Challenges in Islet Transplantation and Strategies to Improve Beta-Cell Function

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Abstract

The incidence of type 1 diabetes is increasing worldwide and therapies of islet transplantation and potential cell-based therapies are rapidly evolving. Choosing the optimal site for such therapies is crucial for safety and for obtaining the best possible outcome. The liver is currently the site of choice, but is unfortunately associated with disadvantages for graft survival.

In paper I, intraportally transplanted human islets were evaluated for hypoxia, apoptosis, and beta-cell survival. This revealed a substantial graft loss of approximately 50 % of transplanted islet mass at one month posttransplantation. At the same time, revascularization was increased, yet still lower than that of native islets. The highest rate of apoptosis was associated with prolonged time in culture prior transplantation.

Due to progressive loss of graft function, repeated islet transplantation is often performed. A mouse model, used in paper II, demonstrated an increased survival rate of islets transplanted one week after a first transplant. This finding may reflect an improved engraftment environment “primed” by the first islet injection. No difference in islet vascular density could be ascribed to it.

As stem cell-based therapies improve, graft monitoring possibilities and retrieval are of importance for safely introducing these techniques into the clinic. Islet grafts to omentum and muscle cured diabetic mice in paper III. Gene expression was unaltered or increased for genes important for beta-cell function.

Decidual stromal cells (DSCs) have immunomodulatory properties that could prove useful for treatments of autoimmune or inflammatory conditions. In paper IV, DSCs were found to be easily isolated from human placenta. The cells were characterized by surface markers, differentiation capacity and gene expression during culture. Co-culture with human pancreatic islets was also conducted. DSCs were observed to be very similar to other types of mesenchymal stromal cells. Greatest change in gene expression was seen between passage 2 and 5. The effect on human islet function may depend on islet viability prior to co-culture.

Keywords: Islet transplantation, Type 1 diabetes, Mesenchymal stem cells

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All things are so very uncertain,
and that's exactly what makes me feel reassured

- Tove Jansson, Moominland Midwinter
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATMP</td>
<td>Advanced therapy medicinal products</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>bmMSC</td>
<td>Bone marrow derived mesenchymal stromal cells</td>
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<tr>
<td>BS-1</td>
<td>Bandeiraea simplicifolia</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CMRL</td>
<td>Connaught Medical Research Laboratories</td>
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<tr>
<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>DSC</td>
<td>Decidual stromal cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
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<tr>
<td>GABA</td>
<td>Gamma aminobutyric acid</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GCK</td>
<td>Glycokinase</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GLUT2</td>
<td>Glucose transporter 2</td>
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<tr>
<td>GPD2</td>
<td>Glycerol-phosphate dehydrogenase 2</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor 1</td>
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<tr>
<td>IAPP</td>
<td>Islet amyloid polypeptide</td>
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<tr>
<td>IBMIR</td>
<td>Instant blood mediated inflammatory reaction</td>
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IEQ  Islet equivalent  
IL   Interleukin  
ISCT International Society of Cellular Therapy  
IT   Islet transplantation  
ITT  Insulin tolerance test  
IVGTT Intravenous glucose tolerance test  
KRBH Krebs-Ringer bicarbonate Hepes buffer  
LSR  Laser selective reflow  
MHC  Major histocompatibility complex  
MSC  Mesenchymal stem cell  
PBS  Phosphate-buffered saline  
PCR  Polymerase chain reaction  
PDX1 Pancreatic and duodenal homeobox 1  
RPMI Rosewell Park Memorial Institute  
SEM  Standard error of the mean  
T1D  Type 1 diabetes  
T2D  Type 2 diabetes  
TNF-α Tumor necrosis factor-α  
VEGF Vascular endothelial growth factor
Introduction

Islets of Langerhans

The pancreas is an unpaired gland that has both exocrine and endocrine function. Clusters of endocrine cells distributed throughout the pancreas form the islets of Langerhans, named after the German physician Paul Langerhans, who was the first to histologically describe them in 1869. Today, we know that these clusters consist of different hormone-producing cells which make up the endocrine function of the pancreas and play an indispensable role in the homeostasis and metabolism of glucose.

The major hormone-secreting cell types are alpha-cells, beta-cells, delta-cells, PP-cells, and epsilon-cells which, produce glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively. In healthy human adults, the pancreas contains 3.2-14.8 million islets\textsuperscript{1-3}. A loss of beta-cell function or mass leads to insulin deficiency, causing elevated blood glucose levels and resulting in the condition known as diabetes mellitus.

Diabetes

Diabetes mellitus is a heterogeneous disease, which can arise due to a patient’s genetic load and/or environmental risk factors associated with inflammation, autoimmunity and metabolic decompensation. Type 1 diabetes (T1D) usually presents in childhood or adolescence and is characterized by an almost complete autoimmune destruction of the pancreatic islets\textsuperscript{4}. Type 2 diabetes (T2D) is characterized by hyperglycemia and insulin resistance often associated with obesity. There are also other variants of diabetes displaying mixed phenotypes with different degrees of autoimmunity and insulin resistance, i.e. late autoimmune diabetes in adults (LADA), and maturity onset diabetes of the young (MODY)\textsuperscript{5}. According to the International Diabetes Federation, diabetes is one of the fastest growing global health emergencies of the 21\textsuperscript{st} century with more than 463 million people suffering from the disease. T1D, one of the most common chronic diseases affecting children, accounts for 5-10 %, whereas T2D represents around 90 % of diabetes worldwide.

The incidence of T1D is increasing, but there are considerable regional variations\textsuperscript{6,7}. To date, Sweden has the second highest incidence rate of T1D in the world (after Finland) in children aged 0-14 years (43.2 per 100,000 per year)\textsuperscript{8}. 
Treatment of Type 1 Diabetes

The standard treatment of T1D includes life-long insulin administration, after groundbreaking experiments by Fredrick Banting and Charles Best in 1921. New insulin formulas and technical devices, such as continuous glucose monitoring and closed loop systems, have substantially improved treatment options and contributed to better health\textsuperscript{9,10}. For the majority of patients with T1D, standard insulin therapy is effective for maintaining euglycemia and minimizing diabetes-associated complications (cardiovascular disease, nephropathy, retinopathy, and neuropathy)\textsuperscript{11-13}. It is difficult to mimic the extremely precise glucose control of a healthy pancreas. Patients with T1D have an increased mortality and a reduced life expectancy compared to the general population, in certain studies by more than 10 years\textsuperscript{14-16}.

Additionally, a small number of patients, despite intensive treatment, show extreme glycemic variability and suffer repeated and unpredictable hypoglycemic episodes. This leads to an unawareness of hypoglycemia and is often associated with defective counter-regulatory mechanisms and autonomous neuropathy\textsuperscript{17}. For this group of patients, it is possible to achieve a restoration of pancreatic endocrine function through transplantation of either a whole pancreas or islets of Langerhans only. However, the clinical utility is limited because of the necessity of life-long immunosuppressive treatment, which is afflicted with adverse side effects such as opportunistic infections, malignancies, and drug toxicity\textsuperscript{18}.

Pancreas and Islet Transplantation

Currently, the outcome after whole pancreas transplantation is an average graft survival rate of 50 % after 10 years. Pancreas transplantation can either be performed alone or as a simultaneous pancreas-kidney transplantation in patients with T1D and chronic renal failure\textsuperscript{19}. The procedure is associated with risks of technical failure, thrombosis, infections, pancreatitis and acute or chronic graft rejection\textsuperscript{20}. However, the success rate is improving and patient survival rate is 90 % at 4 years\textsuperscript{20,21}.

For patients where insulin therapy is unsuccessful and it is not suitable to receive a whole pancreas, islet transplantation (IT) has become an optional treatment. The islet isolation technique using collagenase, was developed in the 1960s by Lacy et al.\textsuperscript{22}, and the first experimental trials performed in the 1970s\textsuperscript{23}. It is, as opposed to whole pancreas transplantation, a minimally invasive procedure in which a radiologically guided percutaneous transhepatic injection allows islets to reach the liver via the portal vein. The procedure is associated with low morbidity and low risks of adverse effects, such as bleeding (7 %) and portal thrombosis (3.7 %)\textsuperscript{24}. Ever since Shapiro et al. introduced the Edmonton protocol\textsuperscript{25} with a greater transplanted islet mass and steroid-
free immunosuppression, IT got a revival and outcomes have since become increasingly better, owing to ongoing improvements in isolation techniques and refined immunosuppressive regimes\(^2\). The number of patients who achieve insulin independence after IT has dramatically improved during the last two decades (44\% at 3 years after transplantation) and is currently almost on par with the results of whole pancreas transplantation\(^2\).\(^6\).

**Islet Isolation and Engraftment**

Studies have revealed that, although islets only constitute 1-2\% of the pancreatic mass\(^2\), they receive 10-15\% of the total pancreatic blood flow. The vessels inside and surrounding the islet cells are fenestrated, further implying a close connection between the vasculature and the endocrine function of the islets\(^2\). These integrated vessels account for 8-10\% of total islet volume\(^3\) and are believed to play a role in paracrine signaling\(^3\), beyond supplying islets with oxygen and nutrients.

As islets are isolated, they are inevitably deprived of their native vascular network. Consequently, the isolated islets must solely rely on diffusion of oxygen and nutrients during culture and during the initial phase after transplantation. Beyond the fact that islets are already subjected to hypoxic conditions during culture, additional factors (islet size, reduced viability, and thrombus formation) can further aggravate the hypoxia, resulting in an anoxic islet core\(^3\).\(^2\). Hence, reestablishment of vasculature is a vital part of the engraftment process, i.e. the adaptation of islets to the surrounding tissue at the implantation site. Engraftment also encompasses reinnervation and reorganization of adjacent stroma, and the conditions for successful engraftment differ between implantation sites\(^3\).\(^4\).

**Islet Transplantation to the Liver**

Recent advances in IT outcome have been accomplished through enhanced procurement of islets and refined immunosuppressive therapies. However, IT often needs to be repeated and even in autologous or in experimental, syngeneic transplantations, a great amount of islet mass is quickly lost\(^3\). Factors affecting islet quality, and thus transplant success, are: donor condition, ischemic time, isolation procedure, degree of exocrine contamination, and culture time\(^3\).\(^6\)-\(^3\)\(^9\). Later on, allogeneic islets grafts are further exposed to autoimmunity, alloimmune rejection and toxicity of immunosuppressive treatment.

Both before and after transplantation, islets are exposed to hypoxic and mechanical stress, as well as activation of inflammatory cytokines (IL-1\(\beta\), TNF-\(\alpha\) and HIF-1), leading to impaired function and beta-cell death\(^4\).\(^0\).\(^4\).\(^1\). Inflammation is induced by both innate immune cells, islet resident macrophages, and
specialized macrophages in the liver (Kupffer cells). Studies have shown that anti-inflammatory treatment and strategies to increase levels of antioxidants inside the islets have improved islet function. Treatment of islets with mesenchymal stem/stromal cells are suggested to mediate their effect in a similar way.

The use of liver as implantation site is associated with further disadvantages linked to islet infusion and engraftment. First, when islets are injected through the portal vein, an immediate blood mediated inflammatory reaction (IBMIR) occurs, which has been shown to damage the endocrine cells and cause a loss of islet mass by up to 50% in the earliest phases after transplantation. Second, once the islets have settled in the liver, glucotoxicity, amyloid formation, and immunological attack may all influence graft survival to various extents. Hyperglycemia per se has also been shown to hamper the islets, if euglycemia is not fully reached after transplantation.

Furthermore, the liver does not provide optimal conditions for revascularization and islet oxygenation. Even under normal physiological conditions, the oxygen tension of the liver is only 5-10 mmHg, as compared to 40 mmHg in native islets. In the liver, islets have been shown to get revascularized from the hepatic artery. A new vascular network is gradually established from the first week after transplantation. However, even after revascularization is complete, the oxygenation of intraportally transplanted islets remains low.

Islet grafts implanted in liver in mice have, in previous studies, exhibited an altered gene expression compared to control islets. These genes, vital for beta-cell function, include PDX-1, a marker of differentiation. This marker was decreased in islets transplanted to liver as well as to pancreas. Similar findings have recently been demonstrated in humans, where all beta-cells in allogeneic islet grafts were lacking the differentiation marker urocortin-3. In the same report, cells were found positive for both glucagon and insulin which indicates that islet cells exhibit some plasticity. It is unclear exactly what causes these changes. Of note, treatment with GABA has been shown to induce alpha-to-beta-cell conversion in vivo.

Alternative Implantation Sites

The renal capsule has historically been readily used for islet transplantations in experimental models, but translating studies into humans has failed. In humans, the renal capsule is not suitable due to volume restriction. Despite the numerous sites that have been evaluated, in both experimental and clinical setting (muscle, omentum, bone marrow, pancreas, gastric submucosa, kidney and spleen), none have in humans reported improved outcomes when compared to the intraportal site. Table 1 lists comparative studies of islet transplant sites in mice.
Each site is associated with specific technical complications, volume capacity and engraftment conditions. Furthermore, studies show that the liver appears superior to bone marrow and renal capsule from an immunological point of view\textsuperscript{73,74}. Also, the liver is the target organ for insulin, and choosing sites with a portal drainage is presumably preferable to systemic insulin release in order to mimic a physiological pancreatic insulin secretion\textsuperscript{75}.

**Table 1.** Comparative studies of islet transplant sites in C75BL6J, modified from review in Cell Medicine\textsuperscript{76}.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Implantation sites</th>
<th>Islet type</th>
<th>Islet volume tx</th>
<th>Major outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korsgren O et al. 1993\textsuperscript{77}</td>
<td>Liver, spleen and renal capsule</td>
<td>Murine</td>
<td>300 islets</td>
<td>Better nerve in growth in renal capsule compared to liver and spleen</td>
</tr>
<tr>
<td>Lau J et al. 2007\textsuperscript{82}</td>
<td>Pancreas vs liver</td>
<td>Murine</td>
<td>200 islets</td>
<td>Glucose stimulated insulin release and oxidation rates were markedly decreased in liver</td>
</tr>
<tr>
<td>Kim HI et al. 2010\textsuperscript{34}</td>
<td>Renal capsule, liver, muscle and omentum</td>
<td>Murine</td>
<td>marginal mass</td>
<td>Renal capsule, liver, muscle and omentum required 100, 600, 600, 200 islets to cure 50% of engrafted diabetic mice, respectively. Kidney had shortest time to reach euglycemia (3 days), muscle the longest (27 days)</td>
</tr>
<tr>
<td>Christoffersson G et al. 2010\textsuperscript{78}</td>
<td>Muscle vs liver</td>
<td>Human and murine</td>
<td>300 islets</td>
<td>Improved revascularisation and response to glucose challenge in intramuscular transplantation, on par with intrahepatic transplantation</td>
</tr>
<tr>
<td>Espes D et al. 2016\textsuperscript{79}</td>
<td>Omentum vs liver</td>
<td>Human and murine</td>
<td>200-300 islets</td>
<td>Normalized vasculature and better response to IVGTT 1-month post-transplant in the omentum</td>
</tr>
<tr>
<td>Stokes RA et al. 2017\textsuperscript{80}</td>
<td>Renal capsule, muscle, liver, spleen capsule, liver capsule</td>
<td>Human and murine</td>
<td>220-250 islets / 2000 IEQ human islets</td>
<td>Renal capsule best site for murine and human IT (Renal capsule used as control). Muscle and intraportal site had similar cure rate for human islets, however both inferior to renal capsule</td>
</tr>
<tr>
<td>Cantarelli E et al. 2017\textsuperscript{73}</td>
<td>Bone marrow vs liver</td>
<td>Murine (two different strain)</td>
<td>450 IEQ</td>
<td>Treating the animals with anti-CD3, islet rejection is prolonged when transplanted to liver compared to bone marrow</td>
</tr>
</tbody>
</table>

**Stem Cell Therapy in Type I Diabetes**

The development of stem cell research has enabled cellular therapies in various disorders, including diabetes. The term “stem cells” is wide and includes
cells found in the embryo as well as adult stem cell populations present in most tissue, on standby, ready to participate in tissue regeneration. Of current interest, protocols for induced pluripotent stem cells have come into practice, opening for personalized stem cell treatments.

Stem cells are capable of self-renewal and possess either totipotency, pluripotency (embryonic stem cells) or multipotency of differentiation into various tissues from one or more germ layers. Ideally, these cells could provide a source for beta-cell replacement, and thus resolve the issues of both immunosuppression and lack of donors\textsuperscript{81,82}. Nevertheless, the underlying autoimmunity of diabetes would not be avoided.

The biology of stem cells is complex, and there is no common standardization and few guidelines. Before safely introducing stem cells into clinical trials, investigations need to be conducted regarding mechanism of action, and protocols developed for isolation, culture, and administration, all of which affect the phenotype of stem cells.

**Mesenchymal Stromal Cells**

A commonly used type of stem cell is the mesenchymal stromal cell (MSC), first described in the 1960-1970s\textsuperscript{83,84}. MSCs are classified as multipotent stem cells due to their capacity to differentiate into various lineages that develop from mesoderm\textsuperscript{85,86}. MSCs have been used in a great number of animal models for human disease as well as in veterinary medicine\textsuperscript{87}. Furthermore, they have been evaluated for clinical cell-based therapies in a wide range of ailments, such as osteogenesis imperfecta, GVHD, myocardial infarctions, autoimmune diseases, and diabetes\textsuperscript{88-91}.

These cells were first identified in bone marrow\textsuperscript{84}, but today we know that cells fulfilling MSC criteria can be isolated from most tissues. The International Society of Cellular Therapy (ISCT) proposed the following criteria in 2006\textsuperscript{92}:

1. Plastic adherence
2. >95 % of the cells must express surface molecules such as CD105, CD73, or CD90
3. <2 % expression of CD45, CD34, CD14 and MHC II
4. Multipotent differentiation potential

The initial hypothesis for the regenerative properties of MSCs was based on their multipotent capacity. Early on, MSCs were shown capable of differentiating into non-mesodermal cells\textsuperscript{93,94}, and studies have even demonstrated that MSCs can develop into insulin producing cells\textsuperscript{95}. Currently, MSCs are believed to mediate their main effect through soluble trophic and immunomodulatory factors, regulating the surrounding environment\textsuperscript{96} (Figure 1).
Contributing reasons for the frequent use of these cells are the property of self-renewal (although only for a limited time), permitting *in vitro* expansion, and that they are “immunologically privileged”, allowing allogeneic MSC transplantation without the need for immunosuppression

**Tissue regeneration**

**Figure 1.** Simplified model of potential mechanism of actions facilitated by MSCs. When tissue injury occurs (due to damage or disease), MSCs can either home, if systemically injected, or be locally administered to the site of event. Inflammatory cytokines may then stimulate/activate MSCs, which will respond by releasing growth factors, producing immunoregulatory chemokines and interleukins, and remodeling the extracellular matrix, all of which will affect the inflammatory process.

**MSC Safety and Legislation**

Registered data from clinical trials show few and minor adverse events of treatment with MSCs. Their genomic stability and the fact that MSCs do not seem to engraft inside the recipient may explain why few cases of ectopic tissue formation or malignancy are reported. Human stem cells can be extensively expanded in culture, and several studies show maintained karyotype for >30 passages. After too many passages, however, the biological function has been reported to be lost. Notably, only 1% of systemically administered MSCs persist longer than a week following injection. Still, an
immunomodulating effect may be lasting, a phenomenon known as “hit-and-run”. Even if intravenously administered MSCs have been shown to end up in the pulmonary bed, they can still mediate a systemic action\textsuperscript{104}. The route of administration is not unimportant, considering that some MSC sources express tissue factor, and thereby are much more prone to thrombus building and to induce IBMIR, resembling the response seen in islet lodging. However, bmMSCs express relatively low levels of tissue factor, and when used for coating of islets, and subsequent exposure to blood (in an \textit{in vitro} tubing system), no increased reaction was found compared to uncoated control islets\textsuperscript{105}.

MSCs are considered to be an advanced therapy medicinal product (ATMP), and thus guidelines from European Medicines Agency (EMA) require potency assays that can predict clinical effect. This demands an understanding of the mechanism of action.

**MSC and Islet Transplantation**

Additional positive effects beyond immunomodulation are seen in several studies using MSCs in IT research\textsuperscript{106,105}. In rodents, MSCs have contributed to enhanced engraftment of islets implanted to muscle, renal capsule, and omentum, with reduced formation of fibrosis and increased vascularization\textsuperscript{107-109}. The mechanism for this effect is MSC production of angiogenic factors, including VEGF, IL-6 and IL-8, which supports islet graft revascularization\textsuperscript{108,110-113}. Also, MSCs express markers important for homing\textsuperscript{114}, which has enabled intravenous administration where the cells migrate to implanted islets (renal capsule) and contribute to neovascularization and islet cell proliferation\textsuperscript{110}. Islets and MSCs have also successfully been co-transplanted to the liver\textsuperscript{111,115}. However, donor and recipient species (for both islets and MSCs) vary, as well as administration routine, culture condition, number of passages, and site of implantation, which makes comparisons difficult. Furthermore, MSC and islet co-culture can be performed allowing physical cell-cell interactions or in an indirect fashion where islets only come in contact with MSC-secreted factors, alternatively in conditioned medium\textsuperscript{116-118}. A review on the topic presented that a higher viability was obtained when choosing an indirect contact system as opposed to culture with direct cell-cell contact\textsuperscript{119}. However, results are not conclusive and specific MSC source is likely to affect the results\textsuperscript{120,121}.

**Placenta as a Source of MSCs**

During pregnancy, the decidual layer is formed from the uterine lining (the endometrium). The process of decidualization creates a specialized tissue composed by glands, immune cells, blood and lymph vessels, and decidual
stromal cells (DSCs)\textsuperscript{122}. It forms part of the placenta, the functions of which are to provide exchange of nutrients, oxygen and excretion, whilst maintaining the fetomaternal tolerance.

DSCs are a type of MSCs that, just like bmMSCs, are easily expanded and exhibit potent immunosuppressive effects\textsuperscript{123,124}. Studies comparing bmMSCs and DSCs have found many similarities with regards to appearance in light microscope, size, and surface marker phenotype\textsuperscript{125}. The ability of mesodermal differentiation has been described as equally good or somewhat reduced compared to bmMSCs\textsuperscript{125,126}. DSCs are currently used to improve acute inflammatory disorders such as steroid-refractory GVHD and radiculomyelopathy, where no other effective therapy exists\textsuperscript{127,128}. The placenta, otherwise discarded after a birth, may provide eligible MSCs in large quantities for evaluation of their possible cell-therapeutic utility and mechanism of action in islet research.
Aims

The overall aim of the work presented in this thesis was to investigate central factors in islet transplantation and finding strategies to improve beta-cell function and transplant outcome. The specific aims for each study were:

Paper I
To investigate human islet engraftment and graft survival within the first month after intraportal transplantation

Paper II
To investigate the possibility of enhancing islet engraftment to the liver through repeated islet transplantation

Paper III
To compare islet graft function and gene expression following transplantation to greater omentum or abdominal muscle

Paper IV
To isolate and characterize decidual stromal cells from human placenta, their alterations in culture, and investigate their effect on human islets
Materials and Methods

Experimental Animals

Papers I-III
The animal housing and all experimental procedures were approved by the local Animal Ethics Committee at Uppsala University, Sweden. All animals were housed under standardized conditions with free access to food and water. Adult male immunodeficient C57BL/6 (nu/nu) mice, obtained from Taconic M&B, Bomholtgaard, Denmark, were used as recipients for human islets in paper I. In paper II, a transgenic mouse model that expresses green fluorescent protein (GFP), under control of the mouse insulin I gene promoter (MIP) on C57BL/6 background (Taconic M&B), was used as donors for islet isolation for the first transplantation. C57BL/6 male mice, wild type and transgenic, purchased from Taconic M&B, Ejby, Denmark, were used for transplantation and islet isolation in papers II and III.

Isolation and Culture of Human Islets

Papers I & IV
The regional ethical board in Uppsala approved the use of human pancreatic tissue. Pancreata from donors after brain death were generously provided by the Nordic Network for Clinical Islet Transplantation and isolated at the human islet isolation facility, Rudbeck Laboratory, Uppsala University Hospital. The human islets were cultured according to the standard protocol at the Rudbeck laboratory. For the first 24 hours, islets were incubated in 95 % O₂ and 5 % CO₂ at 37°C, thereafter at 25°C until the islets were transferred to our laboratory and to CMRL 1066 medium (Gibco, Grand Island, NY, USA) containing 10 % vol/vol fetal calf serum, 50 mmol/L L-glutamine (Sigma-Aldrich, St Louis, MO, USA) and 5 U/mL Penicillin Streptomycin (Roche, Sigma-Aldrich). The mean culture time before transplantation was 6.2 ± 0.5 days.
Isolation and Culture of Murine Islets

Papers II & III
Pancreatic islets from non-diabetic C57BL/6 mice were isolated using collagenase digestion and density gradient purification as previously described\textsuperscript{61} (Figure 2). Briefly, mice were anesthetized with sodium pentobarbital (200 mg/kg, Apoteket AB, Stockholm, Sweden). Collagenase A from Clostridium histolyticum (2.5 mg/mL Roche Diagnostics, Mannheim, Germany) suspended in cold HBSS (National Bacteriological Laboratory, Stockholm, Sweden) was injected via the common bile duct. The pancreas was inflated, surgically removed and placed in 37°C for 18 minutes. Density gradient centrifugation by adding Histopaque-1077 and serum-free RPMI 1640, both purchased from Sigma-Aldrich, was performed for separation of islets from exocrine tissue. Islets were handpicked and cultured free-floating at least overnight in 5 mL of culture medium consisting of RPMI 1640 (Sigma-Aldrich) supplemented with L-glutamine (2 mmol/L; Sigma Aldrich), 10% vol/vol fetal calf serum (Sigma-Aldrich) and Penicillin Streptomycin (100 U/mL and 0.1 mg/mL, respectively; Roche Diagnostics) in paper II. In paper III, the antibiotic used was Benzylpenicillin (100 U/mL; Roche Diagnostics), otherwise the procedure was the same as detailed above.

Figure 2. Islet isolation through density gradient purification after retrograde injection of collagenase I digestion solution through the common bile duct. In papers I and II, islets were transplanted after having recovered for 1-2 days in culture before transplanting them to the liver of recipients through the portal or appendicular vein. In paper III, islets were instead implanted to either the greater omentum or abdominal muscle.
Induction of Diabetes

Paper III
Diabetes was induced in C57BL/6 mice by a single intravenous injection of alloxan (75 mg/kg, Sigma-Aldrich). Mice were defined as diabetic when repeated blood glucose concentrations exceeded 15 mmol/L on two consecutive days, using blood glucose reagent strips (Freestyle Lite, Abbot, Alameda, CA, USA). Blood glucose concentrations were monitored daily for the first week posttransplantation and then every fifth day.

Islet Transplantations

In papers I, II, and III, all animals were anesthetized by an intraperitoneal injection of 0.02 mL/g body weight Avertin (Kemila, Stockholm, Sweden). In paper II, IT through the appendicular vein to the liver was made under spontaneous inhalation of isoflurane (first transplantation). All transplantations to the liver were made after a midline abdominal incision and islets were infused into the portal vein system through a butterfly needle. In paper I, islets were transplanted selectively into the right liver lobe by temporarily clamping other portal vein tributaries during the islet infusion. This procedure enabled us to evaluate a greater proportion of islets transplanted, and is not believed to affect the engraftment negatively.

Paper I
Human islets (300 islets per transplantation) were injected in the livers of C57BL/6 (nu/nu) mice, as detailed above. Two hours before animals were killed by cervical dislocation, the hypoxia marker pimonidazole (60 mg/kg; Hypoxyprobe, Burlington, MA, USA) was injected intravenously through the tail vein of awake animals.

Paper II
Two groups of animals received IT twice, one week apart, while mice in a control group received a sham transplantation with saline infusion only, followed by one IT a week later (Figure 3). The first islet transplant consisted of 125 syngeneic GFP-expressing islets, while the second transplant consisted of 125 syngeneic non-GFP-expressing islets of similar size (50-100 µm). Islets were infused into the appendicular vein or portal vein with a butterfly needle (25G). The total volume of infusion at each time was ≤ 100 µL. The animals were killed 1 day or 1 month after the second transplantation. Animals in the control group were killed 1 month after IT.
Paper III

200 mouse islets were transplanted under aseptic conditions into the abdominal muscle or omentum of recipient non-diabetic or alloxan-diabetic C57BL/6 mice. Islets were transplanted to the abdominal muscle after a midline incision in the skin, as previously described. In order to transplant islets to the greater omentum, a midline incision in the skin and the abdominal muscle was made and the stomach was exposed. The greater omentum was localized and carefully extended by holding the omental fat and a ligature was placed, but not fixed, around the omentum. An opening between the sheets of the omentum was made with a cannula, a braking pipette filled with islets was inserted, the islets were infused into the pouch, and the ligature was thereafter closed. The abdominal muscle was carefully sutured, followed by suturing of the skin.

![Figure 3.](image)

**Figure 3.** Experimental design paper II. A first transplantation was performed with either GFP-positive islets or a shame saline infusion to a control group. After one week, a second transplantation to the liver with non-GFP C75BL/6 murine islets was performed in all groups.

**Function of Transplanted Islet Grafts**

Paper III

Thirty days posttransplantation, an intravenous glucose tolerance test (IVGTT, 2 g/kg) was performed in cured animals (defined as a non-fasting blood glucose concentration <12 mmol/L for two consecutive measurements on separate days). Blood glucose concentrations were monitored for two hours following glucose injection. On the following day, an intravenous insulin tolerance test (ITT; 2 U/kg NovoRapid, Novo Nordisk, Bagsvaerd, Denmark) was performed, during which blood glucose concentrations were monitored for two hours. Non-diabetic C57BL/6 mice were used as controls for both the IVGTT and the ITT. When the animals subsequently were killed, the pancreas
was surgically removed, placed in acid ethanol (0.18 mol/L HCl in 95 % vol/vol ethanol), sonicated in order to disrupt the cells and subsequently frozen. The remaining insulin content in the pancreas was measured with an insulin enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden) in order to exclude endogenous beta-cell regeneration. An insulin content > 10 % of that of the pancreas in control non-diabetic animals was used as an exclusion criterion for the study.

**Immunohistochemistry**

Sampled tissue was either fixated in 4 % vol/vol paraformaldehyde before paraffin embedding or snap-frozen for cryosectioning. Frozen tissue was imbedded in TissueTek OCT (Sakura Finetek, Torrance, CA, USA) and sectioned using Leica CM 1950 cryostat (Leica Biosystems, Germany).

**Paper I**
The paraffin embedded liver lobes were sectioned to a thickness of 7 µm and every fifth slide was stained for insulin. Adjacent slides were stained, respectively, for blood vessels, amyloid, hypoxia and apoptosis according to Table 2 and counter-stained with Mayor’s hematoxylin (HistoLab Products AB, Gothenburg, Sweden). Insulin and caspase-3 were dye developed using 3,3’-Diaminobenzidine (DAB) (Dako, Glostrup, Denmark).

Sections from human pancreata were simultaneously stained with the lectin Bandeiraea simplicifolia (BS-1) and evaluated for vascular density, as previously described\textsuperscript{130}, for comparison between native and transplanted islets.

**Paper II**
Graft bearing livers were initially fixated in 4 % vol/vol paraformaldehyde overnight at 4°C, transferred to 15 % wt/vol sucrose in PBS (2 h) and finally to 30 % wt/vol sucrose in PBS overnight at 4°C, to preserve GFP-expression. The livers were sectioned (10 µm) to a depth of 4.0 mm. Islet grafts were identified in sections stained with hematoxylin, while consecutive sections were saved for later performed immunostainings (Table 2). Only islets with an area exceeding 50 µm\textsuperscript{2} were evaluated in order to avoid misestimations based on single cell residues.

**Paper III**
Tissue sections were collected from the same tissue block for insulin staining of the islet graft. Cryosections and paraffin sections were prepared for immunohistology using antibodies outlined in Table 2. ProLong Gold Antifade reagent with 4’,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Rockville, MD, USA) was used for mounting and nuclei staining for frozen
sections. Hoechst 1:10,000 (Life Technologies) was used for nuclei staining of paraffin embedded pancreas and grafts from muscle and omentum.

Table 2. Stainings and antibodies used for histological evaluations

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig anti-insulin</td>
<td>1:400</td>
<td>Fitzgerald Industries International</td>
<td>Paper I</td>
</tr>
<tr>
<td>Rabbit anti-caspase-3</td>
<td>1:100</td>
<td>Cell Signaling Technology, Danvers, MA, USA</td>
<td>Paper I</td>
</tr>
<tr>
<td>Lectin Bandeiraea simplicifolia</td>
<td>1:100</td>
<td>Sigma-Aldrich</td>
<td>Paper I</td>
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<tr>
<td>Mouse anti-pimonidazole</td>
<td>1:100</td>
<td>Bioscience Research Reagents, Temecula, CA, USA</td>
<td>Paper I</td>
</tr>
<tr>
<td>Alkaline Congo Red</td>
<td></td>
<td>VWR International, Radnor, PA, USA</td>
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<tr>
<td>Rat anti-mouse CD31</td>
<td>1:100</td>
<td>BD Biosciences, San Jose, CA</td>
<td>Paper II</td>
</tr>
<tr>
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<td>Ken-EN-Tec Nordic, Tåstrup, Denmark</td>
<td>Paper II</td>
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<td>Abcam, Cambridge, UK</td>
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<td>Paper III</td>
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<tr>
<td>Mouse monoclonal anti-glucagon</td>
<td>1:800</td>
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<td>Paper III</td>
</tr>
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<td>Anti-mouse FABP4</td>
<td>10 µg/mL</td>
<td>R&amp;D systems</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Anti-human Aggrecan</td>
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<td>R&amp;D systems</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Anti-human Osteocalcin</td>
<td>10 µg/mL</td>
<td>R&amp;D systems</td>
<td>Paper IV</td>
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</table>

<table>
<thead>
<tr>
<th>Secondary antibody</th>
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<th>Manufacturer</th>
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<td>Southern Biotech, Birmingham, AL, USA</td>
<td>Paper I</td>
</tr>
<tr>
<td>TrekAvidin-AP Lable and Vulcan Fast Red</td>
<td></td>
<td>Biocare Medical</td>
<td>Paper I</td>
</tr>
<tr>
<td>Biotinylated goat anti-mouse</td>
<td></td>
<td>Southern Biotech</td>
<td>Paper I</td>
</tr>
<tr>
<td>Alexa Flour 647 donkey anti-rat</td>
<td>1:300</td>
<td>Jackson ImmunoResearch Laboratories, West Grove, PA, USA</td>
<td>Paper II</td>
</tr>
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<td>Alexa Flour 488 donkey anti-guinea pig</td>
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<td>Jackson ImmunoResearch Laboratories</td>
<td>Paper II</td>
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<tr>
<td>Alexa Flour 488 donkey anti-rabbit</td>
<td>1:300</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>Paper II</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-guinea pig</td>
<td>1:1000</td>
<td>Invitrogen, Carlsbad, CA</td>
<td>Paper III</td>
</tr>
<tr>
<td>Alexa Fluor 594 donkey anti-mouse</td>
<td>1:250</td>
<td>Invitrogen</td>
<td>Paper III</td>
</tr>
<tr>
<td>Alexa Fluor 594 donkey anti-goat</td>
<td>1:300</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Alexa Fluor 594 donkey anti-mouse</td>
<td>1:300</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>Paper IV</td>
</tr>
</tbody>
</table>
To evaluate differentiation capacity of DSCs in paper IV, cells were stained with Oil Red O (Santa Cruz Biotechnology, CA, USA), Alizarin Red (Sigma-Aldrich), and Alcian Blue (Sigma-Aldrich) following fixation with 4 % or 10 % vol/vol paraformaldehyde. Parallel fixated cells or, for chondrogenic differentiation performed in 3D-culture, sections of the fixated pellet, were stained with mFABP4, osteocalcin, and anti-aggrecan antibody (R&D Systems, McKinley Place, MN, USA).

Cell death assessment was performed by staining with Live-or-Dye NucFix Red Staining Kit (Biotium, Fremont, CA, USA) and bisbenzimide 20 µg/mL (Hoechst 33324, Sigma-Aldrich).

Image Analysis

Paper I
Images were captured with Leica Leitz DMRBE microscope (Leica Microsystems, Wetzlar, Germany). Assessment of islet vascular density and evaluation for percentages of caspase-positive cells were made using ImageJ (National Institutes of Health, Bethesda, MD, USA). In order to determine the intra-islet capillary density, only vessels surrounded by islet tissue were counted and compared to native human pancreas, assessed with the same criteria.

The percentage of pimonidazole-positive islets was assessed. Islet amyloid was evaluated by fluorescence microscopy and polarized microscopy, in the latter amyloid displays an apple-green birefringence.

Paper II
Images were captured using the laser scanning confocal microscope Zeiss LSM 780 (Carl Zeiss AG, Oberkochen, Germany) with Plan-Apochromat 10x/0.45 M27 and 20x/0.8 M27 objectives. For image analysis of islet area and vascular density, a macro was built in Fiji software with the assistance of staff at the BioVis facility platform of Uppsala University.

Paper III
Light microscopy images were acquired with a Leica LMD6000 laser microdissection microscope (Leica Microsystems). Fluorescent immunohistochemistry images were acquired with Zeiss LSM780 confocal (Zeiss).

The respective areas of insulin and glucagon were calculated using the image software Imaris (Bitplane AG, Zurich, Switzerland) with a fixed intensity cut-off value for each staining. The percentage in each image was combined into an average for each animal and considered as one experiment.
Paper IV
A macro using Fiji software performed threshold-based calculations of the ratio of dead cells, taken as a relative measure of islet resistance to cytokine stress.

Glycogen Assay

Paper III
Glycogen concentration in muscle and liver was measured from 10 mg of homogenized tissue from cured and control animals by a Glycogen Assay Kit II (Abcam, Cambridge, UK). Reading of the plate was performed using a Spark Microplate Reader (Tecan, Männedorf, Switzerland).

Decidual Stromal Cell Isolation

Paper IV
Human placentas were obtained from mothers at Uppsala University Hospital. The following inclusion criteria were used: healthy women planned for routine caesarian section in week 37-42 of gestation. Exclusion criteria: positive test for HIV, hepatitis or Treponema pallidum, smokers, patients suffering from diabetes, preeclampsia, placenta accreta or known tumor disease. After collection of the placentas, the isolation process started within four hours. For decidual stromal cell isolation, the protocol from Pelekanos et al was used\textsuperscript{132}. The placenta was washed inside a biosafety cabinet and the decidua was dissected into pieces of 5 g. The tissue was washed in HBSS, minced, divided into portions of 10 g and incubated in 25 mL of the following digestion solution: Dispase 2.4 U/mL (Thermo Scientific, Waltham, MA, USA), DNase I 15 µL, $\geq$ 2500 U/mL (Thermo Scientific) and Collagenase I 100 U/mL (Thermo Scientific). Incubation was sustained until the solution assumed a cloudy appearance, approximately 1.5 hours at 37°C during vigorous shaking. The tissue was then washed, pulse centrifuged and passed through a 100 µm filter. The single cells were seeded in T75-bottles (Coming, New York, NY, USA) in DMEM culture medium with low glucose from Thermo Scientific, supplemented with FBS (Sigma-Aldrich) and Penicillin Streptomycin (100 U/mL and 100 µg/mL respectively, Thermo Scientific). Medium was changed every three days. When 80-90 % confluency was reached, cells were passaged by trypsinization with TrypLE (Thermo Scientific). Dissociated cells were pelleted by centrifugation 200 rcf for 5 minutes, the supernatant discarded and cells resuspended in culture medium.
DSC Characterization

STR Analysis
Short tandem repeats (STR) were analyzed to confirm the origin of expanded DSC culture. DNA was recovered from blood samples and from cell cultures by Qiagen DNA Mini Kit (Qiagen, Hilden, Germany) and STR analysis was performed by Uppsala Genome Center.

Differentiation Capacity
Multilineage differentiation capability was evaluated by a differentiation kit (R&D Systems). For differentiation experiments, cells from passage 2 were seeded in 24-well dishes (Sarstedt, Nümbrecht, Germany) and differentiation medium was changed every third day. As controls, cells seeded or pelleted at the same cell density and cultured in standard culture medium were used.

Flow Cytometry
Cells from passages 1-10, isolated from different donors (n=15), were detached from flask using TrypLE, washed in DPBS (Thermo Scientific) and stained with a multifluorescent panel for MSC markers CD29-APC, CD90-FITC, CD73-BV605, CD105-BV421 (Biolegend, San Diego, CA, USA); hematopoietic markers CD45-PE, CD34-PE; endothelial marker CD31-PE, immunological markers HLA-ABC-PE-Cy7, HLA-DR-BV786; and the co-stimulatory molecule CD80-PE. All antibodies except CD105 were purchased from BD Biosciences (San Jose, CA, USA). The stained cells were assessed on the LSRFortessa (BD Biosciences) at the core facility BioVis at Uppsala University. Results were analyzed in FlowLogic software (Inivai Technologies, Australia).

Islet and DSC Co-Culture

Human islets were co-cultured with DSCs in either direct contact conditions upon a DSC monolayer (group 1) or in an indirect setting (group 2), where islets were placed inside a cell insert with pore diameter 0.4 µm, which allowed medium exchange but not cell-cell contact with a DSC monolayer. DSCs (group 3) and human islets (group 4) were also cultured separately.

Analysis of Inflammatory Biomarkers
Inflammatory biomarkers were measured in medium and lysates from DSCs and human islets after 48 hours of co-culture. Analysis was performed with an inflammatory biomarker panel provided by Olink (Uppsala, Sweden).
Cytoprotective Properties of DSCs on Human Islets

Islets were cultured in the presence of cytokines IL-1β 50 U/mL, IFN-γ 1,000 U/mL, and TNF-α 1,000 U/mL (PeproTech, London, UK) for 24 hours in the different co-culture conditions detailed above.

Islet Function

Duplicates of 50 islets, after DSC co-culture or not, were evaluated for insulin release in response to glucose and forskolin perifusion. The islets were initially perifused with KRBH-buffer, then primed to low glucose solution (2.0 mM) before exposure to high glucose (20.0 mM). The same procedure was repeated with addition of forskolin 1 µM. Samples were assessed for their insulin content by a human insulin ELISA (Mercodia, Uppsala, Sweden).

Gene Expression Analysis

Papers III and IV

For complete descriptions of preparation of tissue for microdissection, RNA isolation, PCR, and microarray analysis, see respective paper. Genes analyzed in paper III are specified in Table 3.

Table 3. Specifications of genes analyzed in paper III.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCK</td>
<td>Glucokinase</td>
<td>Glucose metabolism</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose transporter type 2</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GPD2</td>
<td>Glycerol-phosphate dehydrogenase 2, mitochondrial</td>
<td>Mitochondrial enzyme, glycolysis</td>
</tr>
<tr>
<td>INS1</td>
<td>Insulin 1</td>
<td>Peptide hormone</td>
</tr>
<tr>
<td>INS2</td>
<td>Insulin 2</td>
<td>Peptide hormone</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase A</td>
<td>Anaerobic glycolysis</td>
</tr>
<tr>
<td>PCX</td>
<td>Pyruvate carboxylase</td>
<td>Mitochondrial enzyme</td>
</tr>
<tr>
<td>PDX1</td>
<td>Pancreatic and duodenal homeobox 1</td>
<td>Transcription factor, beta cell differentiation marker</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>Glycolytic enzyme, reference gene</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
<td>Metabolic enzyme, reference gene</td>
</tr>
</tbody>
</table>
Statistical Analysis

Statistical analysis was performed using GraphPad Prism. All values are expressed as means ± SEM. Comparisons between two groups were performed by unpaired or paired two-tailed Student’s t-test. For all comparisons, p-values < 0.05 were considered statistically significant.

Paper I
Pearson’s product moment correlation was used for linear regression analysis. Multiple comparisons between data were performed using analysis of variance (ANOVA) and Bonferroni post-hoc test. Kruskal Wallis test was used for non-parametric data (the data was considered non-parametric based on Shapiro-Wilk normality test).

Paper II
Comparisons between three groups were performed using ANOVA and Tukey’s multiple comparisons test. For the multiple comparisons regarding the angiogenic markers a two-way ANOVA was performed with Sidak's multiple comparisons test.

Paper III
For comparison of relative gene expression data, a non-parametric one-way ANOVA was applied with Dunn’s post-hoc test using native pancreatic islets as control. For comparison of blood glucose measurements between the three groups, a one-way ANOVA with Tukey’s post-hoc test was applied. Comparison of time to normoglycemia was performed as a survival curve using Log-rank test (Mantel-Cox).

Paper IV
For comparisons between more than two groups, one-way ANOVA was performed using Dunnett’s correction for multiple comparisons. One-way ANOVA was calculated on AUC for islet function. Inflammatory biomarkers were analyzed with multiple t tests using False Discovery Rate (FDR) approach, where FDR was set to 1% and using the two-stage step-up method of Benjamini, Krieger and Yekutieli.
Results and Discussion

In this thesis, factors affecting islet transplantation and methods for improving its outcome were investigated. Human islet engraftment and surviving beta-cell mass after intraportal transplantation, the site of golden standard, was evaluated in paper I. Islets expressing GFP were used in paper II for repeated IT. Abdominal muscle and greater omentum were assessed as alternative sites in paper III. Finally, DSCs, a type of MSC isolated from human placenta, were isolated and characterized for possible use in IT.

Beta-Cell Loss after Intraportal Transplantation

The question if the liver is the optimal site for islet transplantation has yet to be answered. Understanding which factors affect engraftment are of importance in order to optimize intraportal transplantation or to find a site more suitable for islet transplantation. In paper I, we investigated the engraftment of human islets intraportally transplanted to nude mice. We have previously investigated the oxygenation of intraportally transplanted islets in mice\textsuperscript{60}, and comparing those results to human islets were of interest since the architecture of human and murine islets differ\textsuperscript{133,134}. When performing an islet transplantation to the liver, an instant loss of graft tissue occurs due to IBMIR. We compared beta-cell mass 1 day and 30 days after transplantation and investigated the degree of hypoxia.

We found that human islets undergo substantial graft loss beyond the immediate transplantation phase. The graft loss in our study was found to depend on both necrotic damage of islets and a high rate of apoptosis at 1 day, which remained at 30 days posttransplantation (21 % and 34 % respectively). Necrotic areas were common at 1 day posttransplantation and were also found to affect the surrounding liver parenchyma. The high rate of caspase-3, an apoptosis marker, at 30 days correlated with the time the islets had spent in culture, in line with what has previously been reported\textsuperscript{39}. Consequently, a negative correlation was observed between a high rate of apoptosis at 30 days posttransplantation and a low retained islet mass. The rate of apoptosis was even higher at 30 days than 1 day posttransplantation, which indicates that more regulated cell death persists in intraportally transplanted human islets than what was observed in murine islets, using the same apoptosis marker\textsuperscript{60}. 

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The total graft area was reduced by 52% after 30 days compared to that found at 1 day posttransplantation.

The vascular density doubled within the first month, but still remained only one third of that in native human islets, again demonstrating a hypoxic propensity in the liver. In line with a low rate of revascularization, 44% of islets were found positive for the hypoxia marker pimonidazole at 1 day posttransplantation, which indicates a tissue oxygen tension < 10 mmHg. Since necrotic and apoptotic cells do not accumulate pimonidazole, the degree of hypoxic cells at 1 day may even have been underestimated.

Amyloid Formation in Transplanted Islets

IAPP, predominantly produced in beta-cells, can form amyloid fibrils, known to be toxic for beta-cells. To evaluate amyloid content, consecutive slides of islet-bearing sections were stained with Cong Red. No amyloid or very low amounts were found in islets at 1 day posttransplantation, similar to levels observed in control islets, whereas 27% of islets contained amyloid deposits 1 month posttransplantation. Islets that already did contain amyloid at 1 day might reflect a known tendency for amyloid formation during culture or even preexistent amyloid in the islet donor. The amyloid observed at 30 days supports previous findings of a predisposition for progressive amyloid formation when human islets are transplanted to renal capsule as well as to the liver.

A case report with autopsy material from a patient that received allotransplanted islets showed that 43% of the islets contained amyloid. One possible reason for this may be inadequate drainage, since substantial formation of amyloid has previously been reported in avascular setting studies of both microencapsulated human islets and recombinant human IAPP murine islets. Although mostly associated with type 2 diabetes, extensive amyloid formation in transplanted islets are likely to contribute to graft failure.

Beneficial Effect of Repeated Islet Transplantation

The need of repeated islet transplantation in the clinic is common when islet graft function starts to decline. To evaluate the differences in engraftment between a first and second transplant, we performed intraportal islet transplantation to the same animal twice. We found a consistently better-preserved islet mass (in terms of both remaining number of individual islets and total islet area) from the second islet transplant. A control group, which was injected with a saline infusion instead of a first transplantation, showed an islet mass on par with the first islet injection in the double transplanted group. A tentative explanation that we investigated was whether the first transplantation induced an angiogenic niche through tissue expression of hypoxia, inflammatory
processes and liver microinfarctions. The latter was actually observed in paper I. However, there was no increase in angiogenic factors in serum immediately preceding the second transplantation and also no increase in islet vascular density between the control group and double transplanted group at 30 days post-transplantation.

The reason for the obtained findings is therefore obscure, but it is likely that the lodging of the second transplant in the liver differs from the first transplant due to some of the portal vein tributaries already being obstructed. Moreover, it is possible that gene expression in the liver parenchyma is changed by a first transplantation, inducing hypoxia and survival genes not necessarily reflected in circulating concentrations of angiogenic factors.

It is interesting to note that disruption of islets and compensatory growth (after unilateral nephrectomy or partial hepatectomy) in recipient tissue have previously been reported to have a positive impact on islet implantation. Autologous IT following pancreatectomy has demonstrated increased vascular density.

Islet Transplantation to Muscle or Greater Omentum

Numerous sites have been tested in attempts to improve islet transplantation. Along with new cell therapies and beta-cell replacement by stem cells, the requirements for a site with regards to monitoring and retrieval are even greater.

Islets implanted into muscle and into omentum are known to rapidly revascularize with a restoration of vascular network superior to the liver. Syngeneic transplantation of 200 islets to striated muscle or omentum was sufficient to cure the majority of diabetic animals in paper III.

Of the two investigated sites, islets transplanted to omentum had a slightly more favorable response to both intravenously administered glucose and insulin. This dysfunction in the muscular site might be due to exposure to prevailing hypoxia in the first week after transplantation, as previously reported by our group. Another explanation may be a marginal surviving islet mass in the intramuscular site, resulting in graft exhaustion. Substantial fibrosis formation has previously been reported for IT to striated muscle. In a study comparing different sites in the same mouse model, a higher number of islets were required, and it took longer time to reach euglycemia, for the mice receiving an IT to the intramuscular site as compared to the omentum. Of note, the surgical technique used in that study may differ from ours.

Blood glucose levels two hours after insulin injection were increased in animals with intramuscular islet grafts when compared to controls, indicating an exaggerated counter-regulatory response to hypoglycemia. Previous studies indicate that the glucagon response to hypoglycemia may be site-dependent, and a dysregulation of alpha-cells has been reported for the intraportal
A more recent study reported that glucagon secretion does increase in response to insulin-induced hypoglycemia, although not to the same extent as in healthy controls.

A difference between the sites investigated in this study is their venous drainage. Since the hepatic cells are a major target for insulin, the importance of portal drainage (as in omentum) has been one of the main arguments for using the liver as an islet implantation site. It has also been shown that a systemic venous drainage of islet grafts induces hyperinsulinemia through a decreased insulin clearance rate, causing insulin resistance. Since neither insulin nor C-peptide levels were measured in the current study, it cannot be excluded that the reduced clearance of glucose during IVGTT in animals with intramuscular islet grafts in part reflects an insulin resistant state. The initial glucose lowering response during ITT was identical to controls in both groups of transplanted animals, which at least is indicative of a normal response to insulin in peripheral tissue. Glycogen content in liver and muscle did not differ, indicating a functional glucose uptake regardless of transplantation site.

In contrast to our previous observations of a decreased percentage of glucagon-positive cells in islets experimentally transplanted to the liver, in this study we found that islet composition was unaltered in both islets transplanted to muscle and to omentum.

Gene Expression of Islet Transplants

The studied genes are involved in glucose metabolism within the beta-cells, which in turn secrete insulin, a prerequisite for the glucose uptake of almost every other cell type. Glucose is the major catabolic and anabolic substrate for our cells, and in itself, glucose regulates gene transcription, enzyme activity, and hormone secretion. This explains the huge range of consequences and complications, short- and long-term, that occur when normal glucose control is lost.

Gene analysis demonstrated an upregulation of GLUT2 (glucose transporter), PCX (metabolic enzyme), PDX1 (beta-cell differentiation marker), and INS1 and INS2 in islets transplanted both to omentum and muscle, compared to native islets (as opposed to humans, mice have a two-gene system for insulin transcription). GCK (enzyme), regulating the initial step of glucose-induced ATP synthesis, was unaltered. LDHA (enzyme) was downregulated, indicative of a low production of lactate. When LDHA was overexpressed in a beta-cell line, lactate stimulated insulin secretion even in the absence of glucose. Moreover, in contrast to our findings, previous experimental studies have observed altered gene expression in islets transplanted to liver, renal capsule, and pancreas.

In this study, as well as in papers I-II, normoglycemic recipients were used to avoid differences in blood glucose levels, which may affect islet grafts. We
have previously not observed any differences in engraftment and function of islet transplants to normoglycemic or successfully cured diabetic mice\textsuperscript{156,158,159}.

**Using Placenta as a Source of MSCs**

More than 1,000 studies involving MSCs were registered with ClinicalTrials.gov as of January 20\textsuperscript{th}, 2020. Human MSCs with the desired characteristics (rich secretome, immune-privileged, accessible, demonstrated genomic stability) and that fulfill the ISCT criteria can be obtained from numerous sources\textsuperscript{160}. Bone marrow is a commonly used source where MSCs only represent 0.01–0.001 % of the total amount of cells, and the proportion declines with age\textsuperscript{86,161}. Several studies demonstrate loss of function when using cells from later passages, e.g. better survival when using passages 1-2 instead of passage 3-4 bmMSCs, as a therapy for GVHD\textsuperscript{98}. MSCs from placenta would be a suitable source to avoid unwanted expansion in culture. Prolonged culture is also associated with a risk of malignant transformation, even though this risk appears low in human MSCs\textsuperscript{162}. The almost non-existent proportion of cell engraftment \textit{in vivo} may also contribute to the low occurrence of ectopic tissue formation reported\textsuperscript{98,99}. The genomic stability of adult stromal cells is another major advantage of MSCs as compared to embryonic stem cells and induced pluripotent stem cells.

Few ethical considerations are connected to harvest of cells when using the placenta, a tissue that would otherwise be discarded. Adequate testing of blood-borne disease prior to isolation can easily be performed during routine visits at the maternity ward for safe handling. Using elective cesarian section, instead of placenta after vaginal delivery, minimizes stress on the cells, resulting in a great number of viable cells without the need of too many passages.

**Alterations of DSCs in Culture**

Surface marker evaluation of DSCs was undertaken and demonstrated a typical display of MSC markers, with a decreasing donor variability the longer the cells stayed in culture. The uneven proportion of DSCs (PE-negative population) seen in early passages (1-2) likely reflects additional cell types that came with the isolation procedure but regressed later on. From passage 5-10, the cell populations were quite homogeneous from this aspect. The HLA-DR marker was only expressed in the first 1-2 passages. The generally low alloreactivity mobilized by MSCs is believed to be due to the lack of MHC II molecules, which we also confirmed for DSCs. Of note, this expression may however change \textit{in vivo}. 
Donor variability has been cited as a contributing reason why clinical trials with MSCs have not delivered the hoped-for results\(^{163,164}\). Our characterization of surface marker expression and subsequent gene analysis from different passages is promising for a “steadiness” of DSCs without too large inter-donor variation. The passage number had a greater impact than the donor in regards to the number of differentially expressed genes, and the changes seen were relatively small. The relative limitation of age and gender regarding the donors might also work to our advantage in the quest for an unswerving DSC protocol\(^{163}\). Additionally, cells were shown to be of maternal origin and exhibited a limited capacity of multilineage differentiation, in line with what has been previous described\(^{126}\). The differentiation capacity and the other ISCT criteria do not necessarily predict biological outcome, which is most likely dependent on the DSC secretome\(^{165}\).

**Impact of DSCs on Human Islets in Vitro**

Previously, MSCs have been used to increase both rodent and human islet viability and to improve transplantation\(^{115,166}\). These effects are mainly mediated by anti-inflammatory effects, secreted angiogenic factors, and induction of tolerance for allogeneic grafts\(^{106,111,167}\). The type of co-culture (direct vs indirect cell contact) is of importance, but exact mechanisms remain unclear\(^{119}\). To investigate if similar effects are induced by DSCs, a co-culture experiment using both direct and indirect contact culture, the latter with a transwell system, was set up.

Assessment of medium after 48 hours from all co-culture groups (including control DSCs and islets) revealed consistently elevated levels of inflammatory markers in DSC-containing medium. Co-culture of islets and DSCs resulted in altered levels (compared to control DSCs) of several markers, including VEGF-A, CXCL11 and PD-L1, involved in chemotaxis and T-cell activation. The same markers were analyzed in cell lysates where no differences were observed between islets from the co-culture settings. Meanwhile, DSCs from indirect culture displayed altered levels of 31 out of 65 detectable inflammatory biomarkers.

DSCs had no cytoprotective effect on islets during 24 hours of exposure to a cytokine mixture. Yeung et al. demonstrated a protective effect of MSC-islet co-culture; however, co-culture was initiated 24 hours before cytokine exposure\(^ {48}\). Different culture times, dosage of cells, culture set up, as well as cell and islet source may affect the contrary results obtained in our experiment. The absolute levels of inflammatory markers measured cannot be determined by the arbitrary NPX-unit, used for relative quantification in this study. Nevertheless, incubation in a pro-inflammatory milieu for too long will likely hamper islets, especially if their prior condition adds to a “pro-inflammatory” state of DSCs\(^ {88,168,169}\).
Islet function after co-culture exhibited three different responses to high glucose and forskolin perifusion, likely reflecting the variability of human islet condition and its importance for the interactions that take place between islets and DSCs. However, our results suggest that the choice of co-culture system does not alter the effect of DSCs on islet function. Forward, assessing islet viability beforehand would be useful in order to understand which sets of islets would or would not benefit from DSC treatment.

Gene expression analysis demonstrated no differentially expressed genes in DSCs from co-culture groups, whereas islets from the direct contact culture group demonstrated a greater number of differentially expressed genes compared to control and indirect cultured islets. Further evaluation of these changes is needed to elucidate what categories of genes constitute these changes and how they may alter the islets. Co-culture setup that allows cell-cell contact has demonstrated alterations in MSCs in close connections to the islets, which started expressing insulin and PDX1, a key protein for insulin production. Recent findings demonstrate mitochondrial transfer as a mechanism of MSC effect on beta-cells in co-culture. This exchange is also reported to depend on cellular stressors of human islets, which increased the mitochondrial transfer compared to undamaged murine islets.
Conclusions

Paper I
- Substantial loss of approximately 50% of human islet tissue occurs between 1 and 30 days after intraportal transplantation
- The vascular density increases after 30 days but is only one third of that in native islets
- One third of human islets contain amyloid after 30 days and those islets express a higher rate of apoptosis

Paper II
- Islets from a second transplantation show better survival if performed 1 week after a primary islet transplantation
- This was not due to improved islet vascular density

Paper III
- The majority of diabetic animals were cured by 200 islets transplanted to omentum or muscle
- The islet grafts maintained or increased the expression of genes important to beta-cell function
- The glycogen levels in muscle and liver were not deranged depending on systemic or portal insulin release

Paper IV
- DSCs can easily be isolated from human placenta
- DSCs display similar characteristics and low inter-donor variability
- Gene expression of DSCs is altered in culture, especially between passage 2 and 5
- Changes in medium content as well as gene expression occur in human islets after co-culture with DSCs, and the functional outcome may be dependent on islet viability
Founding

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Att inte alla kan erbjudas transplantation beror på flera faktorer, dels bristen på donatorer, dels att effekten av en transplantation avtar med tiden och att ingreppet därför ofta behöver upprepas, och dels behovet av immunhämmande behandling för att inte transplanterad stötas bort.

Delarbeten

I delarbete II fick samma mus två transplantationer för att se om öarnas infästning i levern förändras. För att kunna skilja på första och andra transplantationen var de första öarna infärgade med en grön markör. I denna studie överlevde genomgående fler öar från andra transplantationen. Vi hittade ingen skillnad i t.ex. kärlinväxt så varför det blev så går inte att säga säkert. Möjligtvis påverkar den första transplantationen van nästkommande öar hamnar, alternativt påverkas levern på ett sätt som inverkar positivt på andra omgångens öar.

I sista arbetet isolerades en typ av stamceller från modernkakor efter kejssnitt. Ett stort antal celler kunde då utvinnas utan några risker för donatorn. Dessa celler har liknande egenskaper som andra stamceller från t.ex. benmärg, men för att isolera stamceller från benmärg krävs ett kirurgiskt ingrepp och cellerna behöver växa till i odling. Varianter av stamceller från människa och mus har tidigare visat sig kunna bidra till bättre resultat vid transplantation av öar men mekanismerna för detta är inte klarlagda. För att ta reda på om stamceller från modernkaka kan användas undersöktes celler från flera donatorer för att se hur de skiljer sig och vad som sker under odling. Cellerna har samodlats med öar varefter genuttryck samt faktorer som frisätts från cellerna har analyserats för att bättre förstå deras funktion.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)