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Auxiliary Cells for the Vascularization and Function of Endogenous and Transplanted Islets of Langerhans

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Abstract

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Type 1 diabetes develops through the progressive destruction of the insulin-producing beta-cells. Regeneration or replacement of beta-cells is therefore needed to restore normal glucose homeostasis. Presently, normoglycemia can be achieved by the transplantation of whole pancreas or isolated islets of Langerhans. Islet transplantation can be performed through a simple laparoscopic procedure, but the long-term graft survival is low due to poor revascularization and early cell death.

This thesis examined the possibility of using different auxiliary cells (Schwann cells, endothelial progenitor cells, and neural crest stem cells) to improve the engraftment and function of endogenous and transplanted islets.

Co-transplantation of Schwann cells with islets improved islet graft function early after transplantation, and caused an increased islet mass at one month posttransplantation. However, the vascular densities of these grafts were decreased, which also related to an impaired graft function.

Islet grafts containing endothelial progenitor cells had a superior vascular density, with functional chimeric blood vessels and substantially higher blood perfusion and oxygen tension than control transplants.

By culturing and transplanting islets together with neural crest stem cells it was found that islets exposed to these cells had a higher beta-cell proliferation compared with control islets. At one month posttransplantation, the grafts with neural crest stem cells also had a superior vascular- and neural density.

The potential of intracardially injected neural crest stem cells to home to the pancreas and ameliorate hyperglycemia in diabetic mice was investigated. During a three-week period after such cell treatment blood glucose concentrations decreased, but were not fully normalized. Neural crest stem cells were present in more than 10% of the pancreatic islets at two days postinjection, at which time the beta-cell proliferation was markedly increased when compared with islets of saline-treated diabetic animals. Three weeks later, a doubled beta-cell mass was observed in animals receiving neural crest stem cells.

In summary, islets can easily be transplanted together with different auxiliary cells. Some of these cells provide the possibility of improving vascular- and neural engraftment, as well as beta-cell growth and survival. Systemic administration of neural crest stem cells holds the potential of regenerating the endogenous beta-cells.

Keywords: Islets of Langerhans, beta cells, diabetes, transplantation, vascularization, Schwann cells, endothelial progenitor cells, neural crest stem cells

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It always seems impossible until it's done.

– Nelson Mandela

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List of Papers

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

I **Grapensparr, L.***, Ullsten, S.*, Drott, C.J., Milbrandt, J., Sandberg, M., Carlsson, P-O. (2017) Influence of islet and peri-islet Schwann cells on vascularity and beta-cell function in endogenous and transplanted islets. *Manuscript.* *Equal contribution.

II **Grapensparr, L.**, Christoffersson, G., Carlsson, P-O. (2017) Bioengineering with endothelial progenitor cells improves the vascular engraftment of transplanted human islets. *Manuscript submitted.*

III **Grapensparr, L.**, Vasylovska, S., Li, Z., Olerud, J., Jansson, L., Kozlova, E.N, Carlsson, P-O. (2015) Co-transplantation of human pancreatic islets with post-migratory neural crest stem cells increases beta-cell proliferation, and vascular and neural regrowth. *J Clin Endocrinol Metab* 2015:jc20144070.

IV **Grapensparr, L.**, Jönsson, S., Ullsten, S., Li, Z., Kozlova, E.N, Carlsson, P-O. (2017) Systemic neural crest stem cell treatment alleviates experimental type 1 diabetes by cellular homing to the damaged islets, inducing beta-cell regeneration. *Manuscript.*

Contents

Abbreviations

CD	Cluster of differentiation
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGFP	Enhanced green fluorescent protein
EPC	Endothelial progenitor cell
EPOC	Endothelial progenitor outgrowth cell
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
IA2	Islet antigen-2
i.p.	Intraperitoneal
i.v.	Intravenous
MMP-2/-9	Matrix metalloproteinase 2/9
NCSC	Neural crest stem cell
NMRI	Naval Medical Research Institute
NOD	Non-obese diabetic
NOD/SCID	Non-obese diabetic/Severe combined immunodeficiency
PGP9.5	Protein gene product 9.5
PNS	Peripheral nervous system
SC	Schwann cell
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
Tfam	Transcription factor A, mitochondrial
TPU	Tissue perfusion unit
VEGF-A	Vascular endothelial growth factor A

Introduction

The pancreas

The human adult pancreas is approximately 12-15 cm long, weighing between 40-175 g. The pancreas is situated behind the stomach and is anatomically divided into the head (caput), body (corpus), and tail (cauda).

A majority of the pancreatic volume (98-99 %) is involved in the exocrine function of the pancreas. The exocrine part of the pancreas is responsible for producing the pancreatic juice, which contains digestive enzymes such as amylase, lipases, and proteases. The produced enzymes are released into the duodenum, where they aid in the digestion of carbohydrates, fatty acids, and proteins [1].

The remaining 1-2 % of the pancreatic volume is involved in the endocrine function of the pancreas.

Islets of Langerhans

The endocrine part of the pancreas is made up of small pancreatic islets, also known as the islets of Langerhans. Paul Langerhans (1847-1888) was the first to discover these islets (1869), describing them as "small cells of almost perfect homogeneous content and of a polygonal form, with round nuclei, mostly lying together in pairs or small groups" [2]. It wasn't until 1893 that their role in glucose homeostasis was first suggested by Edouard Laguesse (1861-1927) [3].

It is now known that a human non-diabetic adult has between 3.6 to 14.8 million islets in their pancreas [4]. Most of these islets are small (<23 μm). When only counting the islets that measure ≥23 μm, the number of islets drop to approximately 1.5 million [5]. These vital pancreatic islets are highly variable in size, with a mean diameter of 108.92 μm [6]. They contain five different endocrine cell types; alpha-, beta-, delta-, polypeptide-, and epsilon cells. These cells produce glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively.

Noteworthy, the cytoarchitecture of pancreatic islets is species dependent. This thesis includes studies of both rodent and human islets. In rodent islets, beta-cells account for >70 % of the total number of islet cells, whilst alpha cells account for <20 %. The beta-cells are clustered at the center, with

alpha-, delta-, and polypeptide cells in the periphery. Meanwhile, human islets contain approximately 60 % beta-cells and 30 % alpha cells. The different endocrine cells are intermingled throughout the human islet, with a majority of them being in close association with endothelial cells and smooth muscle cells [7].

The neural- and vascular network of the pancreatic islets

In addition to the endocrine cells, pancreatic islets contain Schwann cells (SCs), as well as a neural and vascular network. Both of these networks are vital for islet survival and function. Furthermore, beta-cells are dependent on communication with different cell types, such as neural crest stem cells (NCSCs) [8], nerves [9], and endothelial cells [10], for normal development and function.

The neural network of pancreatic islets contains sympathetic-, parasympathetic (vagus nerve)-, sensory (splanchnic nerve)-, as well as peptidergic neurons [11]. Just like the cytoarchitecture of pancreatic islets is species dependent, their innervation pattern is also unique.

Rodent islets have a dense neural network, with a large number of parasympathetic nerves in the core of the islets. The periphery of the rodent islet is rich of sympathetic nerves. Whilst the parasympathetic nerves innervates both alpha- and beta-cells, sympathetic nerves innervate alpha-cells [12].

Unlike rodent islets, with their dense neural network, human islets are sparsely innervated and parasympathetic nerves are rarely observed. Meanwhile, sympathetic nerves run into the center of the islets in parallel with the endothelium [12].

In both rodent and human islets, parasympathetic nerves secrete neurotransmitters that stimulate insulin secretion [13, 14] and sympathetic nerves inhibit insulin secretion [12, 15, 16].

Studies in rats have shown that although the pancreatic islets only constitute 1-2 % of the total pancreas, they normally receive approximately 10 % of the total pancreatic blood flow. This can further be increased to 15 % in response to glucose [17]. These results signify the high metabolic demand for oxygen and nutrients, as well as the importance of the glomerular-like vascular system of the islets.

Apart from facilitating transport of nutrients, oxygen, and hormones, the intra-islet endothelial cells constantly communicate with surrounding beta-cells, aiding in normal beta-cell development, growth, differentiation, and function. Many growth factors involved in this communication have been identified, including hepatocyte growth factor (HGF) [18, 19], thrombospondin-1 [20], endothelin-1 [21], laminins [22, 23], and Collagen

IV [22]. In return, beta-cells express vascular endothelial growth factor A (VEGF-A), which is crucial for normal islet vascularization and vascular function [24, 25].

Diabetes mellitus

Beta-cells release insulin, which is the only hormone in the body capable of decreasing the blood glucose level. Insulin lowers the blood glucose level by stimulating glucose uptake in target tissues, as well as inhibiting hepatic glucose production. A healthy pancreas contains an excess of beta-cells in relation to what is needed for normal glucose homeostasis. Nevertheless, the beta-cell mass is not constant throughout life, it changes based on beta-cell size, proliferation and death, as well as neogenesis from precursors.

Diabetes mellitus is characterized by a decreased insulin secretion and/or insulin sensitivity, resulting in hyperglycemia. The disease can be divided into several subtypes; type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), latent autoimmune diabetes in the adult, maturity onset diabetes in young, gestational diabetes mellitus, and mitochondrial diabetes mellitus. According to the International Diabetes Federation, the global prevalence of diabetes was estimated to 415 million people in 2015. The number is estimated to increase to 642 million by 2040 [26]. Approximately five to ten percent of these patients suffer from T1DM.

T1DM is developed through a progressive and selective destruction of the insulin-producing beta-cells, resulting in a decreased insulin secretion and eventually a near complete lack of endogenous insulin production.

As stated above, the pancreas contains a great reserve of beta-cells. Although it differs greatly between individuals, symptoms of T1DM start to manifest following a 40-95 % reduction in beta-cell mass [27, 28]. At this point, exogenous insulin administration is necessary to obtain a normalized glycemic control. Nevertheless, long-term microvascular and macrovascular complications may gradually develop as a consequence of hyperglycemic episodes [29].

Beta-cell proliferation

Although a majority of the beta-cells are gone at the time of T1DM diagnosis, studies have shown that an intense insulin treatment with close monitoring of the blood glucose level early after diagnosis can result in a temporary partial remission from the disease [30]. A recent study show that islets isolated from T1DM patients initially have an impaired glucose-induced insulin secretion [31], explained by episodes of hyperglycemia [32]. When cultured in a normoglycemic environment for

three to six days, the islets demonstrated a normalized biphasic insulin release [31]. The results from these studies indicate that the disease progress can momentarily be haltered and beta-cell function restored. This begs the question if means of expanding the remnant functional beta-cell mass can be used as the ultimate treatment modality in both T1DM and T2DM.

Although significant human beta-cell proliferation seems to be limited to the first three decades of life [33], it has been reported that a high metabolic demand can stimulate expansion of the beta-cell mass and improve insulin secretion. One such example is the suggested beta-cell proliferation that occurs during pregnancy. Although contradictive results have been published [34, 35], detectable C-peptide levels have been observed during pregnancy in women with longstanding T1DM with no detectable C-peptide prior to pregnancy [36]. Studies have also shown that obese individuals have an increased beta-cell mass and insulin secretion in comparison with lean individuals [37-39], further indicating that a high metabolic demand affects the number and function of the pancreatic beta-cells, at least for some time.

In order to create a treatment for diabetes, it would be necessary to find a stimulus that is both capable of finding the target tissue as well as promoting proliferation of existing beta-cells. Several stimuli (including intrinsic regulators [40], glucagon-like peptide-1 [41, 42], glucose-dependent insulinotropic polypeptide [43], insulin-like growth factor 1 [44], HGF [45], prolactin and placental lactogen [46, 47]) are known to remarkably promote rodent beta-cell proliferation. Nevertheless, the same stimulus is often proven incapable of inducing human beta-cell proliferation.

Islet transplantation

As of this moment, the only curative treatment option for T1DM is to replace the lost beta-cell, either by transplantation of whole pancreas or of isolated pancreatic islets.

Whole pancreas transplantation is a complicated surgery, often performed simultaneously with a kidney transplant. Although the long term outcome is superior that of islet transplantation, with a 72.3 % graft survival at ten years posttransplantation [48], it often leads to longer hospitalization and more severe complications [49].

The alternative to transplanting the whole pancreas is to transplant isolated pancreatic islets. Although it can be performed through a simple laparoscopic procedure, meaning fewer risks and less suffering for the patient, the long-term outcome is poor. At best, only 44 % of the islet recipients are free from exogenous insulin administration at three years posttransplantation [50].

Several implantation sites have been tested experimentally or clinically [51], including liver [52, 53], muscle [54, 55], pancreas [56], omentum [57],

spleen [58], beneath the kidney capsule [59], anterior chamber of the eye [60], and bone marrow [61]. Attempts have also been made to transplant encapsulated islets, shielding them from the hostile environment in the recipient with a physical barrier [62-64].

Clinically, the most common implantation site is the liver. Islets are infused into the liver through a catheter in the portal vein, and thereafter become lodged in distal vein tributaries. The major drawbacks to using the liver as implantation site is the instant blood mediated inflammatory reaction [65], high lipid levels [66], the low intrahepatic oxygen tension [67], and poor revascularization [68]. Several studies have used pimonidazole to study hypoxia in islets transplanted to the liver. Pimonidazole is a marker for hypoxia, as it accumulates in cells at a tissue oxygen tension <10 mmHg [69]. The normal oxygen tension for metabolically active rodent islets is approximately 40 mmHg [70]. The results of these studies show that at one month posttransplantation 45-70 % of the islets stained positive for pimonidazole, thus being severely hypoxic [71, 72]. Further analysis of the grafts revealed that the islet mass had been reduced by 52 % during the first month posttransplantation [71]. By retrieving islets transplanted to the liver, it was found that these islets were irreversibly functionally impaired. They had a lowered insulin content, glucose-stimulated insulin release, and glucose oxidation rate [73].

Islet engraftment: Revascularization and reinnervation

During whole pancreas transplantation, vascular and enteric anastomoses are performed, securing an adequate supply of oxygen and nutrients to the islets [74]. Meanwhile, when islets are isolated prior to islet transplantation, both the neural- and vascular network within islets are disconnected from the surrounding nerves and blood vessels. As soon as the islets are transplanted into the recipient, they must adapt to the implantation site in order to survive. To achieve long-term graft survival it is crucial that this engraftment process is efficient and that both the neural- and vascular networks form new connections rapidly posttransplantation.

Graft revascularization precedes graft reinnervation [75]. Revascularization is achieved through a process known as angiogenesis, which is triggered by proangiogenic factors such as VEGF-A, interleukin 8, and fibroblast growth factor. During angiogenesis, new blood vessels are formed from the preexisting vasculature [76]. It is a complex remodeling process involving proliferation of endothelial cells, cell migration, detachment and recruitment of mural cells, proteolytic breakdown of the basement membrane, tube formation, and vessel fusion [76, 77]. These newly formed blood vessels can arise from either the recipient endothelium or the remnant donor endothelium [78]. Although the process of

angiogenesis occurs rather rapidly posttransplantation due to the proangiogenic factors released from the hypoxic tissue, the vascular density is often lower in transplanted islets compared with native islets.

Neural growth is a slow process that is triggered and supported by the presence of neurotrophic factors. The resulting neural network in transplanted islets differs depending on the implantation site, but the innervation pattern is often dissimilar compared with native islets [75, 79]. Interestingly, islets transplanted to the anterior chamber of the eye develop a neural network similar to that of native islets, with regard to both parasympathetic- and sympathetic nerves [80].

New ways of improving islet engraftment is highly desirable. This may include a combinational cell therapy in order to expand the number of functional beta-cells and give support to transplanted islets by improving the revascularization and reinnervation process. By adding different stem- and progenitor cells – known to enhance vascular and neural growth – when transplanting islets, graft survival and function can be improved [81-85]. In this these, the potential of SCs, endothelial progenitor cells (EPCs), and neural crest stem cells (NCSCs) to improve islet vascularization, innervation, and function was investigated.

Schwann cells

Glial cells provide support for neurons, aid in the maintenance of homeostasis by regulating the concentrations of ions and neurotransmitters, and form myelin. There are several different glial cells, including SCs, oligodendrocytes, satellite cells, astrocytes, microglia, ependymal cells, olfactory ensheathing cells, and enteric glia.

The SC is a type of glial cell present in the peripheral nervous system (PNS). SCs ensheath all axons in the PNS and can be divided into two types; myelinating and non-myelinating. Myelinating SCs form myelin around axons and non-myelinating SCs provide metabolic- and mechanical support for neurons [86].

SCs develop from the neural crest during the embryonic development [87]. It has been shown that these cells are vital for normal neuronal development and regeneration of damaged nerves [86]. Studies have shown that they promote both vascular and neural growth [88-90].

Pancreatic islets are sheathed by SCs. They cover a substantial area of the islet periphery, but also follow the axons into the islet parenchyma. Although the presence of SCs in islets has been known for many decades [91-93], the physiological role of these glial cells has never been investigated.

The cells have been shown to increase in numbers, and migrate into the islet parenchyma in response to islet injury, e.g. in streptozotocin (STZ) treated mice and non-obese diabetic (NOD) mice [94, 95], likely due to their

role in regeneration of damaged nerves. SCs are also present in islet transplants, but in decreased numbers, and have therefore been suggested to be important to attract new blood vessels and nerves [96]. Furthermore, the cells have been implicated to be an early target of the immune process during the development of T1DM [97-99].

In this thesis, their importance for islet function and vasculature was investigated, both by exposure of islets to increased SC numbers *in vitro* and *in vivo*, as well as by studies of mice with dysfunctional SCs.

Endothelial progenitor cells

EPCs were first described by Asahara *et al.* in 1997 [100]. These proangiogenic cells are generally divided into early EPCs and late (also known as endothelial progenitor outgrowth cells (EPOC)) EPCs, depending on their surface markers. However, these markers can differ greatly between studies, making comparisons difficult.

The processes by which EPCs contribute to neovascularization have been widely studied and debated. Most would agree that early and late EPCs contribute to neovascularization in different ways. Early EPCs are suggested to enhance angiogenesis and support endothelial cell proliferation and survival through the release of angiogenic- and pro-inflammatory cytokines, including VEGF, HGF, granulocyte-macrophage colony-stimulating factor (GM-CSF), and matrix metalloproteinase 9 (MMP-9) [101-103]. The release of GM-CSF works as a recruitment signal that increases the release of EPCs from the bone marrow and attracts them to the site of vascular injury. Late EPCs, on the other hand, both produce signals that facilitates in the neovascularization process (such as monocyte chemoattractant protein-1, interleukin 8, and matrix metalloproteinase 2 (MMP-2) [102, 103]) and become building blocks for the developing vasculature by maturing into endothelial cells. While some report that they get incorporated into the damaged endothelium [104-106], others argue that they work as supporting perivascular cells rather than lining the vascular lumen and only work through the release of said factors [107, 108]. In fact, it has been shown that cord-blood derived late EPCs are capable of promoting angiogenesis by mere paracrine effects [109]. Interestingly, Yoon et al. used a hind-limb ischemia model in athymic nude mice and found that early- and late EPCs work synergistically through the release of different cytokines and MMPs, leading to a greater neovascularization than when using early- or late EPCs alone [103].

Due to the large islet endothelial cell death, as well as all the micro- and macrovascular complications, that follow with diabetes, one might hypothesize that EPCs would be recruited from the bone marrow at disease onset to aid in rebuilding the vasculature. In fact, in a study where STZ was used to induce diabetes in mice, EPCs were found to be recruited and homed

to the injured pancreas [110]. However, the EPCs of mice and humans suffering from diabetes are dysfunctional, likely due to the glucotoxicity, inflammation, and oxidative stress that also contributes to the islet vascular destruction. In fact, it has been demonstrated that the decreased number of functional EPCs in diabetic patients correlate with both the occurrence and severity of diabetes related vascular complications [111]. If these EPCs can become functional anew, they may provide support for both native and transplanted islets, as well as for the repair of vascular complications.

Neural crest stem cells

The neural crest emerges from the dorsal neural tube, from the head to the tail, and its cells starts to migrate out into the developing embryo [112], influencing almost every major organ. One of these organs is the pancreas. In mice, it has been shown that the NCSCs populate the pancreas at E10.5-11.5, and that this process is stimulated by glial-derived neurotrophic factor (GDNF) expression in the pancreatic epithelium. Without this expression, the pancreas develops an inferior innervation pattern, with a massive loss of neural- and glial cells, as well as parasympathetic nerves [113]. As already mentioned, these parasympathetic nerves are typically supposed to stimulate insulin secretion [13, 14]. Noteworthy, these GDNF-lacking mice had an equal islet area at 0 and 3 weeks compared with normal controls. In addition, the islet architecture was unaffected and there were no changes in beta-cell proliferation between the groups [113].

Interestingly, Plank et al. [8] have looked at the pancreas of mice completely lacking the NCSCs that would normally migrate to the pancreas, through the deletion of the winged-helix transcription factor FoxD3. When FoxD3 is deleted, there is an increased proliferation of insulin-expressing cells in the developing pancreas. However, when investigating the gene expression of these cells, it was found that the actual maturation of these “beta-cells” was impaired [8]. Since the mice in Muñoz-Bravo et al. study [113] did not completely lack NCSC migration to the pancreas it is likely that the few NCSCs that were present negatively regulated the beta-cell proliferation, explaining the equal islet mass between groups. Since many of the beta-cells in this study are likely immature, functional testing is desirable.

Despite their negative regulation of beta-cell proliferation during development, NCSCs have been shown to stimulate beta-cell proliferation and improve the function of adult cultured and transplanted rodent islets [114, 115]. The beta-cell proliferative effect was mediated through cell-cell interactions. In addition to their ability to induce beta-cell proliferation, NCSCs also secret many factors that may be of further benefit for transplanted islets. This is the suggested pathway for the increased

reinnervation and revascularization observed in rodent islet grafts containing NCSCs [116].

Aims

The overall aims of the work presented in this thesis were to evaluate the potential of auxiliary cells to improve vascularization, innervation, and function of endogenous and transplanted islets of Langerhans. The specific aims of the studies included in this thesis were:

Paper I
The aim of this study was to investigate the importance of SCs for islet function and vasculature. This was studied by exposing islets to increased SC numbers *in vitro* and *in vivo*, as well as by studies of mice with dysfunctional SCs.

Paper II
This study tested the hypothesis that EPCs, isolated from human umbilical cord blood, could be coated to human islet surfaces and be used to promote islet vascular engraftment.

Paper III
This study tested the hypothesis that cellular therapy with NCSCs may be used to induce human beta-cell proliferation, as well as to allow for reconstitution of the innervation and vasculature in transplanted human islets.

Paper IV
The aim of this study was to investigate the potential of intracardially injected NCSCs to home to the pancreas and restore normoglycemia in diabetic mice.

Materials and Methods

Schwann cells (paper I)

The SCs used in this study (cell line sNF96.2) were obtained from a malignant peripheral nerve sheath tumor, isolated from a 28 year old male diagnosed with neurofibromatosis type 1 (NF1; ATCC, Manassas, VA). The sNF96.2 cell line was originally obtained from numerous passages of primary tumor material in culture, until they were a homogenous SC-like population (characterized by their clonal morphology and expression of both cytoplasmic SC markers S100 and p75).

The cells were cultured in T75 flasks (Corning Life Sciences, Corning, NY), with Dulbecco's Modified Eagle's Medium containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate (ATCC). The media was further supplemented with fetal bovine serum (ATCC).

The medium was changed every two to three days. When subculturing the cells, a trypsin (0.25 % (w/vol)) and ethylenediaminetetraacetic acid (EDTA) (0.53 mM) solution was added for five minutes at 37°C.

Endothelial progenitor cells (paper II)

EPCs from human umbilical cord blood (AMS Biotechnology, Abingdon, UK) were used at passage 6-7 in all experiments in paper II. The obtained cells were characterized by their expression of cluster of differentiation (CD) 34, CD146, vascular endothelial growth factor receptor 2, von Willebrand factor, low-density lipoprotein uptake and lectin binding. The cells used in paper II did not express CD133, defining them as late EPCs/EPOC. Their functional capacity was confirmed by tube formation and migration assays.

Cells were cultured in T75 flasks (Corning Life Sciences, Corning, NY), with endothelial progenitor outgrowth cell media (AMS Biotechnology) containing fetal bovine serum and human recombinant growth factors. The medium was changed every two days. The EPCs were subcultured using a 0.05 % trypspin/EDTA solution for 5 minutes at 37°C.

Neural crest stem cells (paper III - IV)

Dorsal root ganglia from C57BL/6-beta-actin enhanced green fluorescent protein (EGFP) transgenic mice at embryonic day 11.5 were isolated and used to generate postmigratory NCSCs from the boundary cap [115]. These cells have previously been shown to remain in an undifferentiated state, being positive for nestin, p75, Bm3A, neurogenin 1, neurogenin 2, and Krox20, whereas only a few cells express markers for mature glia- or neural cells [117]. Cells were cultured in N2 medium (Life Technologies, Carlsbad, CA) containing B27 (Life Technologies), epidermal growth factor (20 ng/mL;R&DSystems) and basic fibroblast growth factor (20 ng/mL; R&D Systems). The medium was changed every other day until neurospheres began to form. The freefloating spheres were cultured in propagation medium (DMEM/F12; Life Technologies) supplemented with B27, N2, basic fibroblast growth factor (20 ng/mL), and epidermal growth factor (20 ng/mL).

In paper IV, the neurospheres were scattered into single cells using TrypLE Express (Thermo Fisher Scientific Inc., Waltham, MA) thirty minutes prior to cell injection. By dispersing the neurospheres into a single cell suspension, the cells could be injected into the left ventricle without causing an obstruction in the vascular system.

Animals (paper I - IV)

In paper I, $Tfam^{loxP/loxP}$ mice in a pure C57BL/6 background [118] were crossed to P_0-Cre mice also in a pure C57BL/6 background [119]. Compound heterozygotes (P_0-Cre/,$Tfam^{+/loxP}$) were then backcrossed to homozygous $Tfam^{loxP/loxP}$ to generate the Tfam-SCKO mice (P_0-$Cre^{+/-}$ /,$Tfam^{loxP/loxP}$) and their control littermates (P_0-$Cre^{-/-}$, $Tfam^{loxP/loxP}$). Tfam is a nuclearly encoded mitochondrial protein that is crucial for mitochondrial DNA maintenance, copy number regulation and transcription [118]. Deletion of Tfam in $Tfam^{loxP}$ homozygous mice expressing cre-recombinase in a tissue therefore results in severe tissue-specific mitochondrial DNA depletion and mitochondrial respiratory chain deficiency causing mitochondrial dysfunction. These mice were studied at three months of age. Adult male C57Bl/6 mice (Taconic M&B, Ejby, Denmark) were used as donors of islets for transplantation, whereas immune deficient non-obese diabetic/Severe combined immunodeficiency (NOD/SCID) mice were used as graft recipients. NOD/SCID mice (Taconic M&B) were also used as graft recipients in paper II.

In paper III, heterozygous embryos from C57BL/6-beta-actin EGFP transgenic mice (The Jackson Laboratory, Bar Harbor, ME), were used to isolate NCSCs. Adult, male Naval Medical Research Institute (NMRI) nu/nu

mice (Taconic M&B) were used as islet graft recipients. NMRI nu/nu mice were also used for all experiments in paper IV.

The animals were housed under standardized conditions (12h light/12h dark cycle), and had free access to water and pelleted food. 'Principles of laboratory animal care' (National institutes of Health, Bethesda, MD; publication no. 85-23, revised 1985) were followed, as well as Swedish national law. All experiments accounted for in this thesis were approved by Uppsala's Ethical Committee on Animal Experiments.

Mouse islet isolation and culture (paper I)

Pancreatic islets were isolated from mice using collagenase digestion and density gradient purification. Briefly, the mice were anesthetized and the common bile duct was exposed. Cold collagenase solution (2.5 mg/mL Clostridium histolyticum (Roche Diagnostics, Mannheim, Germany) in Hanks balanced salt solution (National Bacteriological Laboratory, Stockholm, Sweden)) was injected into the pancreas via the common bile duct. The pancreas was surgically removed and placed in a 37°C water bath for 18 minutes. Density gradient centrifugation using Histopaque-1077 and serum-free RPMI 1640 (Sigma-Aldrich, Saint Louis, MO) was performed in order to separate the islets from the exocrine tissue.

Islets were handpicked and cultured free floating in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 11.1 mmol/L glucose and 10 % (vol/vol) fetal calf serum before islet function assays or transplantation two days later.

Human islet isolation and culture (paper II & III)

Human islets were obtained from the human islet isolation facility for the Nordic Network for Clinical Islet Transplantation (Rudbeck Laboratory, Uppsala University Hospital, Uppsala, Sweden) as previously described [120]. Islets were isolated from 29 brain-dead, heart-beating donors (see Table 1 in paper II and III for donor characteristics). The human islets were cultured at 37°C and 5 % CO2 in CMRL 1066 media (Gibco, Grand Island, NY) containing 10 % (vol/vol) fetal calf serum (Sigma-Aldrich), 2 mmol/L L-glutamine (Sigma-Aldrich), and 5 U/mL Penicillin-Streptomycin (Roche Applied Science, Penzberg, Germany) prior to transplantation. All experiments involving human islets were approved by the Regional Ethical Review Board in Uppsala.

Islet-cell coating (paper I & II)

In paper I and II, islets were coated with SCs or EPCs prior to transplantation. In order to ensure a strong contact between islets and cells, 10,000 SCs or EPCs were cultured together with 400 (paper I) or 200 (paper II) islets for one hour under gentle shaking at 37°C. During this hour, both SCs and EPCs attached to the islet surface, enabling for easy islet-cell co-transplantation. In order to avoid protocol differences, control islets were also subjected to the shaking procedure for one hour prior to islet transplantation.

Islet transplantation (paper I - III)

Mouse (paper I) or human (paper II and III) islets were transplanted either to the renal subcapsular space or the abdominal external oblique muscle of NOD/SCID (paper I and II) or NMRI nu/nu (paper III) mice.

Renal subcapsular space (paper I - III)

When transplanting islets beneath the renal capsule, mice were anesthetized by an intraperitoneal (i.p.) injection of Avertin (0.015 mL/g bodyweight of a 2.5 % (vol/vol) solution of 10 g 97 % (vol/vol) 2,2,2-tribromoethanol (Sigma-Aldrich) in 10 mL 2-methyl-2-butanol (Kemila, Stockholm, Sweden)). A small incision was made at the left subcostal flank, exposing the kidney. Four hundred (paper I) or 200 (paper II and III) control islets, 400 SC-bioengineered islets (paper I), 200 EPC-bioengineered islets (paper II), or 100 islets together with 100 spheres of NCSCs (paper III) were transplanted into the left renal subcapsular space of NOD/SCID (paper I and II) or NMRI nu/nu (paper III) mice.

Abdominal external oblique muscle (paper II)

In separate experiments in paper II, islets were transplanted to the abdominal external oblique muscle. NOD/SCID mice were anesthetized by spontaneous inhalation of 2.6 % isoflurane. The abdominal wall was exposed and a total of 200 control or EPC-bioengineered islets were injected into several locations between abdominal external oblique muscle fibers using a 25-gauge butterfly needle. This transplantation site enabled for easy visualization of the islet grafts within the living animals using a Zeiss LSM 5 LIVE microscope (Carl Zeiss AG, Oberkochen, Germany).

Graft blood perfusion and oxygen tension (paper II & III)

In both paper II and III, graft blood perfusion and oxygen tension was measured at one month posttransplantation. Mice were anesthetized by an i.p. injection of Avertin (0.015 mL/g bodyweight of a 2.5 % (vol/vol) solution of 10 g 97 % (vol/vol) 2,2,2-tribromoethanol (Sigma-Aldrich) in 10 mL 2-methyl-2-butanol (Kemila, Stockholm, Sweden)) and maintained at body temperature on a surgical table. A polyethylene catheter was inserted into the right carotid artery and thereafter connected to a blood pressure transducer (ADInstruments, Dunedin, New Zealand). An incision was made at the left subcostal flank. The kidney was freed from its surrounding tissues and placed in a plastic cup for immobilization, with a clear view over the graft.

Graft- and kidney blood perfusion was measured using laser-Doppler flowmetry with a needle probe (Transonic BLF 21 Series, probe diameter 1.2 mm; Transonic, Ithaca, NY).

Clark microelectrodes (external tip diameter 2-5 μm; Unisense, Aarhus, Denmark) were used to measure the oxygen tension of the graft as well as the kidney cortex. The same microelectrode was used for all experiments.

Six to ten measurements were performed in each graft and kidney, and the calculated mean in each animal was considered to be one experiment. All measurements were performed according to previously established protocols [121].

Diabetes induction and glucose monitoring (paper I & IV)

In paper I, the state of hyperglycemia was established through a single intravenous (i.v.) injection of alloxan (60 mg/kg bodyweight; Sigma-Aldrich, St Louis, MO) in the tail vein three to five days prior to islet transplantation. Blood glucose was measured on the day of alloxan treatment, the day before islet transplantation, as well as on day 3, 7, 14, 21, 28, 30 and 33 posttransplantation, using the FreeStyle Lite monitoring system (Abbott Laboratories, Abbott Park, IL).

In paper IV, the state of hyperglycemia was established through a single i.v. injection of alloxan (75 mg/kg bodyweight; Sigma Aldrich, St Louis, MO) in the tail vein three days prior to cell transplantation. Diabetes was defined as blood glucose above 16.7 mmol/L at the time of cell transplantation. Blood glucose was measured at day 0, 3, 7, 14, 21 and 24 following intracardiac injection in all mice, using the FreeStyle Lite monitoring system (measures blood glucose concentrations up to 27.8

mmol/L). All animals, including those who did not survive for 24 days, were included in the final analysis of the blood glucose change.

The discrepancy in alloxan dose between paper I (60 mg/kg bodyweight) and paper IV (75 mg/kg bodyweight) is due to sensitivity differences between animal strains (NOD/SCID in paper I and NMRI nu/nu in paper IV).

Ultrasound-guided intracardiac injection (paper IV)

Animals were anesthetized by spontaneous inhalation of 2.6 % isoflurane. Ultrasonographic gel (Parker Laboratories, Fairfield, NJ) was applied on the left side of the thorax and the ultrasonographic probe (FujiFilm VisualSonics Inc., Toronto, ON, Canada) was positioned above the heart. The echocardiogram, using the Vevo 1100 imaging system and platform (FujiFilm VisualSonics Inc.), enabled real-time visualization of the left ventricle. A 30-gauge needle was inserted below the ribcage and through the diaphragm. After visual confirmation of the proper needle position, saline (control, 100 μL) or NCSCs (10,000 cells per gram bodyweight, diluted in saline, 100 μL) was slowly injected over 30 seconds. Detection of blood within the syringe, and the observed fluid turbulence during the injection, further confirmed proper needle position. Following injection of the cells, the needle was carefully pulled back, the ultrasonographic probe was lifted and excess ultrasonographic gel was removed. The animals were placed separately in clean cages and observed until full recovery. Animals were monitored daily for the duration of the experiment.

Glucose and insulin tolerance test (paper I)

The functionality of islets transplanted to diabetic recipients, as well as native islets in Tfam-SCKO and control littermates, was evaluated by an i.v. injection of glucose (2.5 g/kg bodyweight; Fresenius-Kabi, Uppsala Sweden). Plasma glucose concentrations were measured with glucose reagent strips (Freestyle Lite) before and at 10, 30, 60 and 120 minutes following glucose administration. The plasma insulin level was determined 10 minutes after glucose administration.

In separate experiments, plasma glucose concentrations were measured before and at 10, 30, 60, 90, 120 and 180 minutes following i.v. injection of insulin aspart (2 U/kg; Novorapid; Novo Nordisk, Bagsvaerd, Denmark).

Glucose-stimulated insulin release (paper I)

Glucose-stimulated insulin release was assessed according to a previously established protocol [23]. Briefly, groups of 10 islets, with or without previous exposure to SCs, or obtained from Tfam-SCKO mice or control littermates, were transferred to glass vials containing 250 µl Krebs–Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES and 2 mg/ml bovine serum albumin (KRBH buffer; MP Biomedicals, Irvine, CA, USA). The islets were incubated with 250 µl KRBH containing 1.67 mmol/L glucose for one hour at 37°C. The media was removed and the islets were incubated with 250 µl KRBH buffer containing 16.7 mmol/L glucose for one hour. Insulin content of the incubation media and homogenates was determined with a mouse insulin ELISA (Mercodia, Uppsala, Sweden).

RNA isolation, cDNA synthesis and TaqMan arrays (paper I)

Total RNA was isolated from mouse islets samples according to the manufacturer's instructions (RNeasy Plus Micro Kit, Qiagen, Venlo, Netherlands). The amount and purity (OD 260/280) of the total RNA was determined using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All RNA samples had OD 260/280 between 2,0 and 2,1, which is in the range for pure RNA. The extracted total RNA was eluted in nuclease free water and stored at -80°C until cDNA synthesis.

The RNA was transcribed to cDNA by Superscript First-Strand Synthesis Super Mix for qRT-PCR (Life Technologies) according to the manufacturer's instructions. Briefly, a mix of random hexamer primers and oligo(dT) primers were incubated with the Superscript III Reverse Transcriptase enzyme mix and RNA at 25°C for 10 minutes, followed by 50°C for 30 min and thereafter 85°C for 5 minutes to inactivate the enzyme. To remove the RNA template from the cDNA:RNA hybrid molecule after first-strand synthesis, the mixture was incubated with two units of RNaseH at 37°C for 20 minutes. The cDNA was stored at -20°C until use.

For TaqMan Array Fast Plates (Applied Biosystems), 5 ng/assay of cDNA was used. Three reference genes and 45 genes of interest were analyzed. The TaqMan array assays were run using ABI 7900HT Fast Real-Time PCR System at Bioinformatics and Expression Analysis Core Facility, Karolinska Institute, Stockholm, Sweden.

Immunohistochemistry (paper I - IV)

In all papers included in this thesis, cryosections (8 µm thick) were incubated with the primary antibodies in a dark and humidified chamber at 4°C overnight. Following washing (three times with washing buffer; Dako, Glostrup, Denmark), the secondary antibodies were added for one hour at room temperature. In some experiments, freshly prepared 5-ethynyl-2'-deoxyuridine (EdU) solution was added for 30 minutes. The nuclei were stained with Hoechst (1:10 000; Life Technologies). The slides were dried and mounted with Fluoromount-G (SouthernBiotech Birmingham, AL).

Primary antibodies:

- Guinea pig antihuman insulin (Kem-En-Tec Nordic, Tåstrup, Denmark; paper I, III and IV)
- Rabbit antihuman Ki67 (Abcam, Cambridge, UK; paper III and IV)
- Rabbit antihuman insulin antigen-2 (IA2; anti-PTPRN; Atlas Antibodies, Stockholm, Sweden; paper II and III)
- Chicken polyclonal protein gene product 9.5 (PGP9.5; Acris Antibody, Herford, Germany; paper III)
- Biotin-conjugated mouse antihuman CD31 (eBioscience, San Diego, CA; paper II and III)
- Rat anti-mouse CD31 (AbD Serotec, Kidlington, UK; paper I, II and III)
- Goat anti-GFP (Novus Biologicals LLC, Littleton, CO; paper III and IV)
- Mouse anti-glucagon (Abcam; paper I)

Secondary antibodies:

- Alexa Fluor 633 goat anti-guinea pig (Life Technologies; paper I and III)
- Alexa Fluor 647 donkey anti-guinea pig (Jackson ImmunoResearch Laboratories, West Grove, PA; paper IV)
- Alexa Fluor 555 goat antirabbit (Life Technologies; paper I and III)
- Alexa Fluor 594/647 donkey antirabbit (Jackson ImmunoResearch Laboratories; paper III and IV)
- Alexa Fluor 488 goat antichicken (Life Technologies; paper III)
- Alexa Fluor 555 goat antirat (Life Technologies; paper II and III)
- Alexa Fluor 488 donkey anti-goat (Jackson ImmunoResearch Laboratories; paper IV)
- Alexa Fluor 647 donkey anti-rabbit (Jackson ImmunoResearch Laboratories; paper II).

In vivo imaging (paper II)

In paper II, some mice were transplanted with control or EPC-bioengineered human islets to striated muscle in the abdominal wall. This transplantation site enabled for easy access and visualization of the islets and vasculature within the living animals.

At two weeks posttransplantation, the mice were anesthetized by spontaneous inhalation of 2.6 % isoflurane and maintained at body temperature on a surgical table. In order to visualize the vasculature, an intravenous injection of Alexa Fluor 488 conjugated rat anti-mouse CD31 (BioRad Hercules, CA) and APC conjugated mouse anti-human CD31 (eBioscience) was performed. The antibodies were allowed to circulate for a minimum of five minutes prior to imaging. The abdominal wall was exposed and the muscle was immobilized for imaging using a vacuum window [122]. A Zeiss LSM 5 LIVE microscope (Carl Zeiss AG, Oberkochen, Germany), with a Plan-Apochromat 20x/0.8 objective was used for all *in vivo* imaging.

Confocal imaging (paper I - IV)

In all papers included in this thesis, confocal imaging was performed using the laser scanning confocal microscope Zeiss LSM 780 (Carl Zeiss AG) with Plan-Apochromat 10x/0.45 M27 and 20x/0.8 M27 objectives.

Image analysis (paper I - IV)

Zeiss Zen 2012 Blue edition software (Carl Zeiss AG) and ImageJ 1.46r (National Institutes of Health, Bethesda, MD) was used for all image analyses.

Vascular and neural density (paper I - III)

In paper I, II and III, the vascular density was assessed by measuring the relative area positive for bandeiraea simplicifolia 1 (Sigma-Aldrich; paper I) and CD31 (paper I-III) within the insulin (paper I) or IA2 (paper II and III) positive area. IA2 is a ubiquitous marker for islet endocrine cells [123].

In paper I, the vascular density of native islets from Tfam-SCKO and control littermates was also assessed by measuring the average blood vessel diameter as well as number of intra-islet capillaries.

In paper II, the islet neural density in NCSC and control islet grafts was assessed by measuring the relative area positive for PGP9.5 within the IA2 positive areas.

When analyzing both the vascular and neural density, a minimum of eight sections from each animal was assessed, and the calculated mean in each animal was considered to be one experiment.

Donor and recipient endothelium within grafts (paper II)

In both paper II and III, human islets were transplanted beneath the renal capsule of mice. This species difference enabled for the evaluation of the donor and recipient contribution to the intra-islet vasculature. Grafts were retrieved at one month posttransplantation and were later cryosectioned. These sections were stained for IA2 as well as human and mouse CD31. The islet vascular density was then assessed by measuring the relative area positive for human and mouse CD31 within the IA2 positive areas.

This species difference was further utilized when studying the functionality of the newly formed vessels in paper II. By *in vivo* imaging, vessels of both human and mouse origin were evaluated for blood perfusion.

Beta-cell proliferation (paper III & IV)

In paper III and IV, the beta-cell proliferation was assessed by counting the fraction of insulin positive cells that were also positive for the endogenous proliferation marker Ki67. This assessment was corroborated in selected experiments by also quantifying the fraction of insulin-positive cells positive for EdU. Beta-cell proliferation was assessed both *in vitro* and *in vivo*.

Islet and beta-cell mass (paper I, III & IV)

In paper I (native islets of Tfam-SCKO and control littermates) and IV, the beta-cell mass was assessed by measuring the relative area positive for insulin within whole sections of the pancreas. In paper I, three levels from the head and three levels from the tail of each pancreas was evaluated. Every 12th-16th section of the pancreas was evaluated in each animal in paper IV.

In paper I and III, the islet and beta-cell mass of each graft was quantified by computerized morphometry. Sections were stained for IA2 (islet mass; paper I and III) and insulin (beta-cell mass; paper III).

Statistical analysis (paper I - IV)

Statistical analysis was performed using GraphPad Prism® version 6.0 (GraphPad Software, La Jolla, CA). All values are expressed as means ± standard error of mean. When only two groups were compared, Student's unpaired or paired t-test (paper I-III), as well as log rank (Mantel-Cox) test (paper IV), was used. Multiple comparisons between data

were performed using analysis of variance (ANOVA) and Bonferroni's post hoc test (paper I). For all comparisons, $P<0.05$ was considered statistically significant.

Results and Discussion

Paper I

Tfam-SCKO versus control littermates

There was no observed difference between Tfam-SCKO and control littermates with regards to:

- Development
- Body constitution and weight at three months of age
- Basal blood glucose
- Peripheral insulin sensitivity
- Beta-cell mass
- Islet composition (80 % beta-cells, <10 % alpha cells)
- Number of islet blood vessels
- Islet vascular density
- Islet glucose stimulation index (*in vitro*)
- Islet DNA content (*in vitro*)
- Islet insulin content (*in vitro*)
- Islet glucose oxidation (*in vitro*)
- Islet gene expression (*in vitro*)

The few observed differences between Tfam-SCKO and control littermates were:

- Impaired glucose tolerance
- Delayed glucose disposal rate
- Decreased insulin peak
- Increased number of islet blood vessels with an exceedingly small diameter (<3 μm)

The effect of excess SCs during islet transplantation

Several studies have shown that SCs have an angiogenic effect [88, 89]. Contrary to these studies, the results in paper I indicate that an excess of SCs during transplantation can hamper the revascularization process. Inferior vasculature further caused an impaired glucose disposal rate following a glucose load at one month posttransplantation.

Noteworthy, recipients of SCs-islet grafts recovered from hyperglycemia faster than those receiving control islets, with a normalized blood glucose level within three days posttransplantation. At one month posttransplantation, these grafts also had a greater islet mass compared with controls, strongly suggesting that SCs have a beta-cell protective effect.

Paper II

Human islets contain endothelial cells even after islet isolation

Both in paper II and III, cultured and transplanted control human islets contained remnant endothelial cells. These cells remained despite prolonged culture (up to fourteen days) and provided a greater pool of endothelial cells to participate in the islet revascularization process posttransplantation. Previous studies of murine islets have shown that islet endothelial cells disappear within the first days during islet culture [78, 124]. In paper II, we considered that the reason for this difference was, besides the species islet difference, the glucose concentration during culture. Endothelial cells are known to be highly vulnerable to glucose toxicity and it has been shown that glucose inhibits angiogenesis of isolated human islets [125]. In paper II and III, as well as in the clinical protocols, the human islet culture media contain a glucose concentration of 5.6 mmol/L, which can be compared with the commonly applied rodent islet culture media with a glucose concentration of 11 mmol/L. Studies in NOD mice show that, during the prediabetic phase, the loss of islet endothelial cells is greater than the loss of beta-cells [126]. This early loss seems to be mediated by decreasing levels of VEGF-A, otherwise produced by healthy beta-cells. As hyperglycemia ensues, glucose toxicity contributes to extensive islet endothelial cell death. Studies of murine islet endothelial cells in culture indicate that their apoptosis rates can substantially be decreased by lowering the glucose concentration during culture [127]. *In vivo* imaging of control grafts revealed that these remnant endothelial cells connect to recipient vessels to form functional (perfused) chimeric blood vessels.

Human islets coated with EPCs have a vascular density comparable to that of native murine islets

Quantification of the vascular density, as well as the respective contribution of mouse and human endothelial cells in the grafts, revealed that EPC-bioengineered islets had a higher vascular density (6.1±0.8 %) than control islets (3.6±0.5 %). The greater vascular density in EPC-bioengineered islet grafts in paper II was observed to result not only from an incorporation of the EPCs into the capillary networks, but also from

an increased ingrowth of recipient vessels (mouse) and proliferation of residual donor (human) endothelial cells.

Several studies, where rodent or porcine islets were exposed to EPCs, have been performed. In these studies, it has been suggested that the increased angiogenesis was triggered by stimulation of VEGF-A expression in the transplanted beta-cells [128] or by the secretion of VEGF-A directly from the EPCs [84]. Kang et al. also suggest that microvesicles from EPCs can transfer RNA, which would then stimulate islet angiogenesis [128].

Grafts containing EPCs have a significantly higher blood perfusion and oxygen tension

Since the increase in vascular density may be explained by expansion of transplanted EPCs or a better survival of remnant islet donor endothelial cells that do not contribute to forming functional and perfused blood vessels, vessel functionality was investigated using three different techniques. Graft blood perfusion (laser-Doppler flowmetry) measurements revealed a massive increase of the blood perfusion level in EPC grafts compared with control grafts (5.5±0.6 tissue perfusion units (TPU) and 2.3±0.2 TPU, respectively). The perfusion levels in EPC grafts were similar to those in the renal cortex (6.8±0.6 TPU and 5.2±0.6 TPU, respectively), and at least in rodents, the blood perfusion of native islets is similar to that in the adjacent renal cortex [129, 130]. Oxygen tension (Clark microelectrodes) measurements revealed that EPC-bioengineered human islets had an almost three-fold increased oxygen tension compared with control islets (20.6±3.3 mmHg and 6.4±1.1 mmHg, respectively), suggesting optimal oxygen delivery to the transplanted beta-cells. And finally, once we had determined superior blood perfusion and oxygen tension in EPC grafts, some animals received i.v. lectin injections. Images of these grafts reveled that vessels, including incorporated EPCs and remnant endothelial cells, were in fact perfused.

EPCs incorporate into functional blood vessels

Unlike previously reported, where EPCs were suggested to only speed the revascularization process and not incorporate into blood vessels [128], paper II show that human islet grafts containing EPCs acquire a much higher vascular density than control grafts, with the inclusion of EPCs into functional vessels. This was determined by *in vivo* imaging of fluorescent EPCs in animals with fluorescently perfused vessels. Like previously mentioned, the incorporation of EPCs into vessels is controversial. Nevertheless, Oh *et al.* also report that donor and recipient endothelium as well as the transplanted EPCs contribute to newly formed blood vessels within mouse islets [84]. Discrepancies between studies are likely explained

by different characteristics of the transplanted EPCs, but perhaps also species differences.

Paper III

NCSCs stimulate human beta-cell proliferation

By exposing human islets to NCSCs in a culture- and transplantation setting, the number of Ki67 positive islet cells, including the beta-cells, was increased. The number of proliferating beta-cells was almost doubled after 48 hours (0.7±0.1 % vs 1.2±0.2 % *in vivo* and 0.2±0.1 % vs 0.6±0.1 % *in vitro*), and at one month posttransplantation a tendency to an increase of both the islet mass (21±7 %; p=0.06) and beta-cell mass (22±7 %, p=0.06) was observed.

We have previously shown that NCSCs are capable of promoting beta-cell proliferation, and thus islet function, in murine islets [114, 115]. When investigating the mechanism behind this, it was found that factors secreted from NCSCs were not enough to cause beta-cell proliferation. Instead, direct cell-cell interactions were necessary for the increased proliferation to occur. Grouwels *et al.* also tested the effect of NCSCs on human islets, but found that they failed to induce beta-cell proliferation [115]. It is important to note that the experimental settings differed between paper III and the study by Grouwels *et al.* Furthermore, there are likely individual differences between donors which will affect the beta-cell capacity to leave the resting (G0) phase and enter the cell cycle.

When comparing the *in vitro* and *in vivo* data, it becomes clear that the increased beta-cell proliferation observed in the transplantation setting cannot merely be attributed to cell-cell interactions with NCSCs. The basal beta-cell proliferation was more than three times higher in control grafts (0.7±0.1 %) compared with cultured control islets (0.2±0.1 %). Thus, there must be something in the transplantation milieu that stimulates beta-cells to proliferate. Based on previous studies, the observed increase may be explained by inflammation or active angiogenesis. Factors secreted by immune cells have previously been reported to promote murine beta-cell proliferation [131, 132], and inflammatory infiltration in the donor pancreas increases human beta-cell replication [133]. We used immune-deficient NMRI nu/nu mice for our experiments and did not observe infiltration of immune cells in our transplants. Nevertheless, unspecific inflammation would be likely to have occurred following surgery. As for active angiogenesis, it has been shown that porcine islet beta-cell proliferation increases during the revascularization phase [128].

Grafts containing NCSCs have an increased neural density

Reinnervation of islet grafts is important, since the nerves regulate beta-cell function [12] and, at least in rodents, also beta-cell proliferation [9]. Furthermore, pulsatile insulin secretion from islet grafts depends on their reinnervation [134]. Islet neural density was assessed as the area percentage of small fibers positive for PGP9.5 within IA2 positive areas. When comparing control grafts with grafts containing NCSCs, it was found that NCSC-exposed islets had a neural density almost four times higher than control islets (1.4±0.1 % vs 0.4±0.3 %). To confirm that the observed increase was not merely a reflection of differentiated NCSCs into neurons, some sections were stained for GFP. Images of these sections show that only a few of the PGP9.5 positive cells were also positive for GFP, supporting the hypothesis that the neural density increase mainly occurred through stimulated neural ingrowth.

In contrast to the cell-cell interaction necessary for the beta-cell proliferative effects by NCSCs, the increased neural density is likely explained by the release of many neurotrophic factors from NCSCs, such as nerve growth factor, brain-derived neurotrophic factor, GDNF, neurotrophin-3 and -5, as well as ciliary neurothrophic factor [116, 135].

Exposure to NCSCs increases the vascular density in human islets grafts

We and others have previously reported on an insufficient or delayed revascularization of pancreatic islets at the renal subcapsular and intraportal site, rendering these islets low blood perfused and low oxygenized for several months posttransplantation [72, 136, 137]. Apart from releasing neurotrophic factors, NCSCs also produce angiogenic factors, including VEGF-A, MMP-2, and MMP-9 [116]. These factors are of critical importance for islet revascularization posttransplantation [24, 136]. It was therefore no surprise when NCSCs improved the vascular density of transplanted human islets. By using separate antibodies for human and mouse CD31, the respective contribution of both donor and recipient endothelium could be analyzed. The human vessel density was doubled in transplants containing NCSCs (4.1±0.8 % vs 1.8±0.5 % in control transplants). Similarly, the mouse vessel density was doubled in NCSC-grafts (2.5±0.5 %) compared with control grafts (1.0±0.1 %). In other words, NCSCs caused an increased recipient (mouse) blood vessel ingrowth, as well as expansion of donor (human) endothelial cells.

NCSCs increase the blood perfusion and oxygen tension in human islets

To investigate whether the greater vascular networks observed in NCSC-containing grafts were in fact functional, blood perfusion- and oxygen tension recordings were performed. Human islets exposed to NCSCs had a more than doubled blood perfusion (7.4±0.6 tissue perfusion units (TPU) vs 3.0±0.3 TPU in control grafts), while there was no difference in blood perfusion of the surrounding kidney cortex (8.4±0.9 TPU in the NCSC-group vs 8.7±1.0 TPU in controls; p=0.8). Similarly, the oxygen tension was three times higher in islet transplants exposed to NCSCs compared to control transplants (24.7±1.4 mmHg and 7.0±2.1 mmHg, respectively), while no difference in the oxygenation of the kidney cortex surrounding the grafts was observed (25.8±4.0 in the NCSC-group vs 24.5±4.3 in controls; p=0.8). To put these values into context, although recording cannot be made in native human islets, rodent studies show that the blood perfusion of native rodent islets is similar to that in the renal cortex [129, 130] and that the oxygen tension of metabolically active islets is approximately 40 mmHg [70].

Paper IV

NCSCs homed to islets in response to a beta-cell injury

Like other stem and progenitor cells, NCSCs are known to home to sites of inflammation [138, 139]. In order to determine if the cells were capable of homing to injured pancreatic islets, GFP-expressing NCSCs were used in all experiments in paper IV. Due to their fluorescence, the cells could easily be traced within the animal following intracardiac injection. When examining the pancreas of NCSC-treated animals at two days postinjection it was found that NCSCs were present in 11.88 ± 1.21 % of the islets. As previously mentioned, this homing towards damaged beta-cells is crucial, since it has been shown that NCSCs require direct contact with beta-cells in order to stimulate beta-cell proliferation [115].

Systemic NCSC treatment ameliorated hyperglycemia

Blood glucose levels were measured at day 0, 3, 7, 14, 21, and 24 postinjection in all animals. At day three postinjection, both saline and NCSC treated animals had a lowered mean blood glucose level when compared with the day of injection. However, whilst the saline treated animals gradually became more and more hyperglycemic after the first three days postinjection, the mean blood glucose level in NCSC treated animals

continued to drop for the initial seven days postinjection and thereafter stabilized.

When comparing the blood glucose level prior to saline or NCSC treatment with 24 days postinjection, NCSCs treated animals displayed a lowered blood glucose level (Δ-value -3.8 ± 1.2 mmol/L). The same analysis in the saline treated group revealed that these animals had an increased blood glucose concentration compared with the day of injection (Δ-value 4.9 ± 1.0 mmol/L. Although systemic NCSC treatment ameliorated the hyperglycemia, the cells were unable to reverse diabetes in the treated animals.

The ameliorated diabetic state was explained by an increased beta-cell proliferation and beta-cell mass

Some animals in paper IV were euthanized at two days postinjection. When examining the pancreas of these animals it was found that NCSC treatment had induced beta-cell proliferation in the injured islets. The percentage of Ki67 positive beta-cells in the pancreas of NCSC treated animals was almost doubled in comparison with saline treated animals.

The total beta-cell mass was evaluated in animals that were euthanized at 24 days postinjection. This analysis further confirmed the increased beta-cell proliferation, as the NCSC-treated animals had a more than doubled beta-cell mass in comparison with animals receiving saline.

Animal survival was prolonged with NCSC treatment

The study period was limited to 24 days postinjection due to restrictions in the ethical approval. Out of the 16 animals that received NCSCs, 15 were still alive on day 24 postinjection. Meanwhile, out of the 16 control animals, only 10 remained alive throughout the study period. This difference in survival rate is likely explained by the ameliorated hyperglycemia observed in the NCSC-treated group. At 24 days, none of the 15 surviving animals of the NCSC group had a blood glucose level >27.8 mmol/L, whilst six out of ten surviving control animals had reached the upper limit on the FreeStyle Lite monitoring system.

Conclusions

Paper I

- Although islet vascular density and blood vessel numbers were similar between Tfam-SCKO and control littermates, a mitochondrial disturbance of SCs results in an abnormal vascular morphology, with slender blood vessels.
- Excess of SCs inhibit blood vessels from growing into pancreatic islet grafts
- An early improved graft function was observed when co-transplanting SCs together with islets.
- Despite having a greater islet mass, grafts containing excess SCs had an impaired graft function at one month posttransplantation.

Paper II

- Human islets contain endothelial cells even after islet isolation.
- EPCs can easily be transplanted together with islets through a simple surface-coating procedure.
- Immunohistochemistry of grafts at one month posttransplantation revealed that grafts containing EPCs had a substantially higher vascular density compared with control grafts. This was true both for donor (human) and recipient (mouse) vessels.
- Oxygen tension and blood perfusion measurements revealed that the blood vessels in EPC-bioengineered grafts were functional. This was further confirmed by lectin injection.
- By labeling EPCs with a far red fluorescent dye, the cells could be traced within the transplants.
- *In vivo* imaging was performed to evaluate the functionality of the newly formed blood vessels. This imaging confirmed that the donor and recipient vessels connect within the grafts to form blood perfused vessels. Furthermore, when co-transplanting islets together with EPCs, the cells also formed functional chimeric blood vessels (i.e. vessels of both human and mouse origin).

Paper III

- NCSCs were capable of inducing human beta-cell proliferation in cultured and transplanted pancreatic islets. After 48 hours of NCSC-exposure, both cultured and transplanted islets had a doubled number of Ki67 positive beta-cells.
- Transplanting human islets together with NCSCs resulted in a three to four fold increased neural density compared with control grafts. This increase was mainly caused by neural ingrowth into the graft, but also through NCSC differentiation into neural cells.
- Co-transplanting islets together with NCSC lead to a doubled graft vascular density, caused by both an increased ingrowth of recipient blood vessels as well as expansion or survival of donor blood vessels.
- The newly formed vasculature within the NCSC-grafts was highly functional. The islets exposed to NCSCs had an increased oxygen tension and more than doubled blood perfusion when compared with control islet grafts.

Paper IV

- NCSCs preferentially home to the injured islets of alloxan-treated mice.
- Alloxan-treated NMRI nu/nu mice receiving NCSCs displayed a lowered blood glucose level in comparison with the increased blood glucose concentrations observed in mice receiving saline.
- Intracardiac injection of NCSCs lead to a doubled beta-cell mass, caused by the early increase of beta-cell proliferation.
- The three-week survival was significantly higher in animals receiving NCSCs (93.8 %) in comparison with control animals (62.5 %).

Sammanfattning på svenska

Typ 1 diabetes (även känt som barn- och ungdomsdiabetes) är en sjukdom som uppstår då kroppens egna immunceller förstör de så kallade beta-cellerna belägna i de Langerhanska öarna i bukspottkörteln. Beta-cellerna ansvarar för att utsöndra hormonet insulin vid förhöjda nivåer av socker i blodet. Insulin är ett livsnödvändigt hormon som gör att kroppens vävnader kan ta upp sockret från blodbanan. Utan insulin blir sockernivåerna i blodet mycket höga och kroppens celler svälter.

Den traditionella behandlingen vid typ 1 diabetes består av kontinuerlig infusion eller upprepade dagliga injektioner av insulin i underhudsfett. Hos de patienter där det är särskilt svårt att kontrollera sockernivåerna i blodet kan insulinproducerande celler i form av en hel bukspottkörtel eller Langerhanska öar transplanteras. Dessa vävnader erhålls från avlidna organdonatorer. Dessvärre slutar många av dessa transplanterade organ att fungera inom några år efter transplantationen och patienterna blir återigen beroende av insulinbehandling. Anledningen till att ö-transplantaten slutar fungera har åtminstone delvis sin grund i öarnas beroende av ett stort blodkärls- och nervnätverk för att fungera normalt. Efter transplantationen måste dessa nätverk återbildas, något som oftast inte sker fullständigt eller tillräckligt snabbt.

I denna experimentella avhandling studeras hur nervstödjeceller, så kallade gliaceller, och förstadier till blodkärlsceller och nervceller kan användas för att förbättra resultaten vid transplantation av insulinproducerande celler, samt även nybilda insulinproducerande celler i bukspottkörteln vid typ 1 diabetes. Genom att stimulera celldelning hos kvarstående insulinproducerande celler, finns möjlighet att få betydligt mer lättbehandlad diabetes och kanske även erbjuda bot. För patienten skulle detta innebära ett liv med begränsad eller ingen tillförsel av insulin genom sprutor, samt en markant minskad risk för kraftiga svängningar i sockernivåerna i blodet med dess kopplade komplikationer.

Gliaceller

I studie I i avhandlingen undersöktes vikten av en typ av gliacell, Schwannska celler, för funktionen av Langerhanska öar. Schwannska celler

omger öarna som en kapsel och finns även i små mängder inuti öarna. Trots deras närvaro är betydelsen av dessa celler för öarnas funktion ej studerad.

Öar med dysfunktionella Schwannska celler visade sig ha ett onormalt kärlnätverk, där blodkärlen var smalare än vad de är i öar med normala Schwannska celler. Vid funktionella undersökningar av dessa öar i bukspottkörteln visade det sig att dysfunktionella Schwannska celler orsakade en nedsatt sockertolerans och insulinutsöndring.

Vid samtransplantation av Langerhanska öar tillsammans med Schwannska celler visade det sig att de Schwannska cellerna bildade en barriär runt de transplanterade öarna, vilket resulterade i att få blodkärl kunde växa in i vävnaden och försörja den med syre och näring. Närvaron av Schwannska celler resulterade dock också i att transplantatet fungerade bättre till en början, blodsockernivån återställdes snabbare och den totala ö-massan var större efter en månad än vid transplantation av enbart öar.

Förstadier till blodkärlsceller

I studie II i avhandlingen undersöktes om förstadier till blodkärlsceller (endoteliala progenitorceller) kunde användas för att förbättra blodkärlsnybildningen och beta-cellers överlevnad vid transplanation av Langerhanska öar. Endotheliala progenitorceller har tidigare visat sig förbättra resultatet vid ö-transplantation i en djurmodell genom att påskynda återbildningen av kärlnätverket och därmed förbättra överlevnaden och celldelningen av de insulinproducerande cellerna i transplanterade öar.

I denna studie användes Langerhanska öar och endoteliala progenitorceller isolerade från människa. Öarna transplanterades tillsammans med de endoteliala progenitorcellerna och en månad senare undersöktes den transplanterade vävnaden under mikroskop. Det visade sig då att öar som hade transplanterats tillsammans med endoteliala progenitorceller hade markant större blodkärlsnätverk än öar som transplanterats utan sådana celler. Det större kärlnätverket resulterade i ett förbättrat syrgastryck och blodflöde i vävnaden.

De endoteliala progenitorceller som användes vid transplantationen märktes in med en färg för att senare kunna detekteras i den transplantanterade vävnaden. Det visade sig att dessa förstadieceller mognar ut till endotelceller och blir en del av de nybildade blodkärlen. Vidare upptäcktes det att blodkärl från ö-donatorn och mottagaren växte samman en kort tid efter transplantationen och bildade funktionella blodkärl. Dessa kombinerade blodkärl var fullt fungerande och visade sig ha ett normalt blodflöde.

Förstadier till nervceller

I studie III i avhandlingen undersöktes om förstadier till nervceller (neurallisstamceller) kunde användas för att öka antalet beta-celler och deras funktion vid transplantation av Langerhanska öar. Neurallisstamceller skickar ut viktiga signaler som reglerar bildning av insulinproducerande celler under den embryonala utvecklingen. Djurstudier har visat att neurallisstamceller innehar förmågan till att stimulera insulinproducerande celler att dela på sig och bli fler.

I studie III studerades effekten av samtransplantation och samodling mellan neurallisstamceller och öar isolerade från människa. Resultaten från dessa försök visar att neurallisstamceller är kapabla att inducera celldelning också av insulinproducerande celler från människa, både i odling och vid transplantation.

En månad efter transplantationen undersöktes öarnas nerv- och blodkärlsnätverk. Öar som hade transplanterats tillsammans med neurallisstamceller visade sig ha ett nervnätverk som var tre till fyra gånger större än det hos öar som transplanterats utan neurallisstamceller. Vidare undersökning visade att även kärlnätverket var större och att dessa nybildade blodkärl var funktionella, med högre syrgastryck och blodflöde i den transplanterade vävnaden.

Neurallisstamceller användes även i studie IV i avhandlingen. I denna experimentella studie behandlades diabetiska möss med en injektion av neurallisstamceller i hjärtats vänstra kammare. Cellerna, som följde med blodflödet ut ur hjärtat, letade sig ut från blodbanan och fram till de skadade insulinproducerande cellerna i bukspottkörteln. Väl framme stimulerade neurallisstamcellerna de kvarvarande insulinproducerande cellerna att dela på sig. Efter tre veckor hade de möss som behandlats med neurallisstamceller dubbelt så många insulinproducerande celler som de som inte fått någon behandling med celler. Denna ökade mängd insulinproducerande celler resulterade i lägre blodsockernivåer. Dessvärre var behandlingen inte tillräckligt effektiv för att helt återställa blodsockernivån.

Slutsats

Denna avhandling består av fyra forskningsprojekt där vi med hjälp av gliaceller och förstadier till blodkärlsceller och nervceller experimentellt har kunnat förbättra resultaten vid transplantation av insulinproducerande celler, samt även återbildat insulinproducerande celler hos diabetiska möss. Målsättningen för forskningen är att framöver kunna erbjuda en botande behandling för typ 1 diabetes.

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