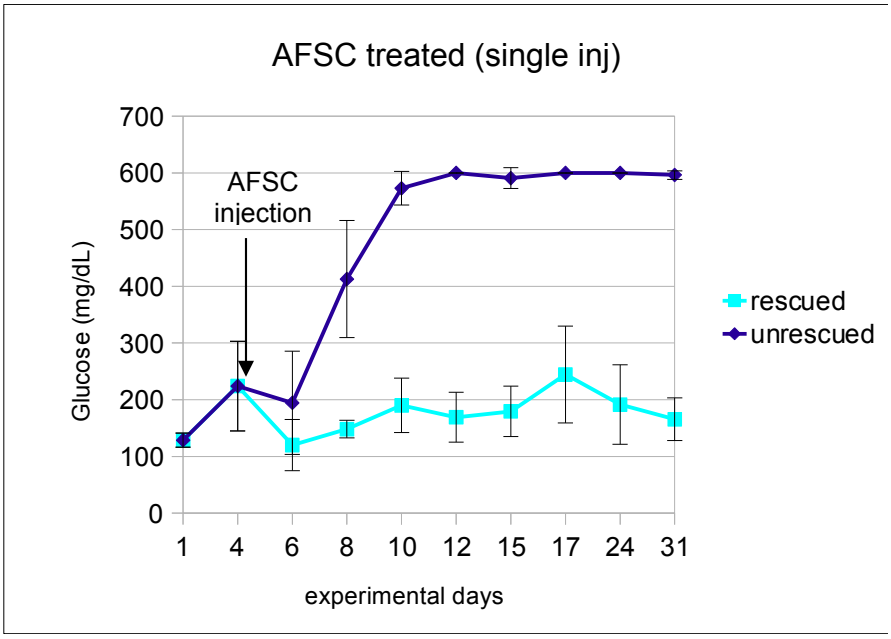


Fig. 4 Time course monitoring of blood glucose levels of rescued versus unrescued mice after one single AFSC injection

3.2 Double injection of AFSC

In order to evaluate whether a higher amount of cells could exert a stronger protective effect on STZ-treated mice, a double intracardiac injection of AFSC was performed on respectively experimental days 4 (onset of hyperglycemia) and 11. Mice received the second cell injection one week apart from the first one so as to let them recover between the two injections. The direct injection into the left ventricle of the heart represents an intensive procedure for the mice.

Monitoring of blood glucose concentrations at defined time points revealed an amelioration of the physiological parameter for the mice that received AFSC (Fig. 5). Two mice died after intracardiac injections. Again, rescued mice (n=2) were able to respond to AFSC treatment, as indicated in Fig. 6.

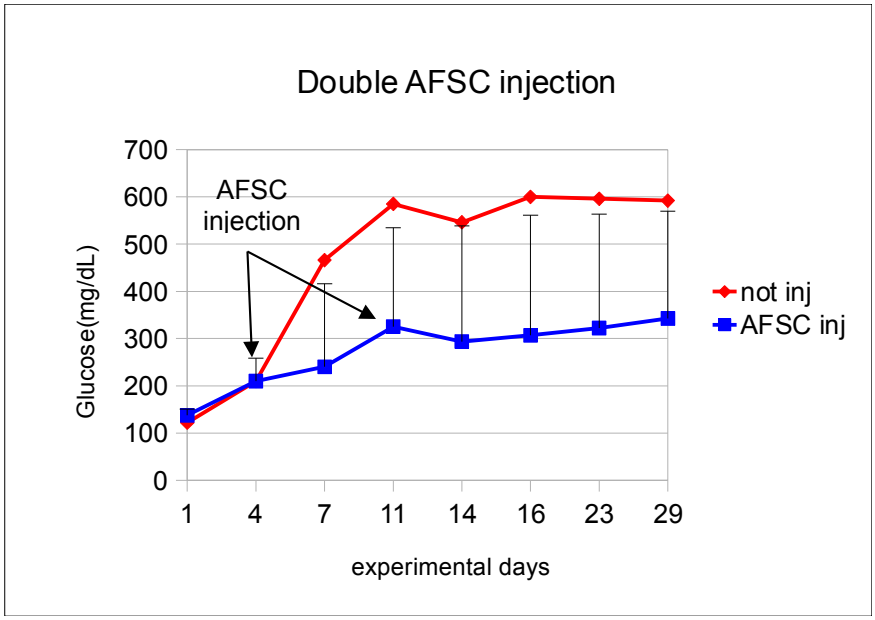


Fig. 5 Time course progression of the disease in STZ-treated control mice compared to STZ-treated mice receiving the double dose of AFSC.

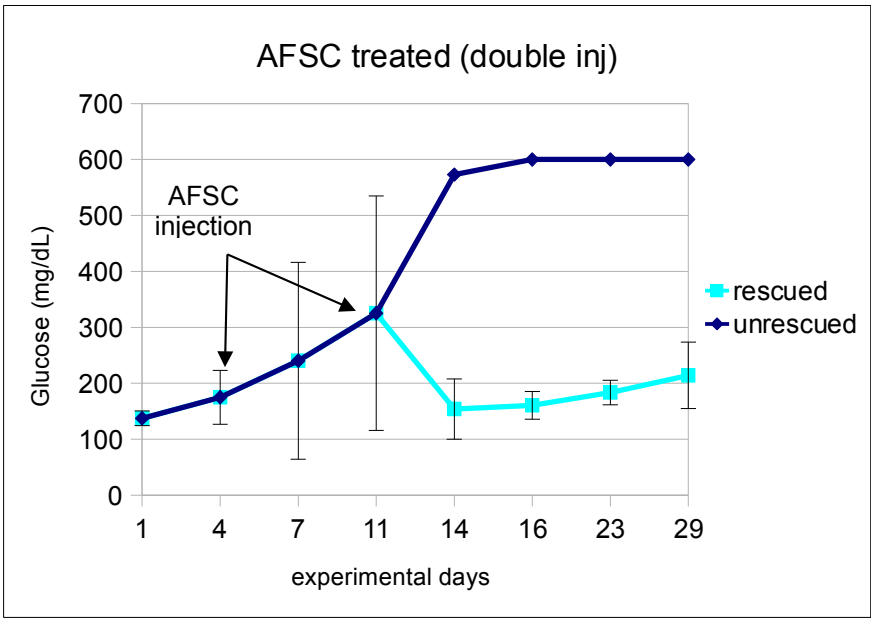


Fig. 6 Rescued mice versus unrescued after a double injection of AFSC. Rescued mice showed amelioration of blood glucose levels throughout the experimental time, maintaining an average normoglycemic state. On the contrary, unrescued mouse developed diabetes as STZ-treated control mice.

Single and double AFSC injection gave a similar result in terms of number of rescued mice that responded to cell treatment and development of the disease. The difference in the outcome among mice could be attributed to variations in the early phase (onset) of the disease progression. Mice are unlikely to be rescued if injected when blood glucose values has already exceeded the threshold value, underlining a preventing and protecting role of AFSC in onset recipient mice.

4. AFSC engraftment

AFSC were labelled with the dye Cm-DiI in order to be traced in the host tissue after injection. AFSC were identified in the exocrine portion of the pancreas of the injected mice 24 hrs post injection, as shown in Fig 7.

However, the amount of AFSC into the host tissue resulted to be very low. On average, ~10/15 AFSC could be detected per each pancreatic section. When mice were sacrificed at 4 weeks post injection, no AFSC were detectable in the pancreas.

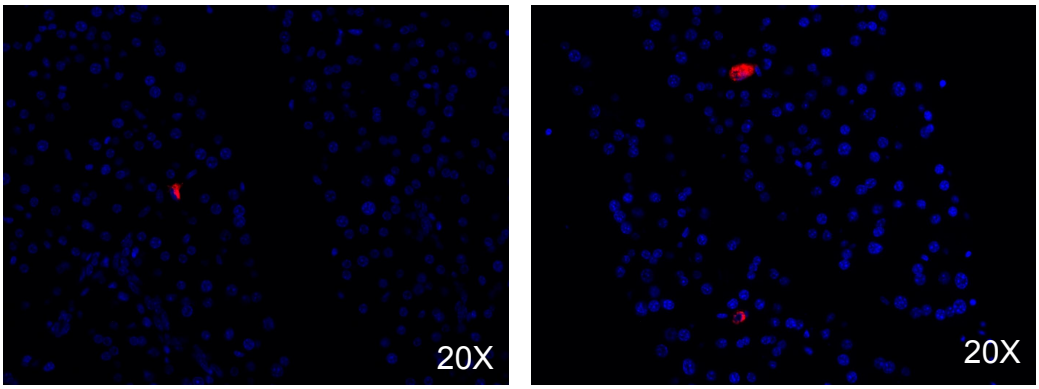


Fig. 7 Detection by immunofluorescent microscopy of Cm-DiI labeled AFSC in the pancreatic tissue of a double injected mouse, 24 hrs post injection. The red staining refers to engrafted AFSC.

5. Histological analysis

At the end of the experimental time (4 weeks p.i for single injected mice, 5 weeks p.i for double injected mice), mice were sacrificed and pancreata harvested for histological and immunohistochemical analysis.

5.1 Morphology

Fig. 8, which reports hematoxylin-eosin staining of pancreatic sections, shows the structure of pancreatic islets in control WT NOD/SCID mice compared to islets of STZ-treated mice and STZ-treated plus AFSC-injected mice.

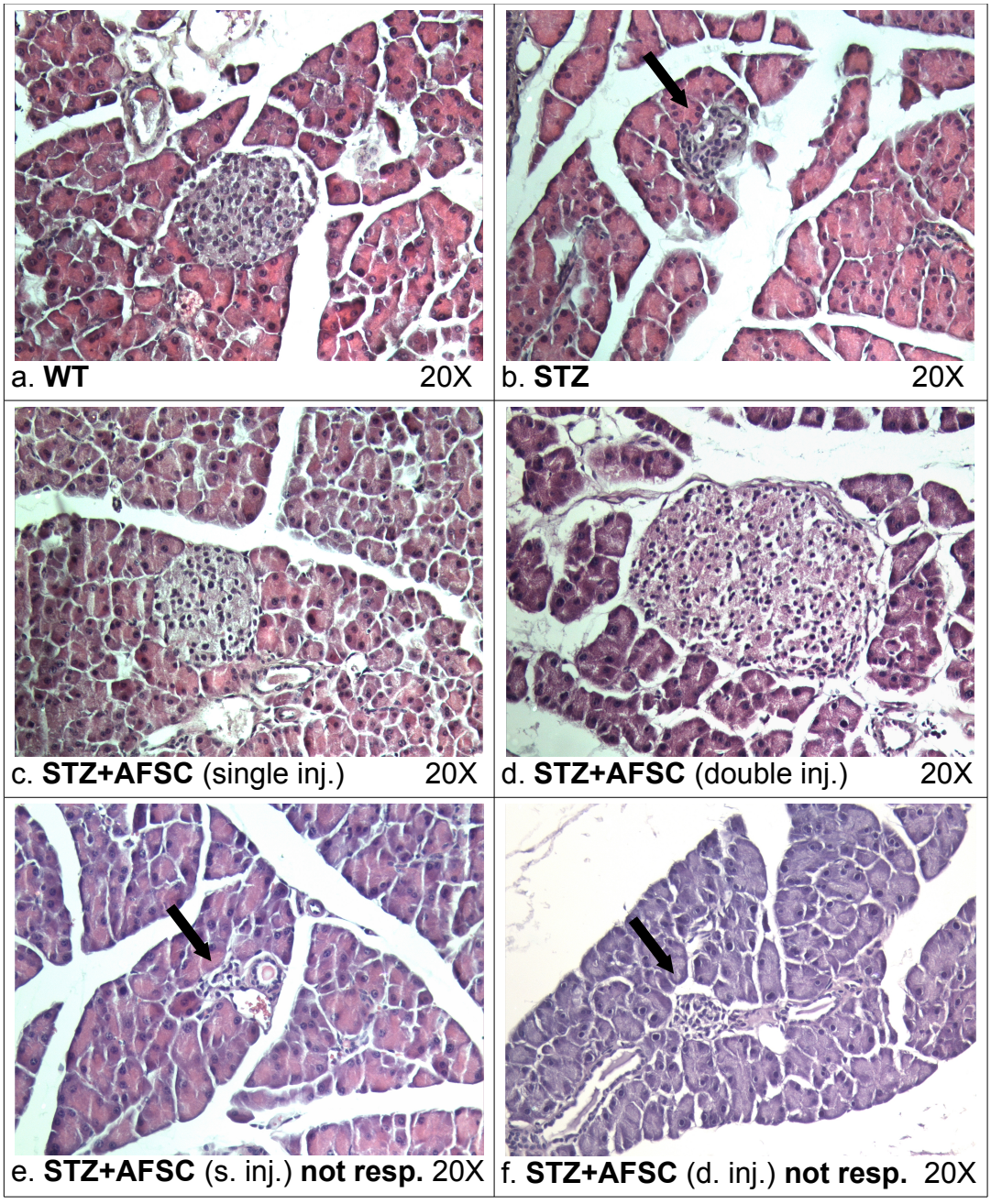


Fig. 8 Morphology of pancreatic islets stained with hematoxylin and eosin, 4 weeks post injection. a Representative pancreatic islet from a WT healthy control mouse. b Mass reduction and shrinkage of cell volume in islet from a diabetic mouse treated with STZ. c and d STZ-treated mice receiving AFSC show, in both cases, a normal islet morphology with no reduction of β cell mass. e and f Islets of unrescued mice that did not respond to AFSC treatment. As indicated by arrows, they present a strong reduction of β cell mass as similarly detected in STZ-treated mice.

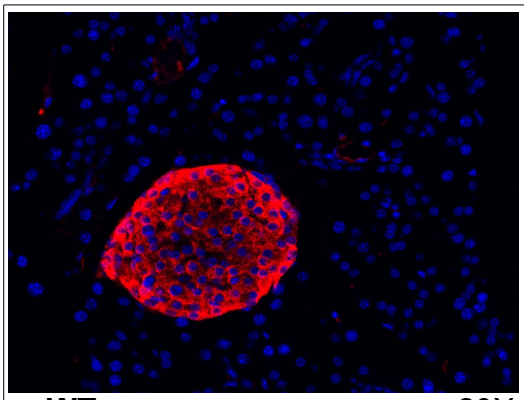
As it can be appreciated, endocrine pancreas from STZ-treated mice (Fig. 8b) showed a strong reduction of islet mass, due to destruction of β cell

caused by drug treatment, compared to WT (Fig. 8a). In contrast, islets from STZ-treated mice injected with AFSC (Fig. 8c/d, respectively single injection and double injection) presented a normal morphology, comparable to that of WT animals.

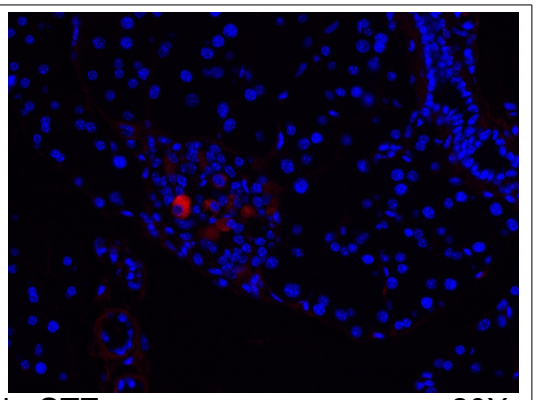
5.2 Expression of pancreatic markers: immunofluorescence

Pancreatic sections from WT, STZ-treated and STZ-treated AFSC-injected mice were immunostained for the endocrine pancreatic markers insulin and glucagon. As shown in Fig. 9, around 90% of the islet structure was characterized by insulin expressing β cells in the wild type (Fig. 9a), while STZ treatment brought to a drastic reduction of insulin expression in the islets (Fig. 9b).

STZ-treated mice injected with AFSC showed stronger expression of insulin compared to STZ-treated mice and very similar to that of wild type mice. STZ-treated AFSC-injected mice that did not respond to cell treatment showed instead reduced insulin expression (Fig. 9 e/f) as detected in diabetic mice.



a. **WT** 20X



b. **STZ** 20X

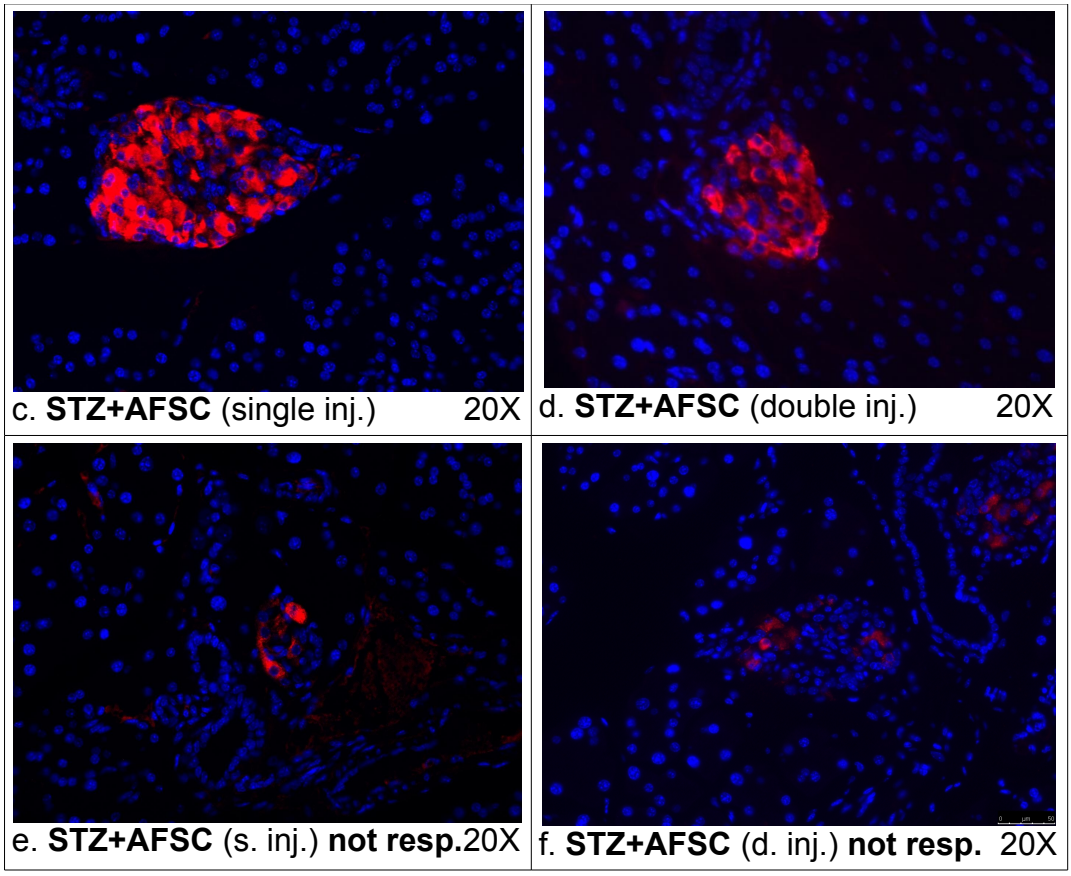


Fig. 9 Detection of insulin expression by immunofluorescence, 4 weeks post injection, in WT (**a**), STZ-treated (**b**), STZ-treated AFSC-injected (**c**, **d**) mice, AFSC-injected but not responsive mice **(e**, **f).** AFSC-injected mice show stronger expression of insulin compared to STZ-treated diabetic mice, where almost no production of insulin is detected.

Moreover, glucagon staining, as reported in Fig. 10, confirmed that AFSC-injected mice had a morphological distribution of hormone expressing cells that markedly resemble that of WT control animals. Glucagon producing cells are usually distributed on the external layer of the islet structure. In contrast, in STZ-treated mice the distribution of glucagon expressing cells resulted highly disorganized. In general, AFSC-injected responsive mice showed a proper islet organization and distribution of hormone expressing cells, architecturally similar to that of WT, with insulin-producing cells in the centre and glucagon-producing cells located peripherally.

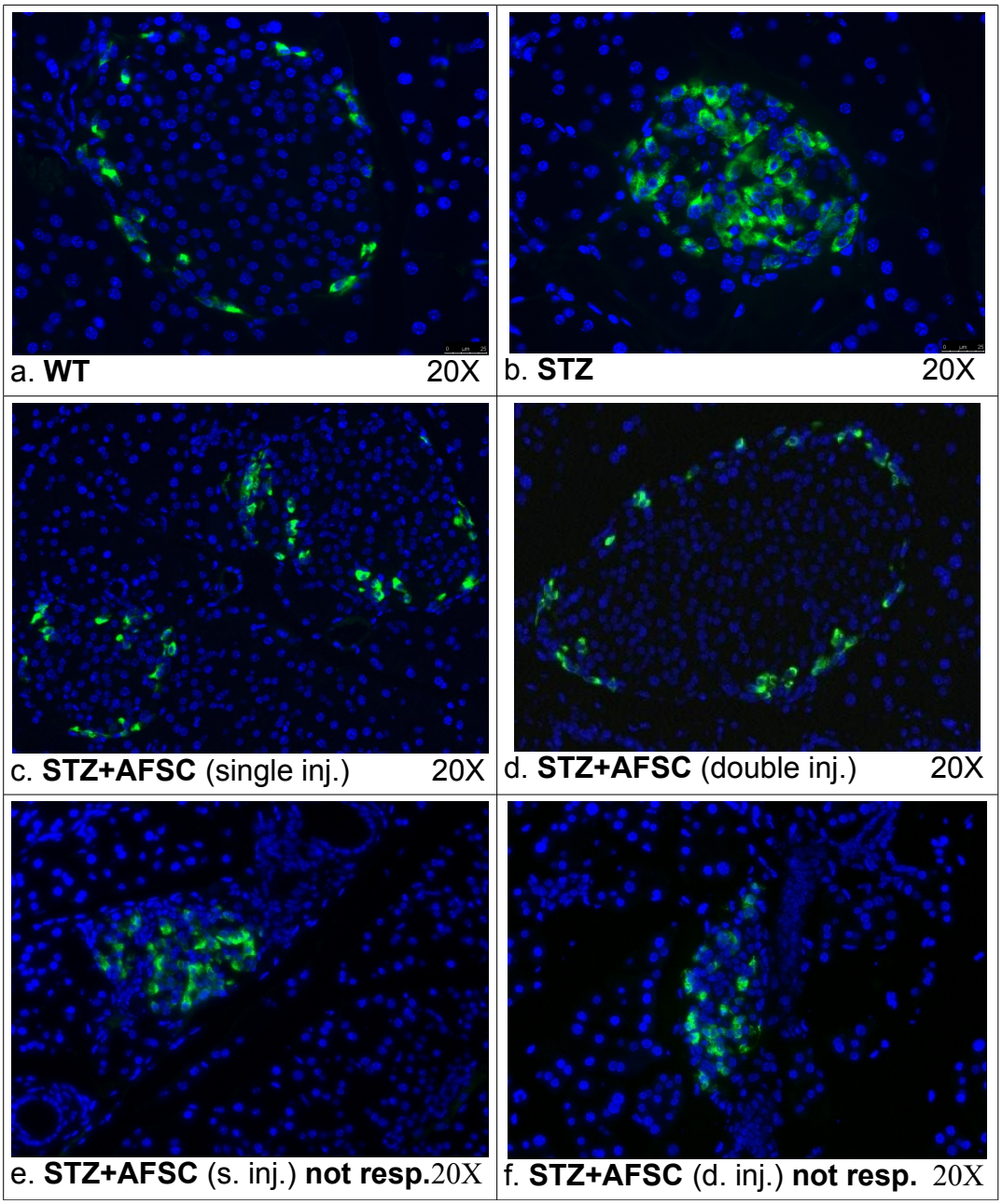


Fig. 10 Detection of glucagon-expressing cells by immunofluorescence: WT (**a**), STZ-treated (**b**) and STZ-treated AFSC-injected (**c**, **d**) mice, AFSC-injected not responsive **(e**, **f).** AFSC injected mice present a normal distribution of glucagon expressing cells as it is in WT animals. Distribution in diabetic mice is highly disorganized. Rescued mice **(c**, **d)** have a regular distribution of α cells, while expression in unrescued mice resembles that of STZ-treated animals.

6. Quantification of islet mass and insulin expression

The mass of pancreatic islets was quantified and result showed that STZ-treated AFSC-injected mice responsive to cell treatment presented a higher total mass when compared to diabetic STZ-treated mice or unrescued mice (Fig. 11). Interestingly, though, STZ-treated mice that received double AFSC injection but not responded to cell treatment and maintained high glucose level show anyway a slightly higher β cell mass than STZ-treated mice, indicating that maybe double injection might have a stronger protection for the preservation of β cell mass than single injection.

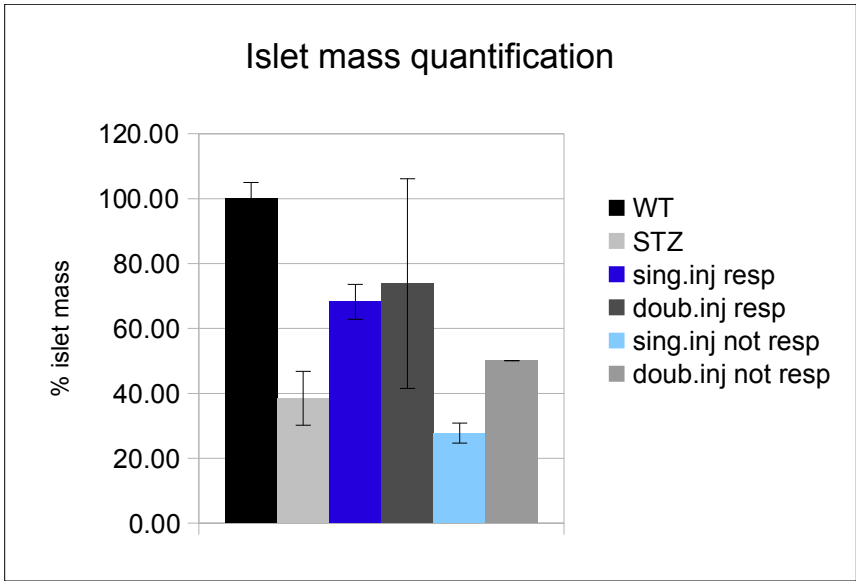


Fig. 11 Quantification of pancreatic islet mass. Values are reported as percentage and were normalized with respect to the total islet mass calculated in WT. Rescued AFSC-injected mice, either single or double, show higher β cell mass compared to STZ-treated and unrescued mice, though less abundant than in WT.

The number of insulin positive β cells within islets of WT, STZ-treated and STZ-treated AFSC-injected mice was quantified. As shown in Fig. 12, injection of AFSC was able to restore the number of insulin expressing cells in responsive mice but not all.

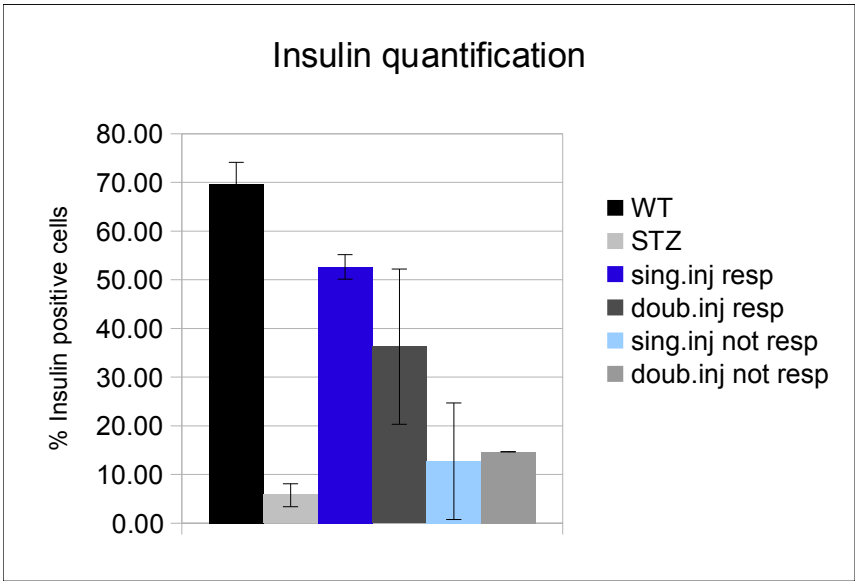


Fig. 12 Quantification of insulin expression in WT, STZ-treated and STZ-treated AFSC-injected mice. Injection of AFSC resulted in stronger insulin expression in rescued mice when compared to STZ-treated animals. Not responsive animals showed lower level of expression but still slightly higher than STZ-treated mice.

Expression of insulin appeared significantly higher in responsive mice that received AFSC injection than STZ-treated mice. AFSC-injected mice that were not rescued showed instead a lower insulin expression, though slightly higher when compared to STZ-treated only diabetic mice. Injection of AFSC resulted in some of the injected mice in an increased number of insulin-expressing β-cells, even though less abundant than in wild type animals, sufficient enough to rescue mice and prevent them from developing strong hyperglycemia.

DISCUSSION

Type I diabetes (T1D) results from a reduction of functioning β cells. It would be therefore of high importance for the diabetic having such loss restored by regeneration of functional insulin-producing cells. The common therapy for the treatment of T1D is the exogenous administration of insulin that so far has allowed many diabetics to survive and live with such disabling disease but whose efficiency can not make of it the ideal definitive therapy. On the other hand, islet transplantation therapy, that would free patients from the burden of exogenous insulin injections, can not meet the high demand of eligible patients and thus can not be regarded as a spreadable treatment for the future.

In the context of regenerative medicine, stem cell therapy has gained much attention as a reliable alternative solution to islet transplantation, whose main obstacle is the limited supply of donor islets. Stem cells have already been applied in animal models of T1D demonstrating that a reversion, even though partial, of the hyperglycemic condition is possible[46,70,71]. However, the mechanism by which *in vivo* pancreatic regeneration occurs by means of stem cell treatment still needs to be fully elucidated.

In the present study we used an *in vivo* approach to evaluate the potential of a novel source of stem cells, amniotic fluid, to reverse and restore functional loss in a murine model of chemically-induced T1D. Amniotic fluid stem cells (AFSC) are pluripotent stem cells capable of high expansion up to many passages and of differentiation to different cell lineages, as already demonstrated[63].

The potential of AFSC for *in vivo* application has already emerged in previous studies for the treatment of acute and chronic injuries in the kidney and lung[64,65,66]. It is therefore to believe that AFSC can also have the potential to reverse functional loss in a model of pancreatic injury.

In this project, we aimed to evaluate the therapeutic potential of human AFSC in an immunodeficient model of T1D. We first established an acute model of pancreatic injury by streptozotocin (STZ) treatment. In literature, different protocols of STZ treatment have been reported to induce diabetes in murine rodents. There are basically two methods, respectively the single high-dose injection of STZ and the multiple low dose (MLD) injections where STZ is administered in lower doses for 3 to 5 consecutive days. It has been demonstrated that while the single high dose injection is able to induce diabetes in a rapid manner due to a direct toxic effect of the drug and consequent necrosis of the tissue, MLD-STZ treatment induces a condition more similar to insulin-dependent type I diabetes in humans. MLD-STZ triggers inflammation processes that cause insulitis and apoptosis of β cells[72,73]. Based on the progression of the disease in diabetic mice we established day 4 as the onset of hyperglycemia. We hypothesized that giving AFSC during this time frame might give better result in term of recovery. Our hypothesis is that AFSC might act mainly by protecting already existing β cells from damage, thus preventing the establishment of the diabetic condition. From previous preliminary experiments it emerged that when mice receive cells at a later stage when blood glucose levels already exceeded the threshold value (250 mg/L), mice are unlikely to be rescued. Therapeutic action of AFSC could be more effective if cells are injected when the disease is at an early stage of progression. This, early intervention of AFSC, was elegantly confirmed by

two major publications in our laboratory. AFSC were able to protect acute kidney damage and restore activity of epithelial damaged renal cells preventing them from apoptosis[64]. In addition, early intervention was able also to rescue mice from progression towards chronic kidney failure[65].

Injections of AFSC, either single or double, were able to protect some of the STZ-treated mice from developing a strong hyperglycemia. From our results we can confirm our hypothesis that AFSC might act immediately after injection to protect and prevent endogenous endocrine cells from damage. When blood glucose already exceeded the threshold value, STZ-treated mice receiving AFSC did not rescue and developed instead hyperglycemia similarly to not injected STZ-treated mice.

Histological analysis of STZ-treated mice injected with AFSC, at 4 weeks post injection, revealed that responsive mice have proper islet morphology with almost no reduction of β cell mass. Such data were supported by direct quantification of pancreatic islet mass. AFSC-injected mice responsive to cell treatment showed increased β cell mass if compared to diabetic STZ-treated mice. Unrescued mice showed instead strong reduction of islet mass, even though mass quantification reveals that unrescued mice receiving double dose of AFSC presented a mild increase in comparison to STZ-treated animals. Moreover, distribution of hormone-expressing cells, α and β, as detected by immunofluorescence, confirmed that STZ-treated AFSC-injected mice that responded to treatment preserved an architectural organization of cells similar to control healthy animals, whose pancreatic islets had insulin-expressing in the center while glucagon-expressing α-cells surrounding the core of β cells.

AFSC injections resulted in a strong expression of insulin within islets of STZ-treated rescued mice, significantly higher if compared to diabetic

mice and STZ-treated mice not responsive to cell treatment. Again, the amount of insulin-expressing cells in rescued AFSC-injected mice did not reach control value of wild type animals but it was enough to prevent mice from developing the disease.

In order to determine the possible mechanism of action of injected cells we wanted to investigate if integration and possible differentiation of AFSC into β cells was occurring. It is already reported in literature that the percentage of cell engraftment in *in vivo* graft transplantation protocols is low[46] and that differentiation of injected cells is hardly detectable[74].

We were able to detect the presence of AFSC in the exocrine portion of the host pancreatic tissue soon after injection. However, AFSC were not detectable when mice were sacrificed 4 weeks post injection. Such results again enhance the hypothesis that AFSC act mainly as protectors of already existing β cells rather than reversing the damage by direct conversion to insulin-expressing cells.

It is possible that protection is not the only way AFSC exert their function. AFSC might as well act as trigger of endogenous regeneration from progenitor cells.

It was already reported that progenitors might reside in the exocrine as well in the endocrine compartment of the pancreas[32,33,75] and that stem cell transplantation is able to promote endogenous regeneration[46,74]. A similar role could be attributed to AFSC. Thus the positive outcome in cell responsive mice might not depend exclusively on a protective action of AFSC but also on activation of endogenous progenitors together at an early phase of the disease progression. In order to test this hypothesis, experiments with injection of Bromodeoxyuridine (BrdU) prior to injection of AFSC are planned. BrdU is a synthetic thymidine analog that gets

incorporated into DNA during the S-phase of the cell cycle and allows identification of cells that slowly divide, usually progenitors, by determining the level of retention by cells. Identification and tracking of stem cell niches by BrdU retention in normal mice as well as in murine models of pancreas injury has been established, bringing important highlights in the role of stem cell niches in the regeneration of damaged tissues[74,76]. We expect that AFSC might activate BrdU positive niches within the pancreatic islets, stimulating endogenous β cell precursors. In addition, we will consider to analyze early time points, 5 days post injection, to evaluate the rate of cell proliferation within the islets and in the surrounding exocrine tissue. High rate of proliferation in AFSC-injected mice would eventually add information to the whole picture. When we performed analysis at late time-point, 4 weeks post injection, proliferation was hardly detected in all experimental groups.

It is likely that stem cells act in the early phase immediately after injection. The fact that we had different responses among mice is probably reflective of variability within the type of diabetes model that we adopted. Despite a general and standardized development of hyperglycemia at a later stage of disease progression, differences among mice occur in the early phase, or onset of the disease development. Based on our experience with other animal models, we consider that experimental day 4, immediately after STZ treatment, represents the correct time window during which mice should receive AFSC injection. Mice are more likely to be rescued but still variations, detectable in terms of blood glucose in the early stage, prevents to have a more generalized outcome. Some mice develop immediately after drug treatment a more severe hyperglycemia compared to others. These mice are thus unlikely to be rescued.

This is why we are also considering for future investigations to adopt a more predictable model, Akita, for the development of the diabetic condition. The Akita mouse (C57BL/6-*Ins2Akita*/J) carries the autosomal dominant mutation *Mody*[77]. In the mutant allele a transition from G to A at nucleotide 1907 causes an aminoacidic change in the A chain of mature insulin[78] and a consequent protein misfolding. Heterozygous mice for the mutation develop hyperglycemia and hypoinsulinemia beginning around 3-4 weeks of age. The diabetic phenotype is more severe and progressive in the male than in the female. We already established the colony and started to study the progression of the disease. Male Akita mice develop strong hyperglycemia already at 8 weeks of age. We performed a preliminary experiment in which 8 week-old heterozygous Akita male mice were injected with $1x10^6$ mouse AFSC. At two weeks post injection, an amelioration of blood glucose levels was detected and immunofluorescence staining for β cells revealed a higher expression of insulin in islets of AFSC-injected mice compared to not treated mice. We confirmed that the level of expression of heterozygous Akita males is nearly undetectable already at 4 weeks of age. Based on these encouraging preliminary results, further investigations will involve a higher number of animals to be injected at different time points in order to evaluate the potential of AFSC to eventually reverse or slow down the progression of the disease, as already demonstrated in a chronic model of renal injury[65].

In conclusion, based on our results we speculate that AFSC holds the potential to protect and restore pancreas functionality in models of type I diabetes, making them a suitable candidate for future clinical application.

REFERENCES

1. Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005; 54 Suppl 2:S97-107
2. Gillespie KM. Type I diabetes: pathogenesis and prevention. CMAJ 2006; 175(2):165-70. Review
3. Akerblom HK, Vaarala O, Hyöty H, Ilonen J, Knip M. Environmental factors in the etiology of type 1 diabetes. Am J Med Genet 2002; 115(1):18–29. Review
4. Cudworth AG, Woodrow JC. HLA system and diabetes mellitus. Diabetes 1975; 24: 345-9
5. Ilonen J, Sjöroos M, Knip M, Veijola R, Simell O, Akerblom HK, Paschou P, Bozas E, Havarani B, Malamitsi-Puchner A, Thymelli J, Vazeou A, Bartsocas CS. Estimation of genetic risk for type 1 diabetes. Am J Med Genet 2002; 115:30–36
6. Knip M, Veijola R, Virtanen SM, Hyöty H, Vaarala O, Akerblom HK. Environmental triggers and determinants of type 1 diabetes. Diabetes 2005; 54 Suppl 2:S125-36. Review
7. Nathan DM. Long-term complications of diabetes mellitus. N Engl J Med 1993; 328(23):1676-85. Review
8. Bliss M. The discovery of insulin. Chicago: University of Chicago Press 1982, 45–58
9. Foulis AK, McGill M, Farquharson MA. Insulitis in type 1 (insulin-dependent) diabetes mellitus in man--macrophages, lymphocytes,

and interferon-gamma containing cells. J Pathol 1991; 165(2):97-103

10. Baekkeskov S, Warnock G, Christie M, Rajotte RV, Larsen PM, Fey S. Revelation of specificity of 64K autoantibodies in IDDM serums by high-resolution 2-D gel electrophoresis. Unambiguous identification of 64K target antigen. Diabetes 1989; 38(9):1133-41
11. Lan MS, Wasserfall C, Maclaren NK, Notkins AL. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A 1996; 93(13):6367-70
12. Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA. Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. Diabetes 1997; 46(11):1701-10.
13. Gale EA, Bingley PJ, Emmett CL, Collier T; European Nicotinamide Diabetes Intervention Trial (ENDIT) Group. European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes. Lancet 2004; 363(9413):925-31
14. Diabetes Prevention Trial–Type 1 Diabetes Study Group. Effects of insulin in relaives of patients with type 1 diabetes mellitus. N Engl J Med 2002; 346:1685-91
15. Skyler JS, Krischer JP, Wolfsdorf J, Cowie C, Palmer JP, Greenbaum C, Cuthbertson D, Rafkin-Mervis LE, Chase HP, Leschek E. Effects of oral insulin in relatives of patients with type 1 diabetes: The Diabetes Prevention Trial—Type. Diabetes Care 2005; 28(5):1068-76.

16. Vajo Z, Duckworth WC. Genetically engineered insulin analogs: diabetes in the new millenium. Pharmacol Rev 2000; 52(1):1-9. Review
17. Bolli GB. Insulin treatment in type 1 diabetes. Endocr Pract 2006; 12 Suppl 1:105-9.
18. Matsumoto S. Clinical allogeneic and autologous islet cell transplantation: update. Diabetes Metab J 2011; 35(3):199-206.
19. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343(4):230-8
20. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JR. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006; 355(13):1318-30
21. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, Shapiro AM. Five-year follow-up after clinical islet transplantation. Diabetes 2005; 54(7):2060-9
22. Matsumoto S, Okitsu T, Iwanaga Y, Noguchi H, Nagata H, Yonekawa Y, Liu X, Kamiya H, Ueda M, Hatanaka N, Kobayashi N, Yamada Y, Miyakawa S, Seino Y, Shapiro AM, Tanaka K. Follow-up study of the first successful living donor islet transplantation.

Transplantation 2006; 82(12):1629-33

23. Matsumoto S, Okitsu T, Iwanaga Y, Noguchi H, Nagata H, Yonekawa Y, Yamada Y, Nakai Y, Ueda M, Ishii A, Yabunaka E, Shapiro JA, Tanaka K. Insulin independence of unstable diabetic patient after single living donor islet transplantation. Transplant Proc 2005; 37(8):3427-9

24. Matsumoto S, Okitsu T, Iwanaga Y, Noguchi H, Nagata H, Yonekawa Y, Yamada Y, Fukuda K, Tsukiyama K, Suzuki H, Kawasaki Y, Shimodaira M, Matsuoka K, Shibata T, Kasai Y, Maekawa T, Shapiro J, Tanaka K. Insulin independence after living-donor distal pancreatectomy and islet allotransplantation. Lancet 2005; 365(9471):1642-4

25. Bara H, Sambanis A. Development and Characterization of a Tissue Engineered Pancreatic Substitute Based on Recombinant Intestinal Endocrine L-cells. Biotechnol Bioeng 2009; 103(4): 828–834

26. Lee JI, Nishimura R, Sakai H, Sasaki N, Kenmochi T. A newly developed immunoisolated bioartificial pancreas with cell sheet engineering. Cell Transplant 2008; 17(1-2):51-9

27. Aguayo-Mazzucato C, Bonner-Weir S. Stem cell therapy for type 1 diabetes mellitus. Nat Rev Endocrinol 2010; 6(3):139-48

28. Friel R, Van der Sar S, Mee PJ. Embryonic stem cells: understanding their history, cell biology and signalling. Adv Drug Deliv Rev 2005; 57(13):1894-903. Review

29. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.

Cell 2006; 126(4):663-76

30. Oh W, Kim DS, Yang YS, Lee JK. Immunological properties of umbilical cord blood-derived mesenchymal stromal cells. Cell Immunol 2008; 251(2):116-23

31. Gee Liew C, Andrews PW. Stem cell therapy to treat diabetes mellitus. Rev Diabet Stud 2008; 5:203-219. Review

32. Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 1999; 48(12):2270-6

33. Street CN, Lakey JR, Rajotte RV, Shapiro AM, Kieffer TJ, Lyon JG, Kin T, Korbutt GS. Enriched human pancreatic ductal cultures obtained from selective death of acinar cells express pancreatic and duodenal homeobox gene-1 age-dependently. Rev Diabet Stud 2004; 1(2):66-79

34. Wen Y, Chen B, Ildstad ST. Stem cell-based strategies for the treatment of type 1 diabetes mellitus. Expert Opin Biol Ther 2011; 11(1):41-53. Review

35. Fiorina P, Voltarelli J, Zavazava N. Immunological Applications of Stem Cells in Type 1Diabetes. Endocr Rev 2011; 32(6):725-54

36. Soria B, Roche E, Berná G, León-Quinto T, Reig JA, Martín F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes 2000; 49(2):157-62

37. Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L,

Wobus AM. Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. Proc Natl Acad Sci U S A 2003; 100(3):998-1003

38. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 2001; 292(5520):1389-94

39. Naujok O, Francini F, Picton S, Bailey CJ, Lenzen S, Jörns A. Changes in gene expression and morphology of mouse embryonic stem cells on differentiation into insulin-producing cells in vitro and in vivo. Diabetes Metab Res Rev 2009; 25(5):464-76

40. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006; 24(11):1392-401

41. Raikwar SP, Zavazava N. Spontaneous in vivo differentiation of embryonic stem cell-derived pancreatic endoderm-like cells corrects hyperglycemia in diabetic mice. Transplantation 2011; 91(1):11-20

42. Blum B, Benvenisty N. The tumorigenicity of diploid and aneuploid human pluripotent stem cells. Cell Cycle 2009; 8(23):3822-30

43. Maehr R, Chen S, Snitow M, Ludwig T, Yagasaki L, Goland R, Leibel RL, Melton DA. Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci U S A 2009; 106(37):15768-73

44. Maehr R. iPS cells in type 1 diabetes research and treatment. Clin Pharmacol Ther 2011; 89(5):750-3. Review

45. de Souza N. iPS cell aberrations. Nat Methods 2010; 7(12):948-9
46. Hess D, Li L, Martin M, Sakano S, Hill D, Strutt B, Thyssen S, Gray DA, Bhatia M. Bone marrow-derived stem cells initiate pancreatic regeneration. Nat Biotechnol 2003; 21(7):763-70
47. Hisanaga E, Park KY, Yamada S, Hashimoto H, Takeuchi T, Mori M, Seno M, Umezawa K, Takei I, Kojima I. A simple method to induce differentiation of murine bone marrow mesenchymal cells to insulin-producing cells using conophylline and betacellulin-delta4. Endocr J 2008; 55(3):535-43
48. Chandra V, G S, Phadnis S, Nair PD, Bhonde RR. Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells. Stem Cells 2009; 27(8):1941-53
49. Chandra V, Swetha G, Muthyala S, Jaiswal AK, Bellare JR, Nair PD, Bhonde RR. Islet-like cell aggregates generated from human adipose tissue derived stem cells ameliorate experimental diabetes in mice. PLoS One 2011; 6(6):e20615
50. Li Y, Zhang R, Qiao H, Zhang H, Wang Y, Yuan H, Liu Q, Liu D, Chen L, Pei X. Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells. J Cell Physiol 2007; 211(1):36-44
51. Sun Y, Chen L, Hou XG, Hou WK, Dong JJ, Sun L, Tang KX, Wang B, Song J, Li H, Wang KX. Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells in vitro. Chin Med J 2007; 120(9):771-6
52. Milanesi A, Lee JW, Xu Q, Perin L, Yu JS. Differentiation of nestin-

positive cells derived from bone marrow into pancreatic endocrine and ductal cells in vitro. J Endocrinol 2011; 209(2):193-201

53. Huang S, Law P, Young D, Ho AD. Candidate hematopoietic stem cells from fetal tissues, umbilical cord blood vs. adult bone marrow and mobilized peripheral blood. Exp Hematol 1998; 26(12):1162-71

54. Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. Blood 2003; 102(10):3483-93

55. Tyndall A, Black C, Finke J, Winkler J, Mertlesmann R, Peter HH, Gratwohl A. Treatment of systemic sclerosis with autologous haemopoietic stem cell transplantation. Lancet 1997; 349(9047):254

56. Farge D, Labopin M, Tyndall A, Fassas A, Mancardi GL, Van Laar J, Ouyang J, Kozak T, Moore J, Kötter I, Chesnel V, Marmont A, Gratwohl A, Saccardi R. Autologous hematopoietic stem cell transplantation for autoimmune diseases: an observational study on 12 years' experience from the European Group for Blood and Marrow Transplantation Working Party on Autoimmune Diseases. Haematologica 2010; 95(2):284-92

57. Steptoe RJ, Ritchie JM, Jones LK, Harrison LC. Autoimmune diabetes is suppressed by transfer of proinsulin-encoding Gr-1+ myeloid progenitor cells that differentiate in vivo into resting dendritic cells. Diabetes 2005; 54(2):434-42

58. Kang EM, Zickler PP, Burns S, Langemeijer SM, Brenner S, Phang OA, Patterson N, Harlan D, Tisdale JF. Hematopoietic stem cell transplantation prevents diabetes in NOD mice but does not contribute to significant islet cell regeneration once disease is

established. Exp Hematol 2005; 33(6):699-705

59. Voltarelli JC, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, Coutinho M, Malmegrim KC, Foss-Freitas MC, Simões BP, Foss MC, Squiers E, Burt RK. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2007; 297(14):1568-76

60. Thorel F, Népote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010; 464(7292):1149-54

61. Chung CH, Hao E, Piran R, Keinan E, Levine F. Pancreatic β-cell neogenesis by direct conversion from mature α-cells. Stem Cells 2010; 28(9):1630-8

62. Prusa AR, Marton E, Rosner M, Bernaschek G, Hengstschläger M. Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research? Hum Reprod 2003; (7):1489-93

63. De Coppi P, Bartsch G Jr, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ, Furth ME, Soker S, Atala A. Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol 2007; (1):100-6

64. Perin L, Sedrakyan S, Giuliani S, Da Sacco S, Carraro G, Shiri L, Lemley KV, Rosol M, Wu S, Atala A, Warburton D, De Filippo RE. Protective effect of human amniotic fluid stem cells in an immunodeficient mouse model of acute tubular necrosis. PLoS One 2010; 5(2):e9357

65. Sedrakyan S, Da Sacco S, Milanesi A, Shiri L, Petrosyan A,

Varizemova R, Warburton D, Lemley KV, De Filippo RE, Perin L. Injection of amniotic fluid stem cells delays progression of renal fibrosis. J Am Soc Nephrol 2012

66. Carraro G, Perin L, Sedrakyan S, Giuliani S, Tiozzo C, Lee J, Turcatel G, De Langhe SP, Driscoll B, Bellusci S, Minoo P, Atala A, De Filippo RE, Warburton D. Human amniotic fluid stem cells can integrate and differentiate into epithelial lung lineages. Stem Cells 2008; (11):2902-11

67. Elsner M, Guldbakke B, Tiedge M, Munday R, Lenzen S. Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin. Diabetologia 2000; 43(12):1528-33

68. Morgan NG, Cable HC, Newcombe NR, Williams GT. Treatment of cultured pancreatic B-cells with streptozotocin induces cell death by apoptosis. Biosci Rep. 1994; 14(5):243-50

69. Takasu N, Komiya I, Asawa T, Nagasawa Y, Yamada T. Streptozocin- and alloxan-induced H2O2 generation and DNA fragmentation in pancreatic islets. H2O2 as mediator for DNA fragmentation. Diabetes 1991; 40(9):1141-5

70. Banerjee M, Kumar A, Bhonde RR. Reversal of experimental diabetes by multiple bone marrow transplantation. Biochem Biophys Res Commun 2005; 328:318-325

71. Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, Prockop DJ. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proc Natl Acad Sci U S A 2006; 103(46):17438-43

72. Leiter EH. Multiple low-dose streptozotocin-induced hyperglycemia and insulitis in C57BL mice: Influence of inbred background, sex, and thymus. Proc Natl Acad Sci U S A 1982; 79(2): 630–634

73. McEvoy RC, Andersson J, Sandler S, Hellerström C. Multiple low-dose streptozotocin-induced diabetes in the mouse. Evidence for stimulation of a cytotoxic cellular immune response against an insulin-producing beta cell line. J Clin Invest 1984; 74(3): 715–722

74. Gao X, Song L, Shen K, Wang H, Niu W, Qin X. Transplantation of bone marrow derived cells promotes pancreatic islet repair in diabetic mice. Biochem Biophys Res Commun 2008; 371(1):132-7

75. Scharfmann R, Xiao X, Heimberg H, Mallet J, Ravassard P. Beta cells within single human islets originate from multiple progenitors. PLoS One 2008; 3(10):e3559

76. Hasegawa Y, Ogihara T, Yamada T, Ishigaki Y, Imai J, Uno K, Gao J, Kaneko K, Ishihara H, Sasano H, Nakauchi H, Oka Y, Katagiri H. Bone marrow (BM) transplantation promotes beta-cell regeneration after acute injury through BM cell mobilization. Endocrinology 2007; 148(5):2006-15

77. Izumi T, Yokota-Hashimoto H, Zhao S, Wang J, Halban PA, Takeuchi T. Dominant negative pathogenesis by mutant proinsulin in the Akita diabetic mouse. Diabetes 2003; 52(2):409-16

78. Wang J, Takeuchi T, Tanaka S, Kubo SK, Kayo T, Lu D, Takata K, Koizumi A, Izumi T. A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the Mody mouse. J Clin Invest 1999; 103(1):27-37