Design of Local Immune-Privileged Microenvironment to Prevent Islet Graft Rejection

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Design of Local Immune-Privileged Microenvironment to Prevent Islet Graft Rejection

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This is to certify that I have examined this copy of a master's thesis by

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ABSTRACT

The development of tolerance induction approaches via immune engineering that can restore and/or replace non-functional tissues and organs represents the leading front of emerging regenerative therapies. Type-1 diabetes (T1D) is an organ-specific autoimmune disease characterized by destruction of pancreatic β cells, which are insulin-secreting cells by autoreactive T cells and other immune cells. Loss of β-cell, thus insulin, makes patients dependent on exogenous insulin or to overcome this need; transplantation of pancreas or intact islets. One of the promising approaches used for treatment of diabetes is the transplantation of islets; however, it also comes with its pitfalls. First and most obvious one is the limitation of donor source. In addition, transplantation of islets or entire pancreas requires suppression of the immune system to prevent graft rejection. This suppression is achieved through immune suppressor drugs, which leaves the body defenseless against infections and increases its susceptibility to other complications such as cancer. Considering all these limitations, immunotherapeutic strategies have focused on restoring immunologic self-tolerance, thus removing the problem at it roots; preventing β cell destruction by patients's own immune system. The main focus of this approach is regulatory T cells (Tregs), which are essential cells in suppression of autoreactive immune responses and maintenance of self-tolerance. Stellate cells (SCs) have various effects on the immune system such as recruitment of Tregs and induction of T cell apoptosis. Besides, they can promote

vascularization, secreting vascular endothelium growth factor (VEGF). Some chemokines are also key modulators in recruitment of Tregs. Macrophage-derived chemokine or C-C motif chemokine ligand 22 (MDC/CCL22) is one of the novel chemokines used for Treg recruitment by binding to CCR4 receptor on their membrane. In this study, we designed an immune privileged microenvironment around implantable insulin secreting islets site to provide local graft tolerance and to overcome limitations associated with donor cells. We focused on achieving local immunosuppression through overexpression of CCL22 proteins by SCs, which recruited immunosuppressive Tregs. We prepared insulin-secreting pseudoislets through aggregation of mouse insulinoma 6 (MIN6) cells as a model system to mimic naïve islet morphology. Our results demonstrated that transfected SCs can secrete CCL22 and recruit a population of Tregs towards the implant *in vivo*. This study is promising to provide fundamental understanding of the SC-islet interaction, ligand synthesis and transport from stellate cells at the graft site for ensuring local immune tolerance to target Type I diabetes. Our results also establish a new paradigm for creating tolerable grafts for other chronic diseases such as diabetes, anemia, cancer, CNS diseases and advance the science of graft tolerance.

ÖZET

Fonksiyonel olmayan doku ve organları yenilemek veya değiştirmek için bağışıklık sistemi mühendisliği ile tolerans geliştirmek üzerine yaklaşımlar yeni gelişen rejenerasyon terapilerine öncülük etmektedir. Tip 1 diyabet organa bağlı bir otoimmün hastalıktır ve insulin salgılayan pankreatik β hücrelerinin otoreaktif T hücreleri ve diğer bağışıklık sistemi hücreleri tarafından yok edilmesi olarak tanımlanmaktadır. β hücrelerinin, dolaylı olarak da insülinin, vücuttan eksilmesi hastaları dışardan alınan insuline ya da bu ihtiyacın önüne geçmek için pankreas veya tüm halde adacık nakline ihtiyaç duyar hale getirir ancak bu işlemin de yan etkileri vardır. İlk ve en bariz olanı organ bağışçısı sınırlılığıdır Ayrıca, işlem sonrası naklin reddedilmemesi için adacık veya pankreas nakli bağışıklık sistemi baskılanmasını gerektirmektedir. Bu baskılama ilaçlar sayesinde yapılır ancak bu ilaçlar vücudu enfeksiyonlara hatta kansere normalde olunduğundan daha duyarlı hale getirir. Bütün bu yan etkiler göz önüne alındığında, hastanın kendi bağışıklık sisteminin beta hücrelerini yok etmesini önlemesi sebebiyle, immünoterapötik yaklaşımların öz-dayanımı yenileyerek sorunun kökenine indiği söylenebilir. Bu yaklaşımın ana odağı regulatuvar T hücreleridir çünkü bu hücreler otoreaktif bağışıklık tepkileri ve öz dayanımın düzenlenmesinde kritiktir. Stellat hücrelerin bağışıklık hücreleri üzerinde, regulatuvar T hücresi çağırmak, T hücresi apoptozunu başlatmak gibi bir çok etkisi vardır. Ayrıca vasküler endotelyal büyüme faktörü (VEBF) salgılayarak damarlaşmayı sağlarlar. Bazı kemokinler de regulatuvar T hücresi çağırılmasında kilit düzenleyici olabilirler. Macrofaj bazlı kemokin ve ya CC kemokin 22, regulatuvar T hücresi çağırılmasında

onların hücre zarındaki CCR4 reseptörüne bağlanarak etki eden, üzerinde az çalışılmış bir kemokindir. Bu çalışmada biz nakledilebilir, insülin salgılayan adacıkların etrafında bağışıklık sisteminden ayrı tutulabilen bir mikroortam dizayn ederek nakledilen doku bölgesinde toleransı sağlayıp nakledilen hücrelerle alakalı sınırlamaların önüne geçmek istedik. Bölgesel bağışıklık baskılamasını CCL22 proteinini stellat hücrelere ürettirerek ve böylece regulatuvar T hücrelerini ortama çağırarak sağlamaya odaklandık. Fare insülinom 6 (MIN6) hücrelerini agregat haline getirerek doğal adacık morfolojisine model olan, insülin salgılayan yalancı adacıklar hazırladık. In vivo sonuçlarımız, transfekte edilmiş stellat hücrelerinin CCL22 salgılayarak regulatuvar T hücrelerini nakil yapılan bölgeye çağırabildiğini göstermektedir. Bu çalışma stellat hücresi ve adacık hücreleri arasındaki etkileşimin temelleriyle ilgili bilgiler sunduğu ve stellat hücrelerde ligand üretimi ve bunların bölgesel immün toleransı oluşturmak adına nakil bölgesine taşınmasını gösterdiği için önemlidir. Sonuçlarımız diyabet için olduğu kadar anemi, kanser, MSS hastalıkları gibi başka hastalıklar içinde kullanılabilecek, hasta tarafından red edilmeyen nakil dokusu örneği teşkil ediyor ve hastalardaki nakil dokuya olan dayanımın geliştirilmesi hakkında olan bilimsel çalışmalara katkıda bulunuyor.

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This thesis is dedicated to my father who is with me forever. You always believed in me and I know that if you were here, you would be proud of me.

Table of Contents

List of Tables

List of Figures

ABBREVIATIONS

T1D	Type-1 Diabetes
Tregs	Regulatory T cells
SCs	Stellate cells
VEGF	Vascular endothelium growth factor
MDC	Macrophage-derived chemokine
CCL22	chemokine C-C motif ligand 22
MIN6	Mouse insulinoma 6
APCs	Antigen presenting cells
HSC	Hepatic stellate cells
PSC	Pancreatic stellate cells
FPG	Fasting blood glucose
DCs	Dendritic cells
NK cells	Natural killer cells
IFN-γ	Interferon gamma
TNF-α	Tumor necrosis factor alpha
RIN5	Rat insulinoma 5
DAMPs	Damage-associated molecular patterns
CCL17	Chemokine C-C motif ligand 17
Th cells	Effector T cells
MSCs	Mesenchymal Stem Cells
IL- ß	Interleukin beta
TGF- ß	Tumor growth factor beta
IL-10	Interleukin 10

CHAPTER 1

Introduction

Type I diabetes (T1D) is an autoimmune disease caused by the destruction of insulin secreting islet cells, resulting in insulin deficiency and high blood glucose [1]. Immune system of patients with T1D recognizes pancreatic β-cells in islets as foreign. This is caused by release of β-cell antigens as a result of stress, viral infection, or proinflammatory cytokines from islet cells. Those antigens are presented by antigen presenting cells (APCs) to $CD8^+$ T cells. Activated $CD8^+$ T cells cause damage on islets through either cytotoxic cytokines or granzymes. In addition, dendritic cells activate $CD4^+$ T cells in pancreatic lymph node, and activated $CD4^+$ T cells migrate to pancreatic islets where they recruit and activate lymphocytes and macrophages. In return, activated lymphocytes and macrophages secrete cytokines and these induce pro-apoptotic signaling in β-cells and kill them [2-5].

There are several approaches to treat T1D such as insulin therapy, pancreas or islet transplantation used in treatment of T1D. Although pancreas transplantation allows long-term insulin independence, suitable donors are in short supply, and there is high risk of complications during and after surgery. In addition, it is required to use immunosuppressive drugs which lead to serious side effects like increasing susceptibility to infections or cancer. As another approach, transplantation of insulin secreting islets from cadaveric human donors is promising; however, systemic suppression of the

immune system of the recipient patient is still required and also there is still donor limitation for islet transplantation. To overcome immunosuppression requirements, immunotherapeutic strategies have been used to restore immunologic self-tolerance thereby preventing destruction of transplanted β cells [1, 6]. Tregs are main actors in this immunotherapeutic strategies and have significant functions in suppression of autoreactive immune responses and maintenance of self-tolerance [4, 7]. It has been shown that $CD4^+CD25^+FoxP3^+$ T cells alleviate progress of type 1 diabetes through diminished autoimmune attack therefore providing graft tolerance [8-10]. To emphasize the roll of Tregs, their loss of function and decrease in number has been observed in diabetic patients[4]. Thus, recruitment of Tregs towards the graft site is a logical approach to overcome graft rejection, and chemokines can be used to achieve this [11-12].

Cancer cells are able to evade immune recognition and suppress immune reactivity by secreting some chemokines, for this task. They can recruit Tregs, and suppress T cells via this property. Therefore the microenvironment in tumors are highly immune suppressive. One of those is CCL22 protein which is a macrophage derived chemokine (MDC) [13-15]. CCL22 is capable of attracting of Tregs by binding to the CCR4 receptor on their membrane [6, 14]. Verchere et al. showed that CCL22 protects islet graft from immune attack and provides islet allograft tolerance [16-17]. In addition, Bischoff et al. demonstrated that CCL22 alleviates T1D by recruiting and activating Tregs, and also invariant natural killer T cells (iNKT) cells and plasmacytoid dendritic cells (pDCs) which are involved in moderation of local autoimmunity [18]. Thus, this molecule, CCL22,

is a considerable candidate to utilize in immunotherapeutic approaches to overcome graft rejection.

For several decades, several approaches such as alternative pancreatic β cell line, different implantation sites, usage of immunosuppressive drugs, encapsulation of graft have been clinically or preclinically applied to prevent loss and dysfunction of islet graft after transplantation. Accessory cells such as mesenchymal stem cells, and stellate cells have already been explored to provide graft tolerance in islet transplantation [19-22]. Both hepatic and pancreatic stellate cells (HSC and PSC) have immunomodulatory activity. HSCs can expand Treg cells, suppress T cell response, and induce T cell apoptosis. Recent studies showed that cotransplantation of HSCs prevent islet allograft rejection via formation of immune barrier [23-27]. PSCs have been reported that they indirectly contribute to immune evasion in pancreatic cancer. They have been shown to sequester $CD8^+$ T cells in pancreatic cancer stroma, migration of myeloid-derived suppressor cells into stroma, and induce apoptosis of T cells [28-30]. However, there are a few studies investigating effects PSCs on pancreatic β cells. Kikuta et al. have showed that co-culture of PSCs with RIN5F rat pancreatic β cells impairs their function by reducing insulin expression and promoting apoptosis [31]. Zang et al have reported similar effects of PSCs on function of mouse islets [32]. Since islet vasculature is degenerated during isolation and transplantation process, oxygen and nutrients must be supplied to islets from culture medium and vessels in transplanted site. However, this situation causes hypoxia at early stage of post-transplantation and it is a main reason of early graft loss [33-34] HSCs and PSCs are also able to promote angiogenesis secreting proangiogenic factors VEGF [35-36].

Qian et al. showed cotransplantation of hepatic stellate cells promoted vascularization in islet allograft after transplantation and prolonged islet survival [37].

In this study, we designed a local immune privileged microenvironment for graft tolerance and focused on achieving this through recruitment of immunosuppressive Tregs. We also addressed challenges of high islet number required for transplantation and repeated islet isolation by forming heterospheroids through aggregation of MIN6. MIN6 cells have been reported as a model system for β-cell to mimic naive islet morphology [38-39]. Here, we are recruiting Tregs via CCL22 protein to provide local immunosuppression. For this purpose, stellate cells were transfected to express CCL22 protein and then pseudoislets were formed through aggregation of MIN6 with transfected stellate cells by hanging drop method. Stellate cells have excellent angiogenesis promotion capabilities along with their immune suppressive properties [23-26, 37]. Here, we combined these characteristics of stellate cells with CCL22 protein to significantly boost the graft tolerance. This approach has potential for clinical transplantation of not only pancreatic islets for type 1 diabetes but also several organ/tissue for other disease without the use of immunosuppressive drugs.

CHAPTER 2

Literature Review

2.1 Type-1 Diabetes (T1D)

2.1.1 Definition

Type 1 diabetes (T1D) is a chronic disease characterized by hyperglycemia due to immune-mediated depletion of pancreatic β cells and cause insulin deficiency [40-41]. Autoimmune destruction of pancreatic β cells may result from not only genetic factors but also environmental triggers even mechanisms are still not thoroughly investigated. Diabetes mellitus is the fourth leading cause of death and incidence of T1D is continuously raising 3% per year worldwide[42].

2.1.2 Symptoms and Diagnosis

It has severe impact on daily life of patients and reduces the quality of patients' life. It causes severe damage, abnormalities and defect in organs such as eyes, kidney, heart etc. [43-44]. Most patients with type 1 diabetes have symptoms of visual damage, weight loss, and extensive thirst and urination [40-41]. Diagnosis of diabetes has included fasting blood glucose (FPG) ≥ 126 mg/dL (7 mmol/L) or random blood glucose value ≥ 200 mg/dL (11·1 mmol/L) with symptoms of hyperglycaemia, or an abnormal 2 h oral glucose-tolerance test [44].

2.1.3 Pathogenesis

Pathogenesis of T1DM is characterized by the loss of immune tolerance against pancreatic β cells. $CD4^+$ and $CD8^+$ T cells are involved in devastation of insulin secreting β cells as shown in Figure 2.1. In addition, other abnormal immune actions such as deficiency in mature dendritic cells (DCs), natural killer NKT cells, and reduced phagocytosis activity of macrophages as well as inflammatory cytokines like IFN-γ and TNF-α are key players in destruction of β cells [4, 45-47]. Immune system recognizes pancreatic β-cells in islets as foreign in patients with T1D. Islets release β-cell antigens as a result of stress, viral infection, or proinflammatory cytokines. Those antigens are presented by APCs to CD8+ T cells. Activated T cells cause damage on islets through either cytotoxic cytokines or granzymes. In addition, immature dendritic cells capture antigens from β-cells and transport them to the pancreatic lymph node. Here, antigens are presented to CD4+ T cells and further lead to clonal expansion of autoreactive $CD4^+$ effector T cells, resulting in clonal expansion. After migration of activated $CD4^+$ T cells to pancreatic islets, they recruit and activate lymphocytes and macrophages which secrete cytokines such as IL-1 and TNF. Those cytokines induce pro-apoptotic signaling in β-cells and also expression of CD95 ligand on membrane of pancreatic β-cells which bind effector T cells expressing CD95L on their membrane and kill β-cells [2-5].

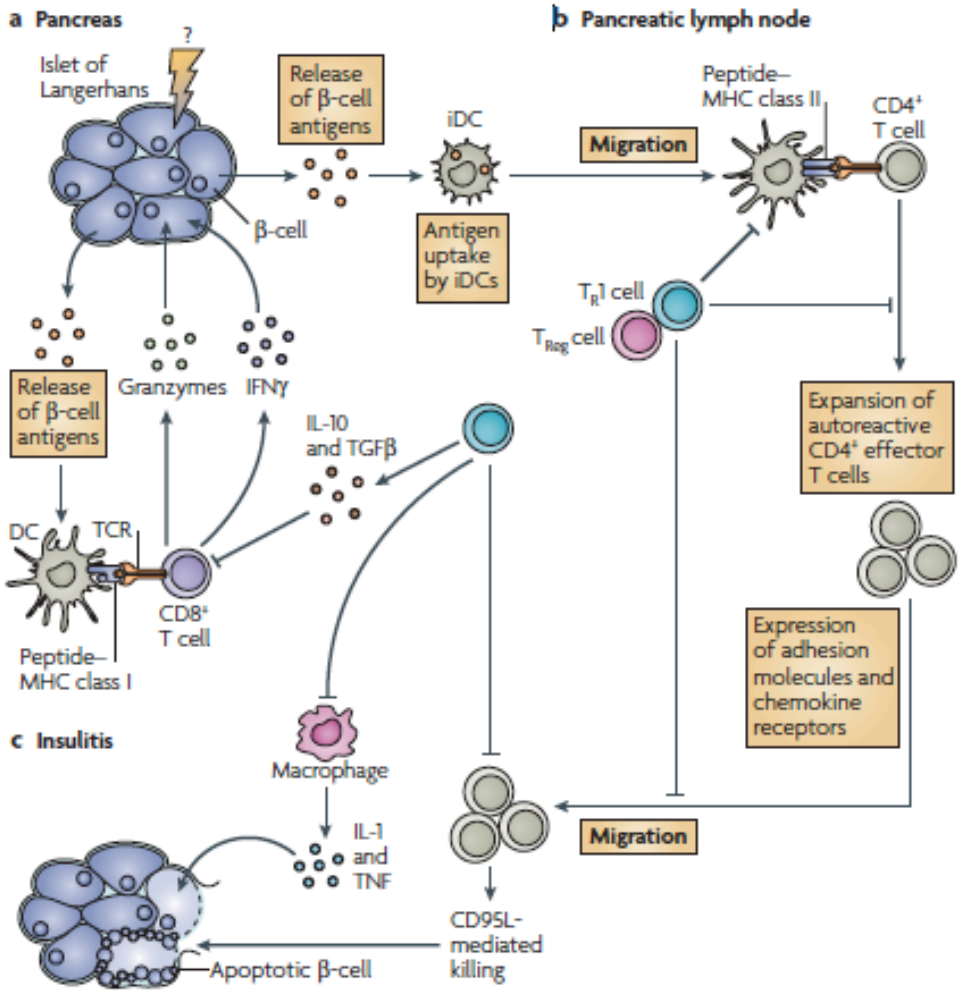

Figure 2. 1 Pathogenesis of type 1 diabetes[2]. Copyright (2017), with permission from Nature Publishing Group.

2.2 Current treatments and Limitations

There is still no effective treatment available for T1D. Injection of exogenous insulin or the use of an insulin pump, which measure blood glucose levels to avoid acute complications, currently comprise the main treatment options. Exogenous insulin treatment requires multiple daily injections and these regular administrations may be inconvenient and painful for patients. It may not sufficient to maintain normoglycemia because it is not same as endogenous insulin. There may be infections at injection site [1, 48-50]. Insulin can be also easily delivered by insulin pump, which provides adjustable insulin delivery by multiple injections, and so enable glycemic control overnight. However, there are some problems in usage of this pump. There may be infection at the injections site, and the regular injections may lead to hypoglycemia. The pump is also not convenient for patients who have to carry an external device [51-53]. Another approaches used for treatment of T1D are pancreas or islet transplantation. Although islet transplantation needs experimental improvement, transplantation of pancreas is a clinical option even there are series complications.

2.2.1. Pancreas transplantation

First transplantation of whole pancreas was achieved in 1960s and 1970s; however, the survival rate was less than 40% [54]. Mortality rate of patients was decreased, and function of graft increased after 2011[55]. It can provide long term complete insulin independence to patients[56]. Although it is improved over the years, transplantation of the whole pancreas is still a very risky operation, as it requires intense immunosuppression. Patients must have immunosuppressive therapy after surgery.

2.2.2 Islet transplantation

β cells in pancreatic islets secrete insulin. Thus, transplantation of islets to patients with T1D has been conceived as an alternative approach. Several animal and clinical trials were conducted, and insulin independence of patients after islet transplantation was achieved in 1990 although islet graft was rejected after 22 day [57]. Unlike pancreas transplantation, transplantation of islets presents a smaller risk during surgical procedure. Thus, this approach still needs experimental improvements to achieve insulin independence and avoid graft rejection after implantation. Although transplantation of pancreatic islet is a promising approach for treatment of type I diabetes, it has some drawbacks like donor limitations and usage of systemic immunosuppressive drugs to prevent graft rejection[58-59] There is a rising unmet need for transplantable islets. They are generally isolated from brain-dead patients [60-61]. However, brain death reduces viability and functionality of pancreatic islets[62]. Various studies have been performed to overcome donor limitation, mimicking naïve pancreatic islets.

2.2.2.1 Formation of Pseudoislets

Since islet transplantation requires a large number of viable islets to maintain normoglycemia and the number of suitable islet sources is limited, alternative tissue engineering approaches to islet transplantation has emerged to mimic naive islets. Thus, three dimensional (3D) islet models have been constructed by aggregation of different β cell lines into spheroid formation called pseudoislets. Even though stem cells or progenitor cells such as induced pluripotent stem cells can differentiate into β cells, reconstruction of islets from single cells is still challenging. It has been shown that 3D

spherical cultures mimicking intact islets have found more functional compared to monolayer cells[63-65]. First, pseudoislet formation from neonatal pig islets was reported at 1981 [66]. It was also established using adult rats[67-70], neonatal rats[67, 71-72], cadaveric pancreata[73] and neonatal humans[74]. Different methods have been carried out to form 3D islet models. β cell lines can self assemble into spheroids when cultured in non-adhesive cell culture petri dishes. In addition, microwells fabricated by various methods have been used to form spheroids due to gravity[65]. Also, hanging drop is another method for pseudoislet formation. Cells are dropped onto lids of cell culture petri dish and then lids are inverted. These cells can then form clusters under gravitational force[75].

2.2.2.2 Pancreatic β Cell Lines

In addition to donor limitation, the process of islet isolation is quite time consuming and costly. Due to these obstacles of using donated islets, insulin secreting cell lines have been the center of attention in transplantation studies. Limited number of cell lines has been derived from insulinomas, which is a small tumor occurred naturally in pancreas and produces an excess amount of insulin. In addition, insulin secretion properties of those cells are permanent in tissue culture since they start to dedifferentiate[76]. Insulinomas have been induced in rat to establish cell lines and RIN cells were the first cell lines obtained by this method. RINm and RIN5F cell are the most commonly used sublines derived from RIN [77]. They have been reported to produce insulin, glucagon, and/or somatostatin. This insulin-secreting cell line has been used to form pseudoislets in several studies[78-81].

Proliferation of β cells, which is derived from naturally-occuring insulinoma occurred naturally, is not easy in cell culture. To overcome this limitation, a gene construct, which includes of the structural region of the simian virus 40 (SV40) T antigen gene driven by the insulin promoter, was injected into mouse embryonic pronucleus to obtain transgenic mice harboring SV40 T antigen. These mice developed β cell tumors and insulin secreting cell lines have been derived from such insulinoma [82-86]. Miyazaki et al. established a β cell line called mouse insulinoma 6 (MIN6) from insulinoma developed in transgenic mice expressing SV40 T antigen[87]. MIN6 cell line has been used in formation of pseudoislets in several researches and viable and functional pseudoislets have been obtained by aggregation of MIN6 cells to spheroids formation. It has been reported that they share similar functional properties with naïve islets [88-91]. As a result, commercially available cell lines are an alternative source of β cells to form spheroids in 3D culture, which are more functional than monolayer and display similar functionality as naïve islets.

2.3. Immunological Tolerance and Transplant Immunology

The immune system contains various mechanisms to protect the body from invasive pathogens and tumors by distinguishing self from non-self. Immunological self-tolerance is crucial to maintain immunological homeostasis and prevent autoimmune attack to body's own cells. [92-93].

Activation of innate and adaptive immune system by allograft lead to rejection of transplant. Release of damage-associated molecular patterns (DAMPs) from damaged graft during transplantation activates innate immune cells. Activated leukocytes release

inflammatory cytokines or chemokines, which cause recruitment of other leukocytes into graft. Complement system, which is a part of immune system and enhances ability of antibodies and phagocytic cells may also be activated by DAMPs and activated complement system lead to further destruction of graft. Thus, transplanted organs or tissue initiates destruction and rejection process. However, innate immune system itself is mostly not enough to reject transplanted tissue or organ. Activation of innate immune system orchestrates adaptive immunity against to graft. Recognition of donor-derived antigens by T cells initiates allograft rejection. Activated T cells undergo clonal expansion, differentiate into effector T cells, migrate to graft site and incite tissue destruction [94-96]. In addition, B cells play a key role in rejection process. They can act as APC interacting with T cells and produce alloantibodies against the graft [97-98].

2.4. Prevention of Islet Rejection

After transplantation, patients' immune system recognizes transplanted islets as a foreign like bacteria or viruses and if immune system is not suppressed, immune cells fight against the transplanted islets and cause damage and further rejection. Thus, several strategies have been developed to prevent rejection of islet graft. In addition, islet vasculature is degenerated during isolation and transplantation process. Thus, oxygen and nutrients must be supplied to islets in transplanted site. Lack of oxygen causes hypoxia at early stage of post-transplantation which is main reason of early graft loss [33-34]

2.4.1 Immunosuppressive drugs

The major drawback of islet transplantation is requirement of lifelong immunosuppressive therapy. This systemic suppression is generally achieved by the

administration of immune suppressive drugs. After surgery, patients must take these drugs every day to prevent implanted islet graft from autoimmune attack. Daclizumab, sirolimus, and tacrolimus are the antirejection drugs used to provide graft tolerance. However, these drugs have serious side effects such as defenseless of patients against infections and even more susceptibility to cancer. In some cases, patients who received islet transplant have to stop using these drugs due to their side effects [99-101].

2.3.2 Local immunosuppression

2.3.2.1 Regulatory Tcells

Tregs are mostly $CD4^+CD25^+FoxP3^+$ cells. Their main function is to suppress and downregulate effector T cells. They can produce immunosuppressive cytokines like IL10 and TGFβ. They also inhibit activation of T cells through APCs by the binding of CTLA4 on Tregs to B7 molecule on APCs. There are large amount of IL2 receptor on Tregs membrane and so they can also indirectly reduce proliferation and differentiation of IL2 dependent cells by binding IL2 due to high level of IL2 receptor on Tregs. Tregs are, as seen in numerous studies, key regulators of immune function in human body. To exert their function, they are generally recruited to the area of need through various ways. Although natural Tregs (nTregs) are cytokine independent and rely on solely contact to function, adaptive Tregs (aTregs) can be directed to different parts of the body[4]. This process usually occurs through ligand-receptor interactions; ligands being antigens, cytokines or chemokines and, receptors residing on the membrane of aTregs. Many studies showed that FOXP3 expression is crucial for Treg suppressive function but in translational studies, immunotherapy centered ones, usually the focus is to find a way to

either recruit or deplete Treg population depending on the disease. Cancer immunotherapy is one of the major topics of immunotherapeutic studies and from these studies, it is seen that most cancer cells have a mechanism to recruit Tregs and bypass the body's immune response, namely effector T cells[102]

2.3.2.2 Chemokines

Many studies showed that $CD25^+$ $FOXP3^+$ $CD4^+$ Tregs are a major part in this process, but recent years revealed a new protein in recruitment of Tregs; C-C chemokine receptor type 4 (CCR4)[103-104]. Ligands of CCR4 are chemokine C-C motif ligand 22 (CCL22) and chemokine C-C motif ligand 17 (CCL17) but studies showed that CCL22 secretion from the tumor tissues recruit FOXP3+ Tregs to the area through this CCR4-CCL22 interaction and effectively suppress self-antigen specific effector T cells[102]. Cancer cells can secrete CCL22 to recruit Tregs and escape from immune cells (Figure 2.2)[105]. Although immunotherapy is one of the major focuses in cancer treatment, it is also used by researchers to find ways against pitfalls of transplantation. Since transplantation procedure's one of the major issues is immune reaction against the donated tissue or organ, successful immune suppression in the receiving patient's body is crucial. However doing so gives rise to new problems, since a body without immune system is susceptible to infections and even some cancer types. The findings on Treg recruitment through CCR4-CCL22 intrigued scientists to exploit CCL22 for many transplantation techniques and auto-immune disease treatments. T1D is one of these diseases. Studies involving CCL22 expressing insulin secreting cells showed increased Treg population [16-17]. Thus, CCL22 is a recently identified for immunotherapy approaches.

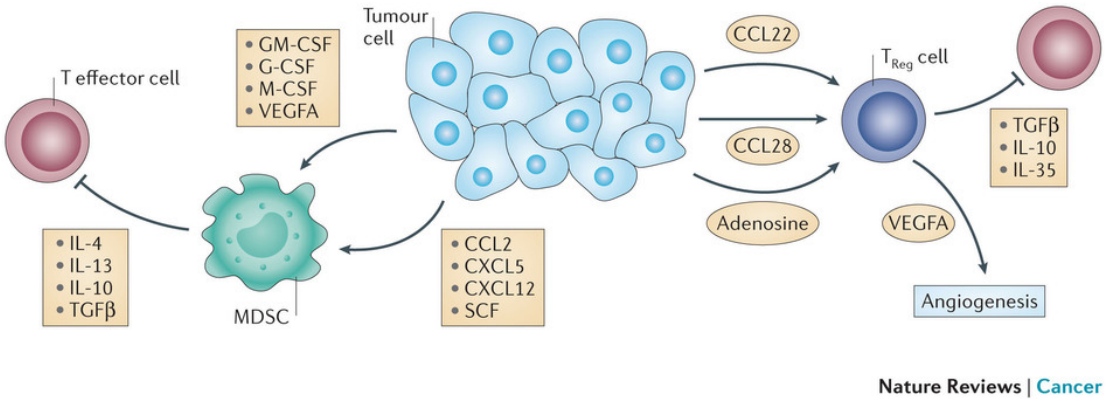

Figure 2. 2. Immunomodulation by cancer cells[105]. Copyright (2017), with permission from Nature Publishing Group.

2.4 Accessory Cells for Islet Transplantation

2.4.1 Mesenchymal Stem Cells (MSCs)

MSCs are known to differentiate into variety of cell types and so usage of them for tissue repair is a novel clinical approach. Numerous studies have been shown their therapeutic potential. They have different mechanisms to achieve MSCs-mediated immune regulation. MSCs have been shown to regulate function of T cell, B cell, natural killer (NK) cells, dendritic cells (DCs), and regulatory T cells (Tregs) by cell- cell contact and through soluble factors secreted from MSCs. They have been reported to alter secretion of cytokine from DCs, effector T (Th) cells, NK cells. MSCs can regulate T cell mediated immune response. MSC may lead to decreased IFN-γ secretion from Th1 and increased IL-4 secretion from Th2. T cells can be inhibited by IFN-γ and IL- ß. MSCs can also induce apoptosis in activated T cells [106-108]. Moreover, MSCs produce NO (in mice), or IDO (in human), and chemokines which play a key role in immunomodulation. T cells are attracted by chemokines into close proximity to activated

MSCs and so NO or IDO suppress immune response by direct T cell inhibition as shown in Figure 2.3 [108]. MSCs have been also shown to suppress B cells with soluble factors released by MSCs and also with direct interaction between MSCs and B cells, blocking proliferation of B cell in G0/G1 phase of cell cycle. In addition, it has been shown that MSCs can suppress proliferation of NK cell although partially inhibit proliferation of activated NK cells. MSCs may cause decreased IFN-γ release from NK cells and also TGF- ß1 have been found to be a key cytokine in NK cell suppression by MSCs. MSCs may lead to decreased TNFα secretion from DCs, and increased IL-10 secretion from DC. In addition, MSCs can regulate immune response by induction of regulatory T cells (Tregs). It has been reported that MSCs can promote differentiation of Th17 cells into Tregs [106-107].

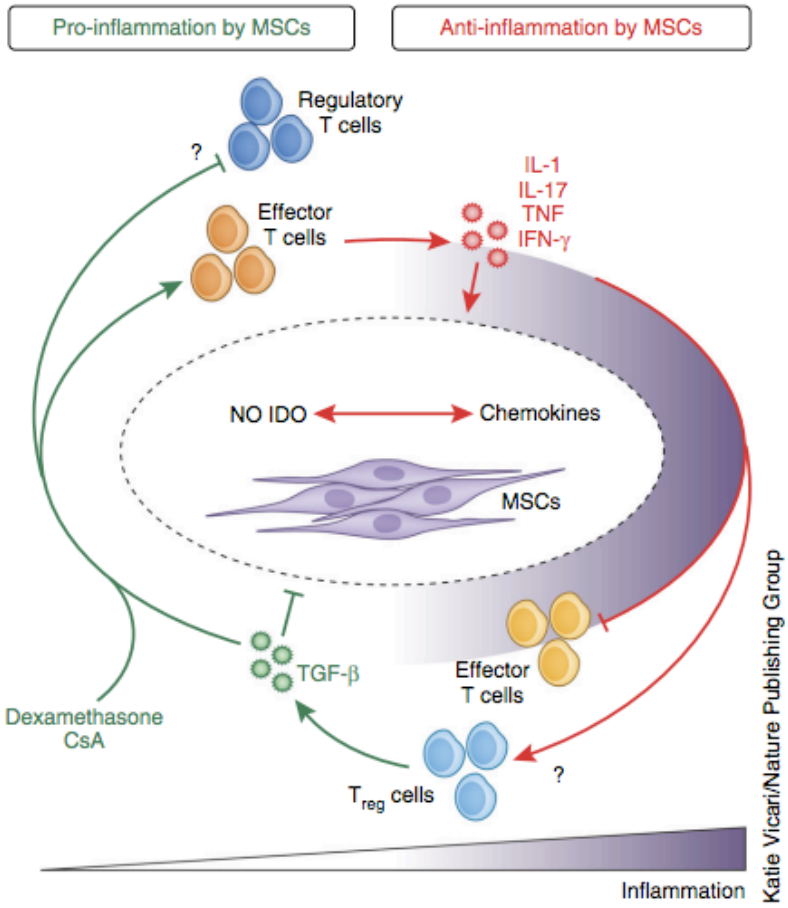

Figure 2. 3. immunomodulation by MSCs [108]. Copyright (2017), with permission from Nature Publishing Group.

MSCs are able to enhance survival and function of islet graft by increasing islet revascularization through secretion of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and matrix metalloproteases (MMPs). Thus, MSCs promotes new vasculature around heterospheroids, providing oxygen and nutrients. Even though MSCs also needs oxygen

and nutrient for their survival after islet transplantation and so compete with MIN6 cell in the beginning, MSCs have excellent angiogenesis promotion capabilities along with their immune regulation properties. Hence, usage of MSCs is a novel clinical approach for islet transplantation [3, 33].

2.4.2 Stellate Cells (SCs)

SCs are star-shaped cells found in some organs such as pancreas, liver etc. PSCs are located adjacent to pancreatic acinar cells, around small pancreatic ducts and blood vessels. PSCs migrate, synthesize, and secrete ECM proteins. These properties of PSC are stimulated during activation. Activated PSCs secretes excessive ECM synthesis and this eventually leads to pathological fibrosis [109].

HSCs are located in the perisinusoidal space of Disse between the fenestrated liver endothelium and epithelial hepatocytes. The key morphological feature of HSCs is presence cytoplasmic droplets containing vitamin A as retinylpalmitate. However, liver injury cause loss of vitamin A droplets and they become activated. Activated stellate cells can produce excessive collogen[110-111].

HSC and PSC have immunomodulatory activity. HSCs are able to expand Treg cells, suppress T cell response, and stimulate T cell apoptosis. Recent studies indicated that cotransplantation of HSCs with islets prohibit rejection of islet allograft via formation of immune barrier [23-27]. PSCs can indirectly help the pancreatic cancer cells to escape from immune system. They sequester $CD8^+$ T cells in pancreatic cancer stroma, migration of myeloid-derived suppressor cells into stroma, and induce apoptosis of T cells [28-30]. Effects of PSCs on pancreatic β cells have been also investigated. Kikuta

et al. have showed that co-culture of PSCs with RIN5F rat pancreatic β cells impairs islet function by reducing insulin expression and promoting apoptosis[31]. Zang et al have found similar effects of PSCs on function of mouse islets[32].

Harnessing SCs as accessory cells for islet transplantation is also promising to supply revascularization around implant site. SCs are able to promote angiogenesis by secreting proangiogenic factors such as VEGF [35-36]. Promotion of vascularization by cotransplanted HSCs have been reported to prolong islet survival[37].

CHAPTER 3

Experimental Part

3.1 Cell culture

Human pancreatic and hepatic stellate cells were provided from Koc University Hospital. SCs were maintained in Dulbeccos's modified Eagles's medium Nutrient Mixture F-12 (DMEM:F12) (Gibco) supplemented with 10 % fetal bovine serum (FBS) (heat inactivated, Gibco) and 1% Penicillin Streptomycin (Sigma) and incubated at 37ºC with 5% CO_2 environment. MIN6 cells were donated by Dr. Donald F. Steiner's Laboratory from University of Chicago. MIN6 cells were cultured in (DMEM) (HG, Gibco), 10 % FBS, 1 % L-Glutamine (Sigma), 1 % sodium pyruvate (Lonza), and 1 % Penicillin Streptomycin (Sigma) and incubated at 37ºC in a humidified 5% CO_2 incubator. Hek293T cells were provided from Dr. Tugba Balci Onder Laboratory from Koc University. Hek293T cells (up to passage 10) were cultured in DMEM (Gibco) with %10 FBS, and %1 Penicillin Streptomycin and incubated at 37ºC, and %5 CO2 for transfection experiments.

3.2 Plasmid constructs

pCMV6-AC plasmid including the CCL22 gene was used as template for PCR based cloning. Primers for PCR were designed to add BamHI and SalI restriction enzyme recognition sites on either end of the gene. CCL22 gene was amplified by PCR. To remove undesired salt, enzyme and nucleotides, PCR products were purified by using Macherey Nagel NucleoSpin® Gel and PCR Clean-up. Then, it was digested with BamHI and SalI to obtain restriction enzyme recognition site at the ends of gene. Retroviral

vector pBABE was also digested with both BamHI and SalI to linearize and then dephosphorylated by Antartic Phosphatase to prevent self-ligation of plasmid. Both insert and_recipient vector were run on 1% agarose gel and extracted from gel by using Macherey Nagel NucleoSpin® Gel and PCR Clean-up kit. Then, CCL22 insert was ligated into the recipient retroviral vector pBABE by T4 DNA ligase overnight at 16 ^{0}C. Then, ligated plasmid was transformed into One Shot™ Stbl3™ chemically competent cells and then inoculated into agar plate containing ampicillin. After 16 h incubation, colonies were picked and inoculated into LB Broth medium containing ampicillin and incubated for 16 h. Plasmid DNA was isolated from bacteria culture by using Macherey Nagel NucleoSpin® Plasmid kit.

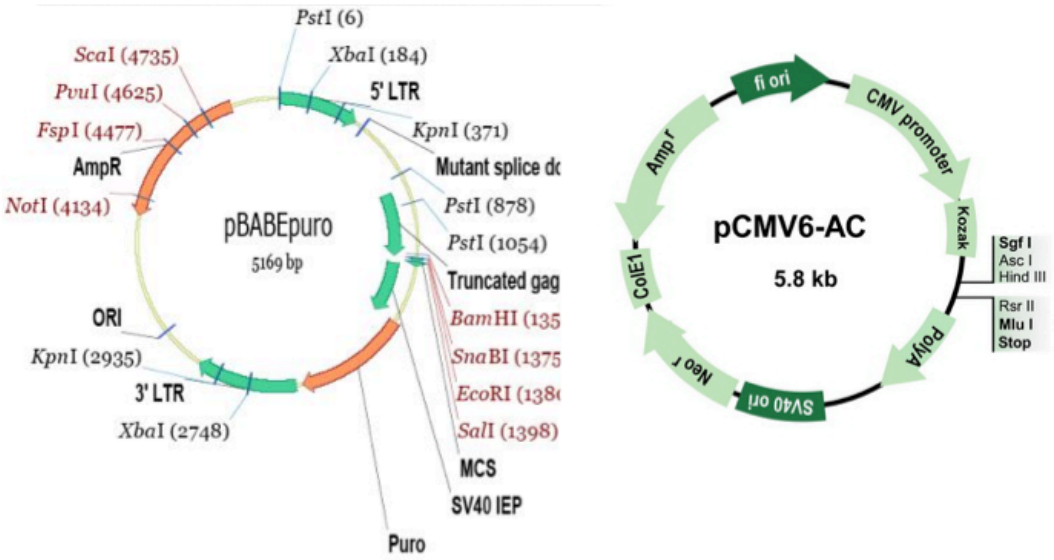

Figure 3. 1. Plasmids used for molecular cloning; retroviral vector pBABE puro as a recipient vector and pCMV-6AC containing CCL22 gene.

3.3 Virus production and infection of pancreatic stellate cells

2.5 x 10^6 Hek 293T cells were seeded into 10 cm petri dish and incubated overnight. pBabe-GFP was used as control vector for transduction. pBabe-GFP or pBABE-CCL22 and other vectors; pUMVC pCMV-VSV-G which are necessary for viral packaging, were transfected to Hek293T cells by using Fugene transfection reagent (Promega). Next day, medium was changed with fresh medium and incubated overnight at 37 ^{0}C and %5 CO_2. Medium containing virus particles were collected and kept at -80 ^{0}C. Viruses were 100X concentrated using % 50 (w/v) PEG solution. SCs were seeded in 6 well plate at a density of 10^4 cells/ well. Next day, cells were infected with retroviruses (GFP or CCL22) containing 10 μg/ml protamine sulfate (PS) and incubated for 2 days at 37 ^{0}C and %5 CO_2. Double infection was performed to enhance transduction efficiency. SCs were selected by adding puromycin at a final concentration of 1μg/ml for 3 days (Figure 3.2).

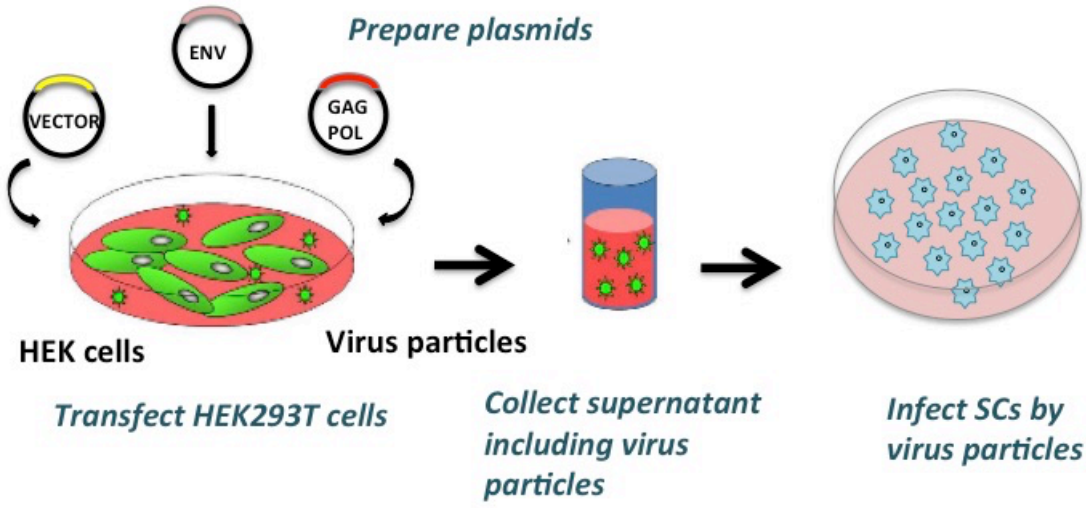

Figure 3. 2. Virus production and transduction of SCs.

3.4 qRT-PCR

Total RNA was extracted using RNA extraction kit from Macherey Nagel according to manufacturer's instructions. cDNA was synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen). Relative gene expression levels of CCL22 were detected by using LightCycler® 480 SYBR Green I Master (Roche). Relative quantification of gene expression was performed using GAPDH as a housekeeping gene. qRT-PCRs was carried out using the primers in Table 3.1. Gene expression levels were calculated using 2^-ΔΔCt method.

Table 3. 1. ***RT PCR primers for CCL22 gene***

Gene	*Sequence*	
CCL22	F	5 -ATTACGTCCGTTACCGTCTG- 3
	R	5 -TAGGCTCTTCATTGGCTCAG- 3
hGAPDH	F	5 -AGCCACATCGCTCAGACAC- 3
	R	5 -GCCAATACGACCAAATCC- 3

3.5 CCL22 Protein Determination

$9x10^4$ transfected stellate cells were seeded on 6 cm Petri dish and medium was changed after 2 day. Then, medium was collected at day 5 after seeding and secreted CCL22 protein levels in serum were determined by Human Quantikine CCL22 ELISA kit (R&D Systems) following the manufacturer's instructions.

3.6 Cell Tracker dye staining and heterospheroid formation by hanging drop method

The hanging drop technique was used to form pseudoislets by agglomerating MIN6 or MIN6:SCs cells into spheroids (Figure 3.3). To monitor cell distribution of MIN6 and SCs within heterospheroids, SCs were stained with 5 μM Cell Tracker red (Invitrogen), and MIN6 cells were stained with 5 μM Cell tracker green (Invitrogen) for

1 h prior to spheroid formation. Different cell densities; 300 and 1000 cells per 30 µl were prepared for MIN6 homospheroid and MIN6:SCs heterospheroid formation. For heterospheroid formation, MIN6 and SCs cell suspension were prepared at the ratio of 1:1, 3:1, 5:1, 7:1, 10:1 (MIN6 to SCs) in half of MIN6 and half of SCs growth medium without β mercatoethanol. 30 µl of cell suspension was pipetted onto lids of 10 cm Petri dishes and inverted lids were placed on 10 cm Petri dish containing phosphate buffered saline (PBS) with %1 penicillin streptomycin. Droplets were incubated for 3 days at 37 ^{0}C and 5% CO2.

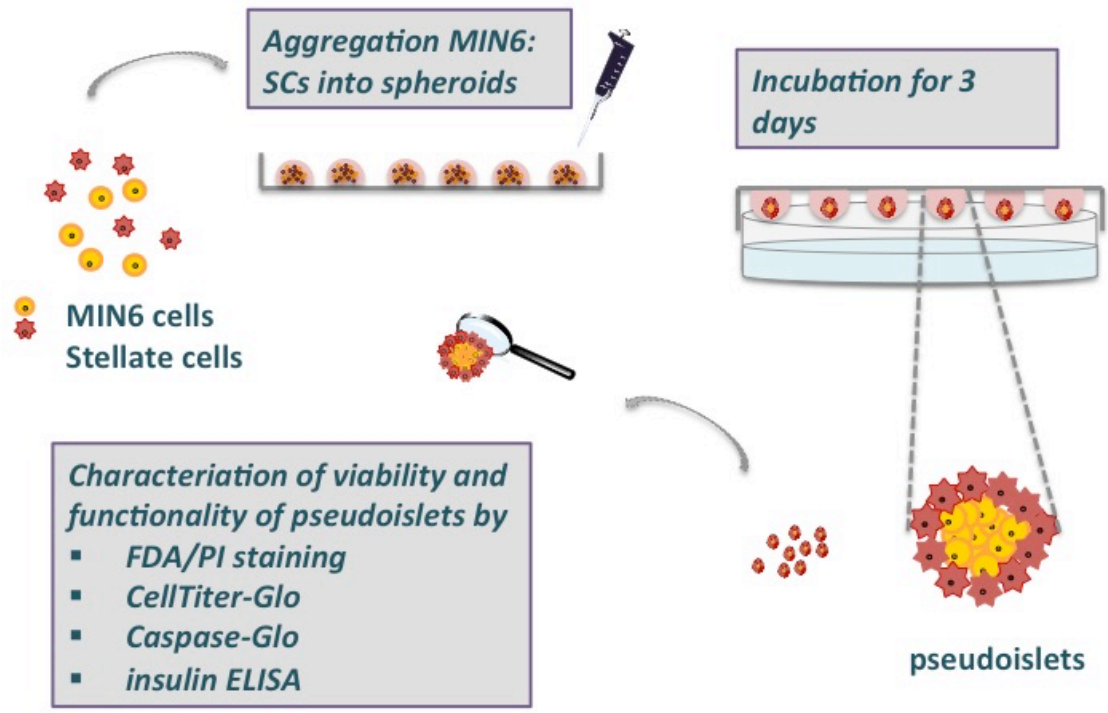

Figure 3. 3. Formation of pseudoislets by hanging drop method.

Viability of pseudoislets was assessed by inclusion dye; fluorescein diacetate (FDA) and exclusion dye; propidium iodide (PI) staining. Briefly, pseudoislets were formed by hanging drop method and incubated for 3 days. Pseudoislets were transferred to phosphate-buffered saline (PBS). Pseudoislets were stained with 8.5 μg/ml PI and 42.4 ng/ml FDA. After 5 min incubation at dark, islets were washed with PBS and then visualized and photographed under fluorescent microscopy. Viable cells were stained green and dead cells were stained red. Percentage of viable cells and area of pseudoislets were estimated by using imageJ software. The mean and standard deviation of viable cell numbers were also calculated.

3.8 Metabolic activity of pseudoislets

Intracellular ATP activity and apoptosis of pseudoislets were measured using CellTiter Glo (Promega), and caspase3/7 Glo (Promega) assay. The CellTiter-Glo is a method to determine number of viable cells in culture based on ATP quantification. The amount of ATP is directly proportional to number of viable cells present in culture. Caspase-Glo 3/7 Assay is also a luminescent assay. It measure caspase 3 and 7 activities by the help of proluminescent caspase 3/7 substrate, which contains tetrapeptide sequence DEVD and it is cleaved to release aminoluciferin used in light production.

Briefly, 15 pseudoislets formed by hanging drop method were picked in 120 μl cell culture medium and transferred to a 96-well plate. Then, 60 μl of each reagent was added to each well. Cell culture medium was contained half of β mercapto free MIN6 growth medium and half of SCs growth medium. Islets were incubated for 15 min 200rpm at dark for ATP activity. To determine apoptosis, incubation for 5 min 200rpm

and then static incubation up to 1.5 h at dark was performed. ATP and caspase activity were read on a microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader; Bio-Tek).

3.9 Functionality of Pseudoislets

Insulin secretion from pseudoislets was measured by static incubation. Briefly, 20 pseudoislets were picked and plated on cell culture insert (12 μm) in 1 ml krebs-ringer buffer (KRB) containing 2.8 mM glucose (low glucose) and incubated for 1 h. Then low glucose treatment was repeated and medium was collected. Inserts containing pseudoislets were transferred to 1ml high glucose KRB containing 28mM glucose (high glucose) and incubated for 1 h. KRB media were collected and stored at -20^{o}C until usage. Insulin was measured using the mouse insulin ELISA kit (Mercodia) and the manufacturer's instructions were followed. To quantify insulin release, stimulation index was calculated as a ratio of high glucose to low glucose state.

3.10 Transplantation of Pseudoislets

All *in vivo* experiments were approved by the institution review boards of Koc University. 8-9 week aged male CD1 mice were used for in vivo experiment. 400 pseudoislets formed by hanging drop method were implanted into abdominal external oblique muscle of recipient mouse. 10 day after transplantation, tissues from recipient mice were isolated and analyzed using flowcytometer (Figure 3.4).

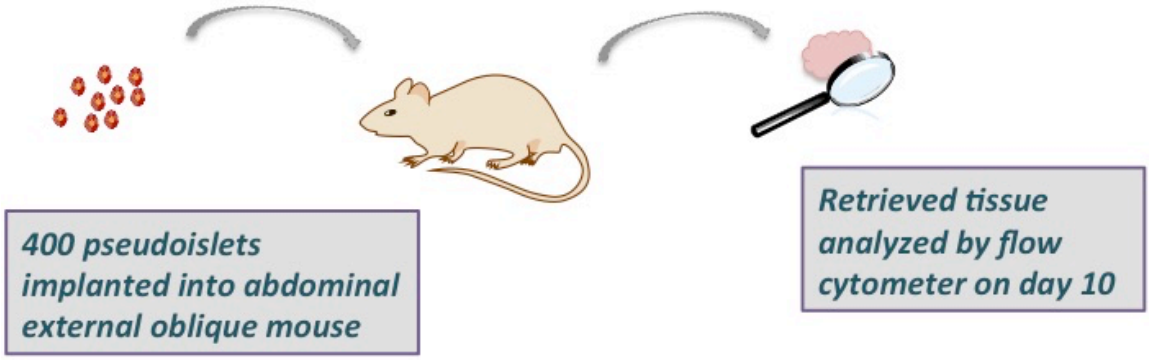

Figure 3. 4. Implantation of 400 pseudoislets into abdominal external oblique muscle of mouse. Tissue was retrieved on day 10 and analyzed by flow cytometer.

3.11 Flow Cytometry

It is a cell analysis technique, which gives highly specific information about individual cells. It can measure the volume of cells, which are labeled with different fluorescent dye, in flowing fluid stream as they passed in front of viewing aperture.

Tissue was retrieved at day 10 after transplantation to mouse and analyzed for Treg recruitment. Briefly, cells were isolated from tissues by mechanically disrupting the tissue in serum free RPMI medium and passed through 70 µm strainers in order to prevent aggregate formation. Cells were counted and adjusted to a maximum of 10000 cells/µl. True-Nuclear FoxP3 Mouse T-Reg Flow Kit (Biolegend) was used to detect $CD4^{+}FoxP3^{+}$ Tregs in tissues. 20 µl of Anti-Mouse CD4 APC/CD25 PE antibody cocktail was added to the bottom of 15x75 mm Falcon tubes and 100 µl of sample was added onto the antibody cocktail, mixed with vortexing and incubated for 15 minutes in the dark at RT. An unstained sample tube was used for autofluorescence detection. 2 ml of staining buffer containing PBS with 0.5% (w/v) BSA was added and tubes were

centrifuged at 400 g for 5 minutes. The supernatant was discarded and cells were fixed and permabilized by adding 1ml of 1X Transcription Factor Perm Buffer and centrifuged for 5 minutes at 400 g. Pellet was resuspended with 100 μl of 1X Transcription Factor Perm Buffer. 5 μl of Anti-Mouse FoxP3-Alexa488 antibody was added to the stained tubes and samples were incubated for 30 minutes in the dark at RT. Cells were washed twice with 2ml 1X Transcription Factor Perm Buffer for 5 minutes at 400 g. Supernatant was discarded and pellet was resuspended with 500 μl of staining buffer. 10^6 cells were analyzed with BD Accuri C6 Flow Cytometer and using Accuri C6 Software (Becton Dickinson). CD25+FoxP3+ cells under CD4+ gate were determined as Tregs.

3.12. Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 6 software and the results were depicted as mean ± standard deviation (SD). Multiple comparisons between groups were analyzed by two-way ANOVA followed by Tukey post hoc testing, $p < 0.05$ was considered statistically significant.

Chapter 4

Results and Discussion

4.1 Results

4.1.2 3D co-culture of MIN6 cells and SCs by hanging drop method

MIN6 β-cells were aggregated into spheroids called pseudoislets to mimic naïve islet morphology. The hanging drop method was applied to form MIN6 homospheroids and MIN6:SCs heterospheroids. 30 µl of cell suspension containing either only MIN6 or MIN6 and SC cells were dropped onto the lids of a cell culture Petri dish. Lids were inverted and cells were incubated for 3 days at 37 ^{0}C. Next, we investigated effects of initial cell number (300 and 1000), effect of stellate cell type (HSC and PSC), and ratio of MIN6 to SC cells in the heterospheroid (1:1, 3:1, 5:1, 7:1,10:1) on the growth and formation of pseudoislets.

To observe MIN6 cells and SCs within heterospheroid, cells were labeled with two different cell tracking dyes prior to spheroid formation and visualized under a fluorescent microscopy (Figure 4.1 and 4.2). We were able to obtain heterospheroids morphologically similar to real islets by hanging drop method for all conditions studied. Heterospheroids composed of MIN6 cells coated with SCs at a 1:1 ratio (MIN6 to SCs) as shown in Figure 4.1 and Figure 4.2. As expected, spheroids that initially contained 1000 cells as initial cell number were larger than those with only 300 cells. In addition, darkening in pseudoislets was observed with increasing cell number (Figure 4.2)

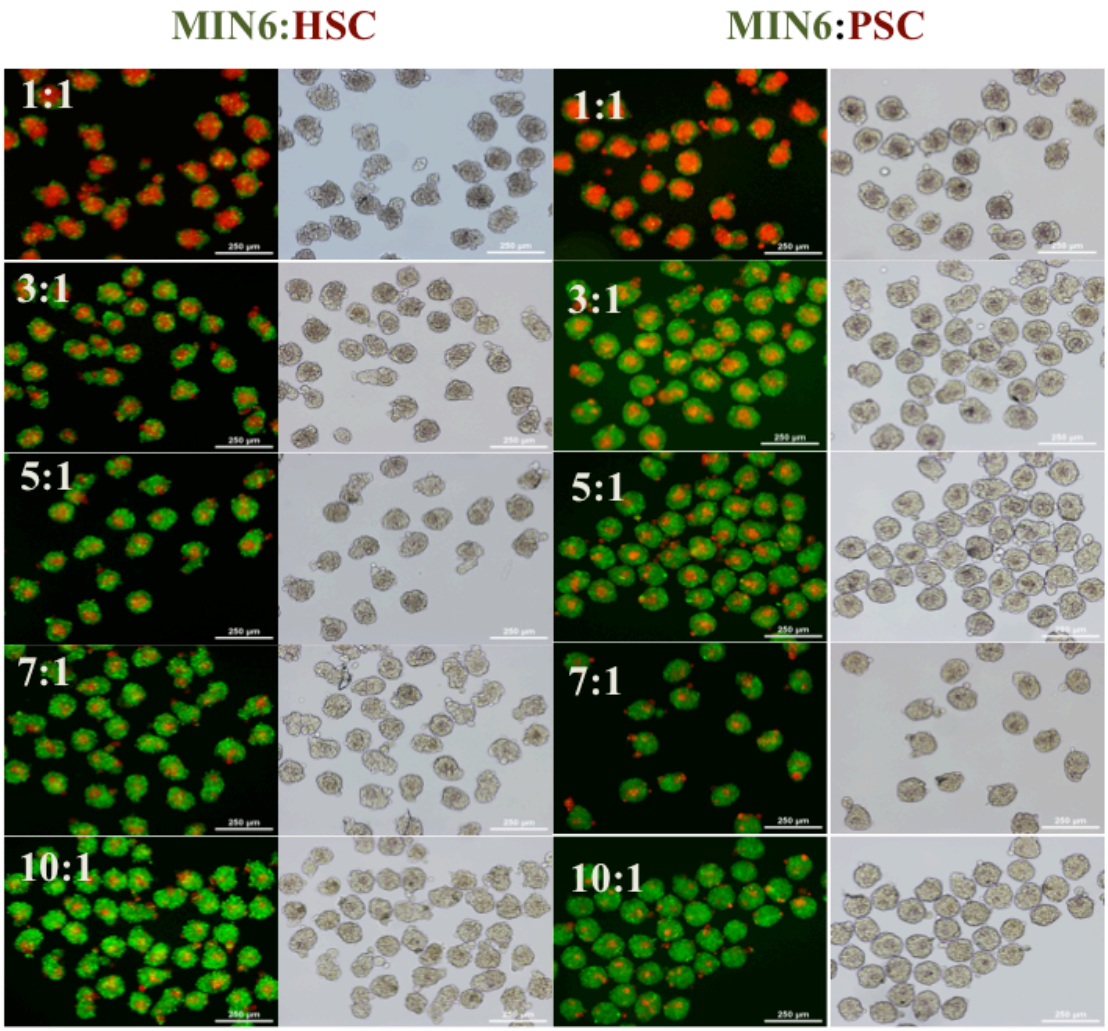

Figure 4. 1. Distribution of MIN6 cells and SCs within 3D spheroids. Cells were stained with 5 µM cell tracker dye for 1h, prior to spheroid formation. Initial cell concentration was 300 cells per 30 µl and different MIN6 cells to SCs ratio (1:1, 3:1, 5:1, 7:1, 10:1) were used for heterospheroid formation. Hanging drop method was carried out and cells were incubated within droplet for 3 days. After 3 days, pseudoislets were visualized and photographed under fluorescent microscopy. (MIN6: green ; SCs: red). (Scale bar: 250 µm).

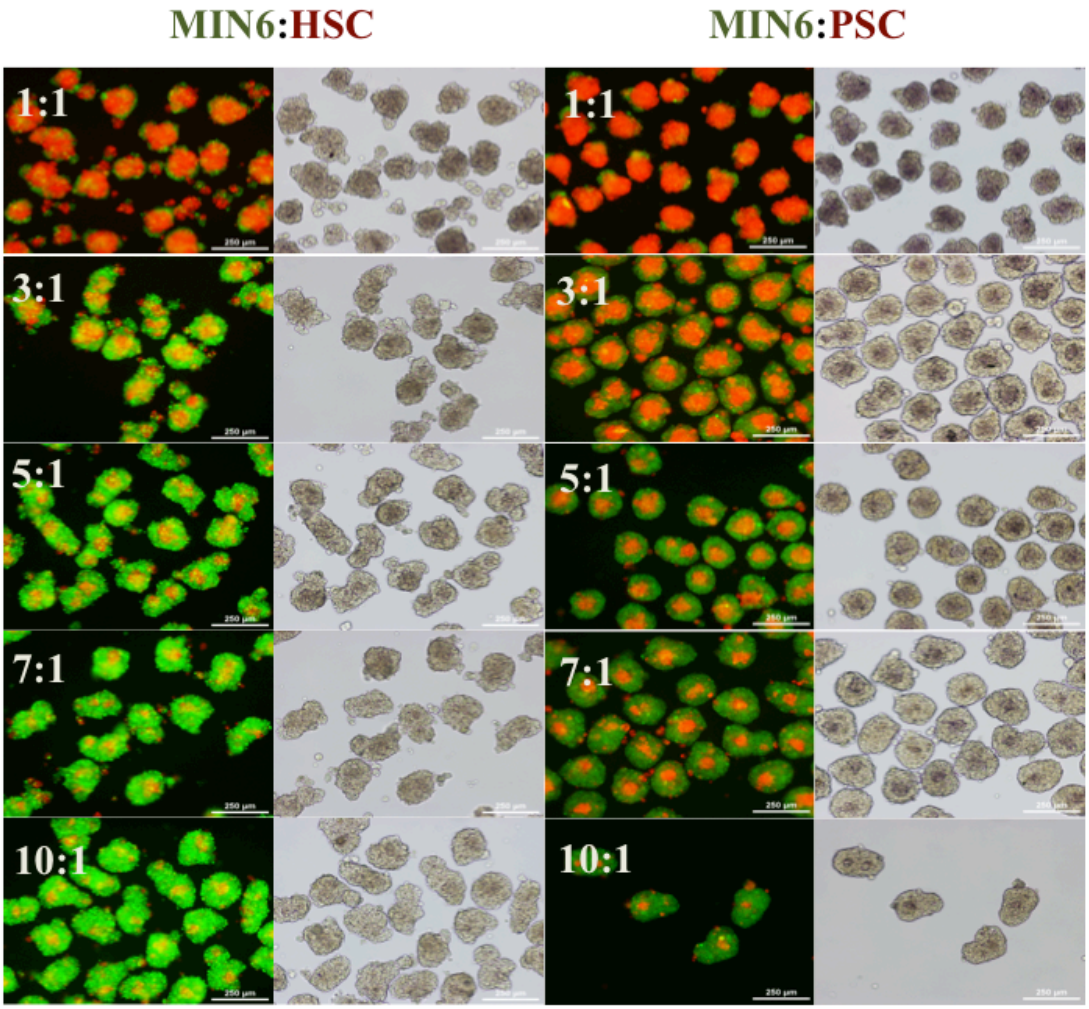

Figure 4. 2. Distribution of MIN6 cells and SCs within 3D spheroids. Cells were stained with 5 µM cell tracker dye for 1h, prior to spheroid formation. Initial cell concentration was 1000 cells per 30 µl and different MIN6 cells to SCs ratio (1:1, 3:1, 5:1, 7:1, 10:1) were used for heterospheroid formation. Hanging drop method was carried out and cells were incubated within droplet for 3 days. After 3 days, pseudoislets were visualized and photographed under fluorescent microscopy. (MIN6: green ; SCs: red). (Scale bar: 250 µm).

4.1.2 Viability of MIN6:SCs pseudoislets

Viability of cells in pseudoislets was assessed by FDA-PI staining assay and visualized under fluorescent microscopy. We obtained high percentage of green area compared to red domains indicating the presence of higher amount of viable cells in the heterospheroid (Figure 4.3 and 4.4). As expected, high amount of red area was observed in larger spheroids, which initially contained 1000 cells.

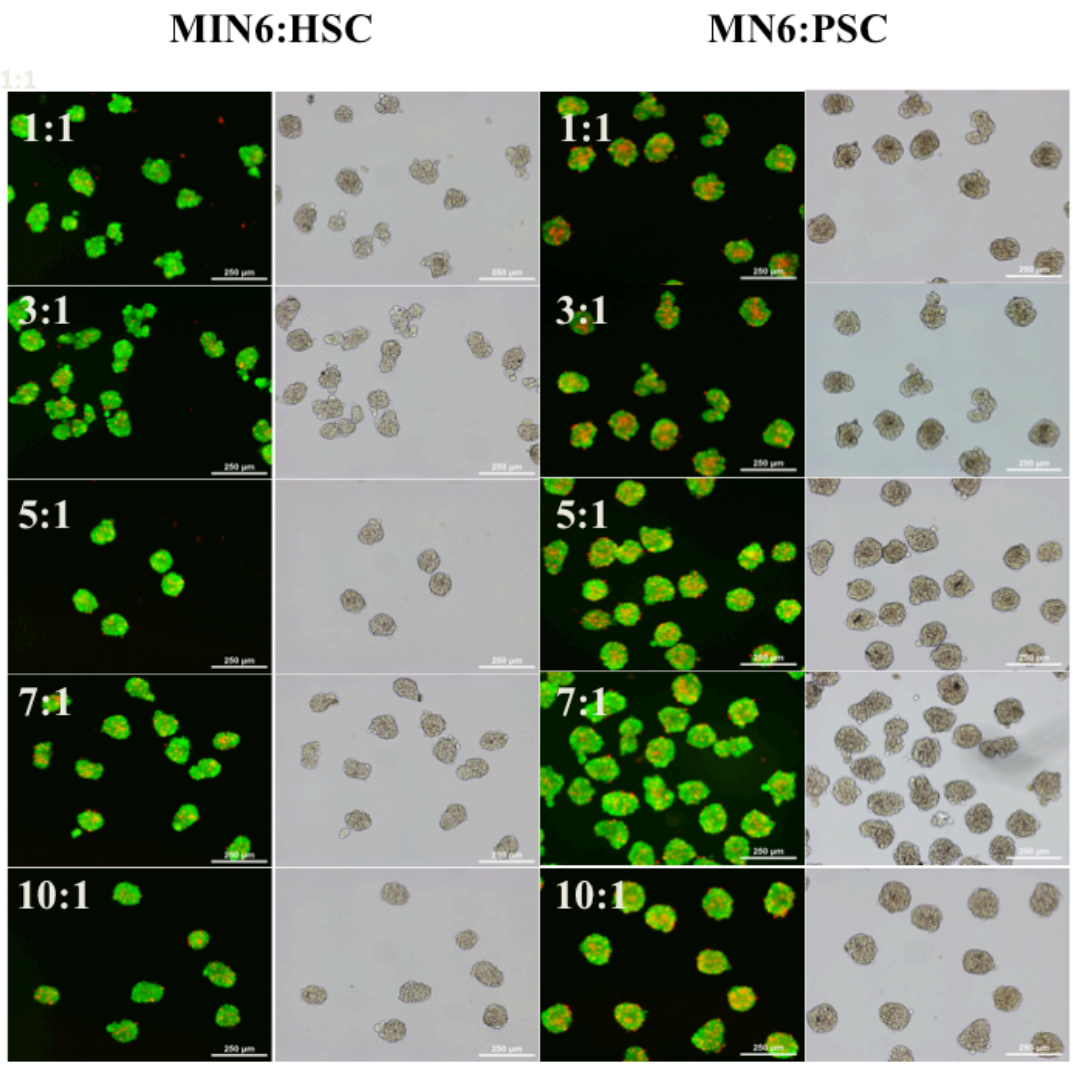

Figure 4. 3. Viability of pseudoislets by FDA-PI staining. Live cells were stained by FDA as green, and dead cells were stained by PI as red and then examined and photographed under fluorescent microscopy. 300 cells per 30 µl as an initial cell number and different cell ratios (1:1, 3:1, 5:1, 7:1, 10:1) MIN6 to SCs were used for spheroid formation. (Scale bar: 250 µm).

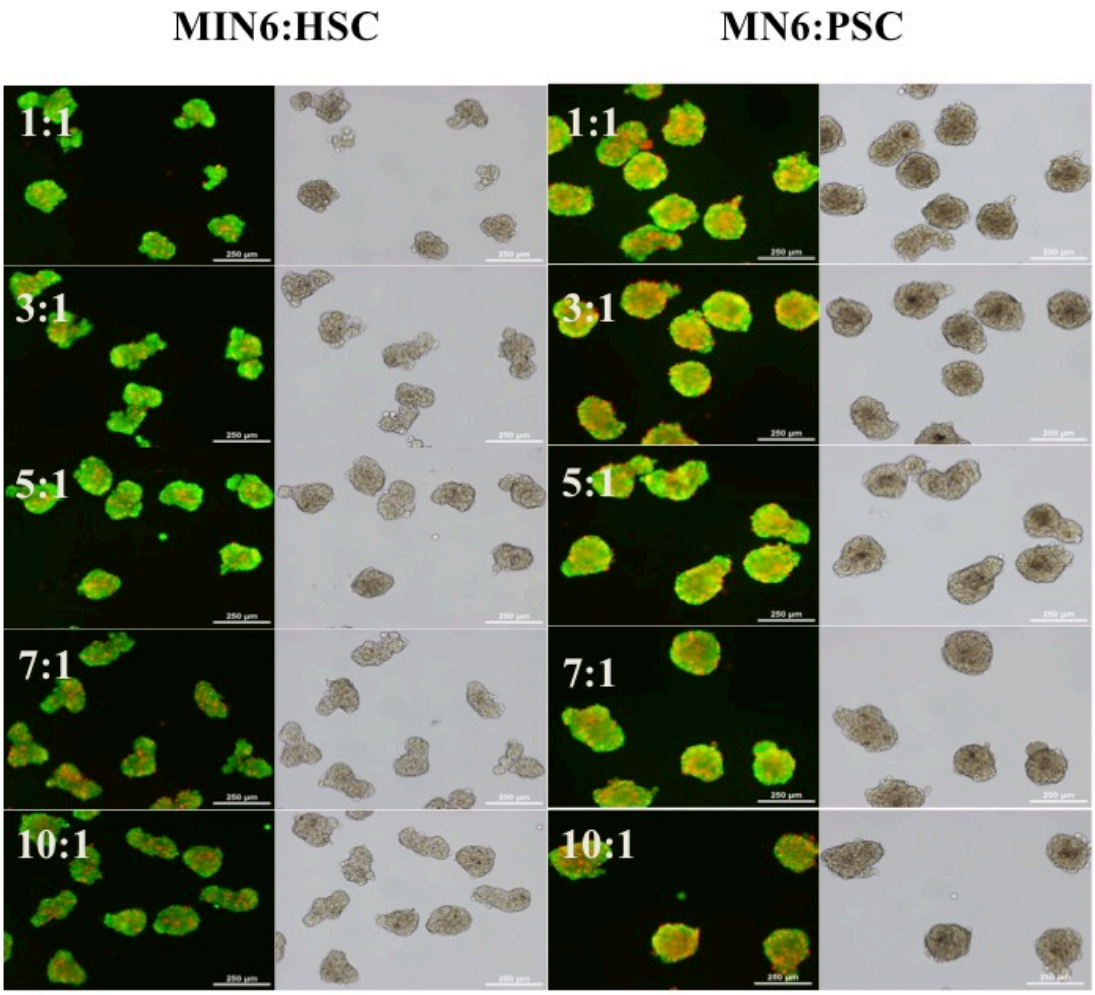

Figure 4. 4. Viability of pseudoislets by FDA-PI staining. Live cells were stained by FDA as green, and dead cells were stained by PI as red and then examined and photographed under fluorescent microscopy. 1000 cells per 30 µl as an initial cell number and different cell ratios (1:1, 3:1, 5:1, 7:1, 10:1) MIN6 to SCs were used for spheroid formation. (Scale bar: 250 µm).

We also studied the comparison of 1:1 and 3:1 ratio of MIN6: SCs ratios to ensure sufficient amounts of SCs required for local immunosuppression. Cell viability in pseudoislets in FDA/PI stained cells was calculated using ImageJ software. (Figure 4.5A and B).

For an initial cell number of 300, we obtained similar viability in MIN6:HSC heterospheroids (92±4% for 1:1 and 91±6% for 3:1) compared to MIN6 homospheroids (92±4%). However, significantly lower viabilities were observed in MIN6:PSC heterospheroids (86±6% for 1:1 and 83±4% for 3:1). Decreased viabilities were observed in larger pseudoislets compared to smaller ones as expected.

For an initial cell number of 1000, decreased viabilities were observed in MIN6:PSC heterospheroids (73±6% for 1:1 and 71±15% for 3:1). Interestingly, slightly higher viabilities were measured with MIN:HSC heterospheroids (87±4% for 1:1 and 3:1) compared to that of MIN6 homospheroids (83±10%). These observations suggested that HSCs slightly promoted viability of pseudoislets, while PSCs negatively affected viability of pseudoislets (Figure 4.5C)

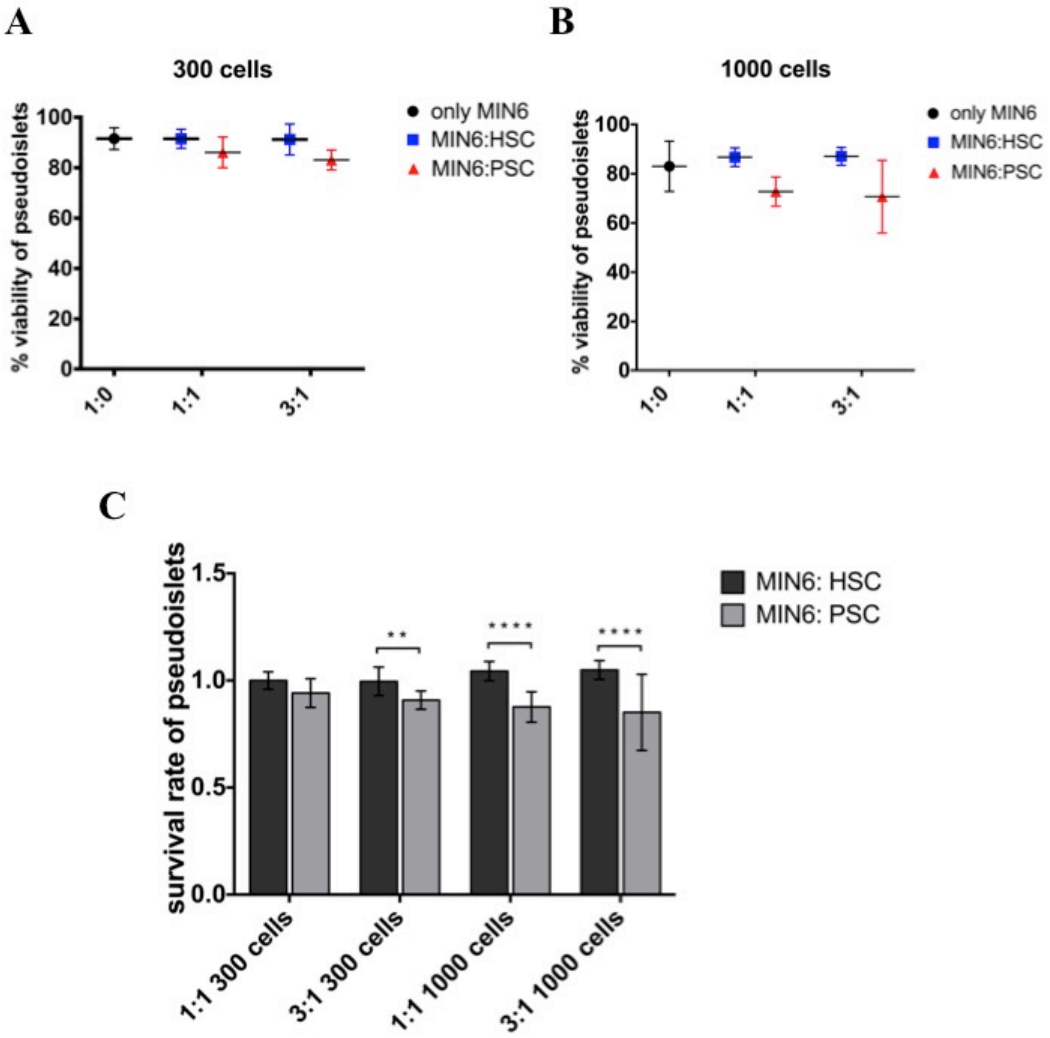

Figure 4. 5. Comparison of pseudoislet viabilities in altered groups. Viability was measured using imageJ program. Initial cell concentration; 300 cells (A) and 1000 cells (B) per 30 µl. Survival rate of pseudoislets were calculates by normalizing viability of heterospheroids to homospheroids. (C) Cell ratios; 1:1 and 3:1 MIN6 to SCs. Mean±SD. ****p<0.0001. ***p<0.001 **p<0.01. *p<0.05.

The sizes of the area and diameter of pseudoislets were also calculated using ImageJ software (Figure 4.6). We obtained significantly larger pseudoislets with higher initial cell numbers as expected. For both initial cell numbers of 300 and 1000, similar morphologies were found in MIN6:HSC heterospheroids and MIN6 homospheroids. Average diameters for 1:0, 1:1 and 3:1 MIN6 to HSC heterospheroid ratios and 300 initial cell number were measured as 109.3 μm, 109.8 μm and 104.4 μm, respectively. We obtained significantly larger heterospheroid diameters for MIN6:PSC compared to that of MIN6:HSC heterospheroids with both initial cell numbers of 300 and 1000. Average diameters measured for MIN6:PSC heterospheroid ratios of 1:1 and 3:1 were 128.2 μm and 123.5 μm, respectively.

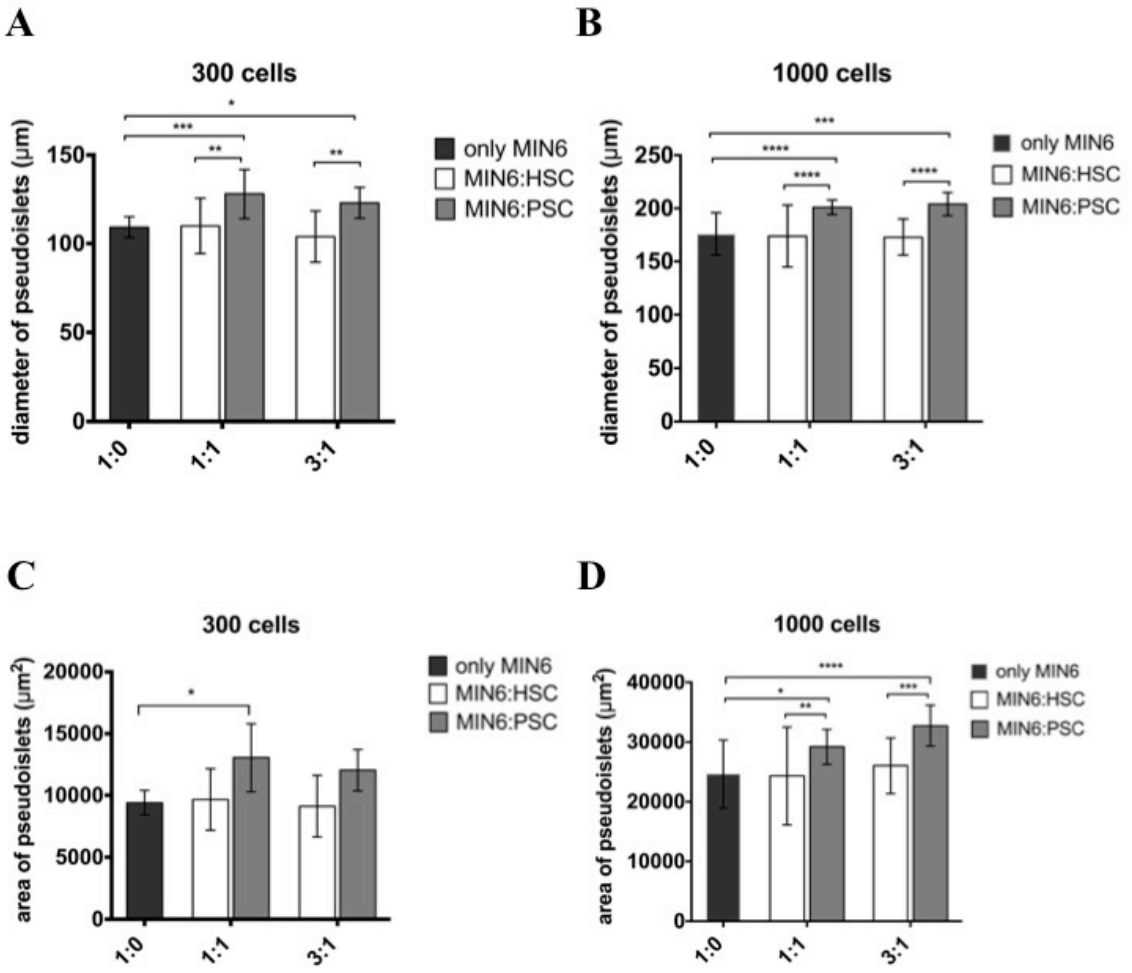

Figure 4. 6. Comparison of pseudoislet sizes in altered groups. Diameters (A-B) and areas (C-D) of spheroids were calculated using imageJ program. Different initial cell concentrations; 300 cells and 1000 cells per 30 µl as an initial cell number and 1:1 and 3:1 MIN6 to SCs ratio were used for heterospheroid formation. Mean±SD.

Effects of different types of SCs (HSC or PSC) on the viability and apoptosis of MIN6 pseudoislets were investigated by measuring intracellular ATP and caspase level. ATP and caspase amounts of heterospheroids were normalized with respect to control group (MIN6 homospheroids). For both spheroid sizes, we observed lower ATP levels in MIN6:HSCs heterospheroids compared to that of MIN6:PSCs heterospheroids (Figure 4.7A). Interestingly, caspase activity was found only slightly higher in MIN6:HSC heterospheroids compared to MIN6:PSC heterospheroids for both initial cell numbers of 300 and 1000. Figure 4.7B indicates normalized caspase activity of heterospheroids to MIN6 homospheroids. When we normalized to caspase activity of MIN6:SC heterospheroids to MIN6 homospheroids, we obtained almost half times increased apoptotic activity in heterospheroids.

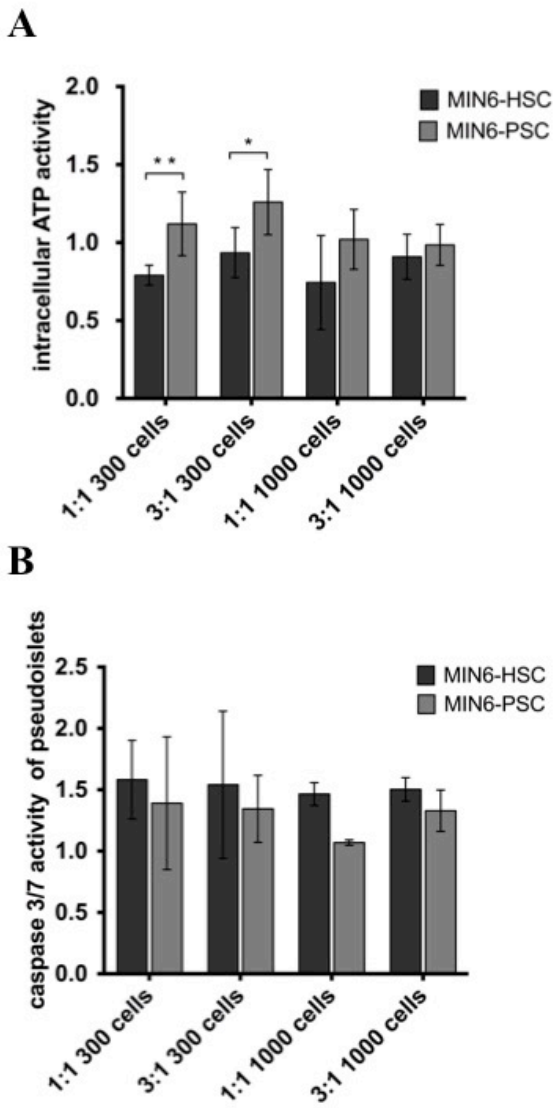

Figure 4. 7. Cellular activity of pseudoislets with different initial cell number and with different type of SCs. For intracellular ATP activity (A) and caspase 3/7 activity (B), 15 islets were picked in 120 µl medium and 60 µl reagent was added on each well. All experiments were performed in triplicate. Both cell survival rate and apoptotic activity in heterospheroids were normalized to homospheroid. **$p<0.01$. *$p<0.05$ statistical significance compared to homospheroid as control. Mean±SD.

4.1.3 Functionality of MIN6:SCs Pseudoislets

Insulin secretion from pseudoislets were analyzed via static incubation of pseudoislets in KRB solution with different glucose concentration. Then stimulation index (SI) was determined by calculating the ratio of insulin secreted by pseudoislet at high glucose buffer to the amount of insulin secreted at low glucose buffer. Stimulation index, which is greater than 1, indicates functional pseudoislets, which can respond to high glucose secreting more insulin compared to insulin secretion at low glucose.

$$stimulation\ index = \frac{insulin\ secretion\ at\ high\ glucose}{insulin\ secretion\ at\ low\ glucose} \quad (4.1)$$

We observed that MIN6:SCs pseudoislets prepared by hanging drop method were functional and could secrete insulin in response to buffers with altered glucose concentrations (Figure 4.8A and B). In general, a slightly higher stimulation index with MIN6:HSC pseudoislets than for MIN6:PSC pseudoislets was measured. We found that pseudoislets prepared with 300 initial cell number in the droplet were more functional and had slightly higher stimulation index values compared to pseudoislets with 1000 initial cells. This result may suggest that increasing cell number in the droplet negatively influences insulin secretion from pseudoislets.

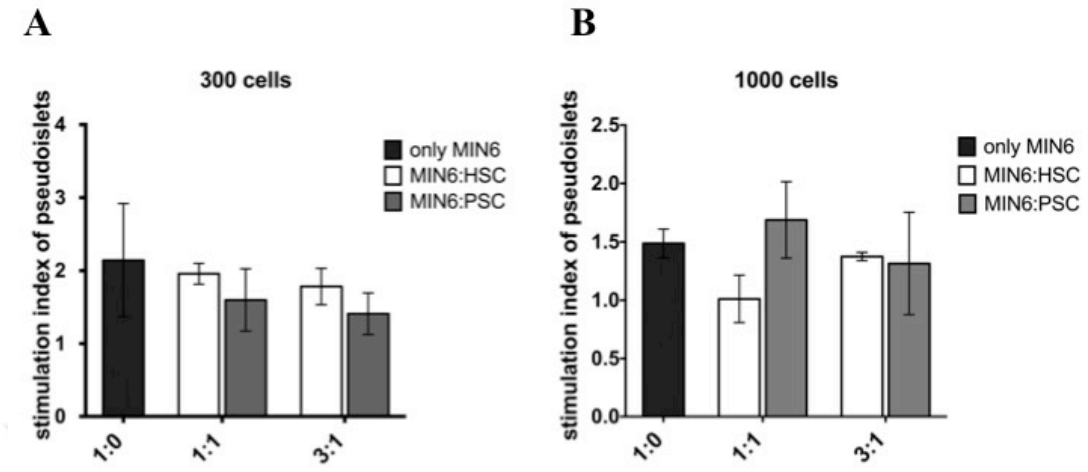

Figure 4. 8. Insulin secretion from MIN6:SCs pseudoislets in response to glucose concentration chance. Pseudoislets were incubated in low glucose (2.8mM) and high glucose (28mM) solutions for 1 h for each. Then, supernatants were collected and analyzed by Insulin ELISA. Stimulation index was calculated as a ratio of insulin secretin in high glucose state to low glucose state in both sizes of pseudoislets; 300 cells (A) and 1000 cells (B). Cell ratios; 1:1 and 3:1 MIN6 to SCs. Mean±SD

4.1.4 Preparation of Pseudoislets containing MIN6 cells transfected HSCs

We managed to observe secretion of CCL22 protein from HSCs via transduction of HSCs by pBABE retroviral vector. We characterized the mRNA CCL22 level by RT-PCR (Figure 4.9A) and protein level from transfected HSCs using human CCL22/MDC Quantikine ELISA kit (Figure 4.9B)

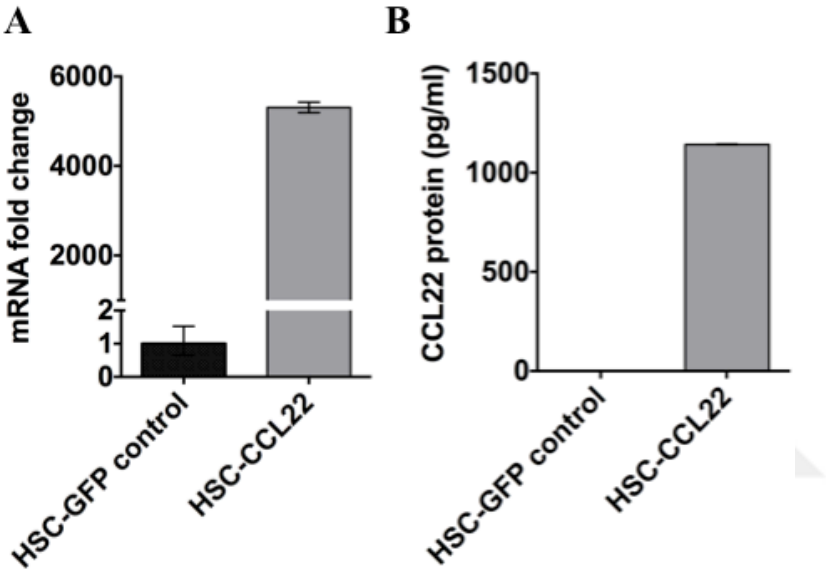

Figure 4. 9. CCL22 expression in transfected HSCs. HSCs were transfected with retroviral pBABE-CCL22 or pBABE-GFP as control. CCL22 mRNA levels in transfected HSCs were analyzed by qPCR and CCL22 expression was normalized to GAPDH (control gene) (n=3)(A). CCL22 protein expression in transfected HSCs was determined by CCL22 ELISA (B).

We obtained more viable and functional pseudoislets with 300 cells as initial cell number and hence continued subsequent experiments with low initial cell number. Viability of cells in pseudoislets was again assessed by FDA/PI staining assay and visualized under a fluorescent microscope (Figure 4.10). We tested the effects of incorporation of transfected stellate cells in the heterospheroid structure on the viability of pseudoislets and calculated percentage viability of pseudoislets using FDA/PI assay. Next, stained cells in heterospheroids were quantified using ImageJ software. We obtained high percentage of green area showing live cells compared to red area indicating dead cells (Figure 4.10).

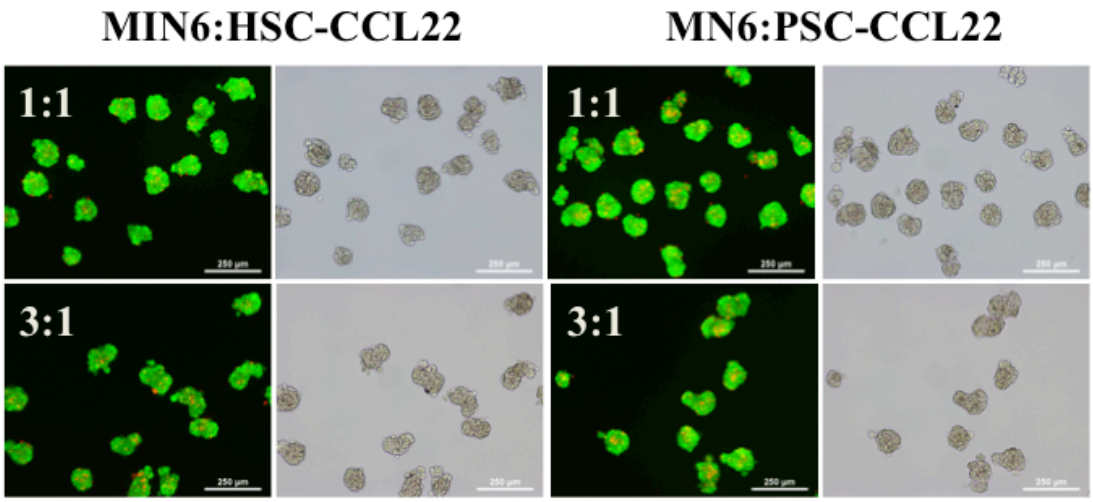

Figure 4. 10. Viability of pseudoislets containing MIN6 cells and transfected SCs by FDA-PI staining. Live cells were stained by FDA as green, and dead cells were stained by PI as red and then examined and photographed under fluorescent microscopy. Initial cell number was 300 cells per 30 ul. Cell ratios; 1:1 and 3:1 MIN6 to SCs. (Scale bar: 250 μm).

We calculated the viability of pseudoislets using imageJ software (Figure 4.11). We obtained similar viabilities with MIN6:HSC heterospheroids where HSCs were transfected for CCL22 secretion. Viabilities were measured as 96±2% for 1:1 and 95±1% for 3:1 heterospheroids, where MIN6 only homospheroids had a viability of 95±2%. However, slightly decreased viability was observed with MIN6:PSC heterospheroids where PSCs were transfected for CCL22 secretion (93±4% for 1:1 and 90±4% for 3:1 ratios).

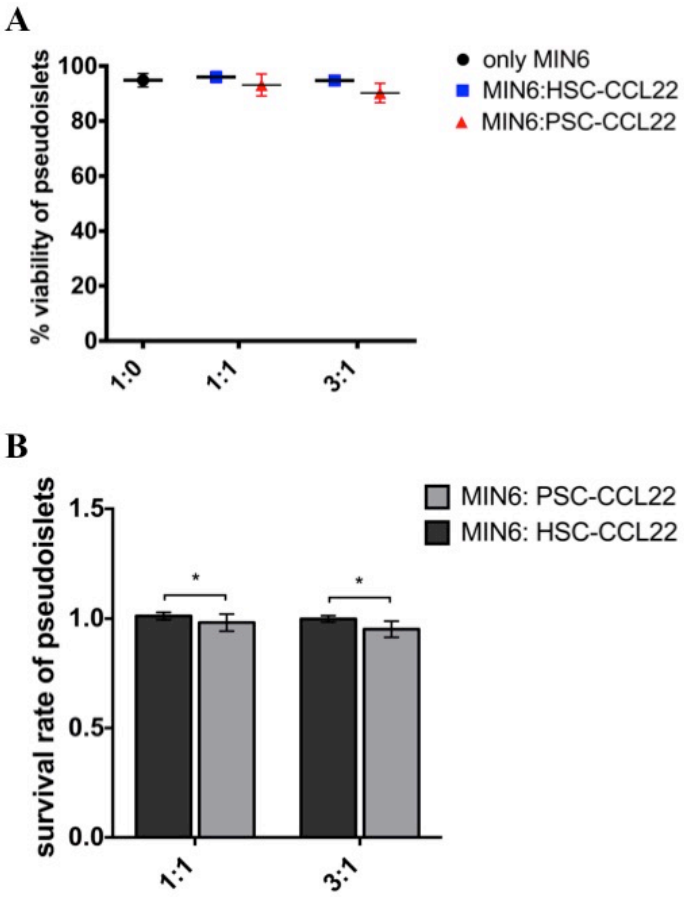

Figure 4. 11. Viability of pseudoislets prepared with MIN6:SC heterospheroids where SCs were transfected for CCL22 secretion. Viability was measured using imageJ program (A). Survival rate of pseudoislets were calculates by normalizing viability of heterospheroids to homospheroids (B). Initial cell number; 300. Cell ratios; 1:1 and 3:1 MIN6 to SCs. Mean±SD. *$p<0.05$.

Next, we investigated morphological changes in transfected pseudoislets compared to MIN6 only control group (Figure 4.12). We observed similar spheroid sizes formed by aggregation of MIN6 cells and transfected SCs same as MIN6 cells and SCs (Figure 4.3). Average of diameters were 110 μm for MIN6 homospheroids, 105 and 111 μm for MIN6:HSC heterospheroids (1:1 and 3:1, respectively), and 109 and 117 μm for MIN6:PSC heterospheroids (1:1 and 3:1, respectively)

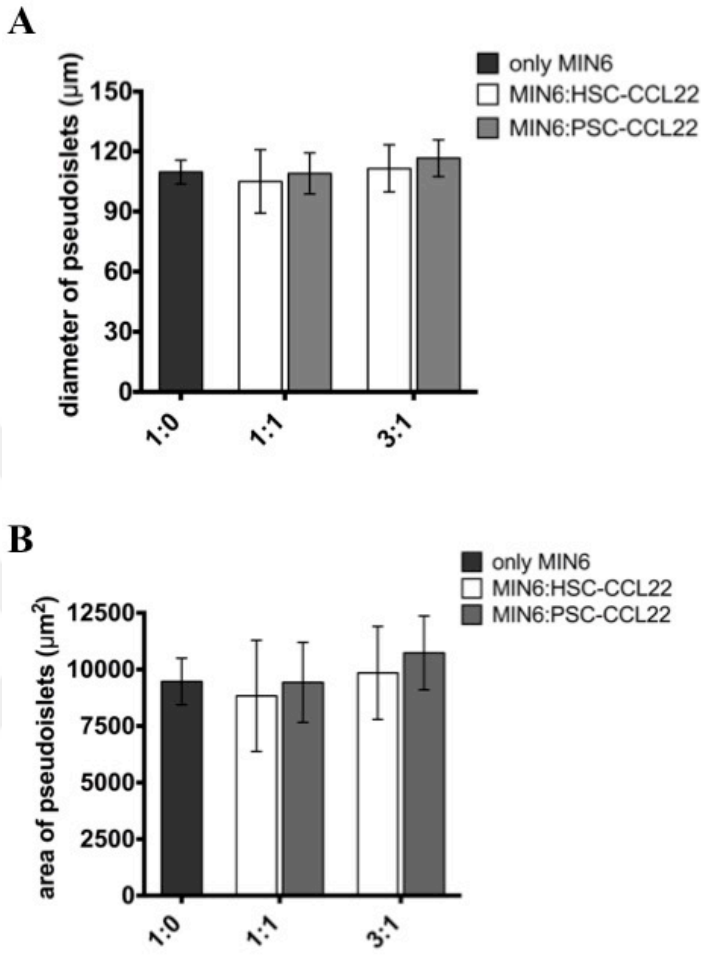

Figure 4. 12. Size of pseudoislets. Diameter (A) and area (B) of spheroids were calculated using imageJ program. Initial cell number; 300. Cell ratio; 1:1 and 3:1 MIN6 to SCs. Mean±SD

Next, we measured intracellular ATP activity as further evidence for cell viability. We normalized ATP activity of MIN6:SCs heterospheroids with respect to MIN6 homospheroids. We obtained increased intracellular ATP levels in heterospheroid group where SCs were transfected with GFP (MIN6:SC-GFP). Intracellular ATP activity for CCL22 transfected SC heterospheroids, either with PSC or with HSC, were similar to the ATP activity measured for MIN6 homospheroids. This suggested that the use of transfected HSCs or PSCs that secrete CCL22 protein did not compromise the viability of heterospheroids. Furthermore, similar effects of HSC and PSC on the viability of heterospheroids prepared with altered initial cell numbers were observed. (Figure 4.13)

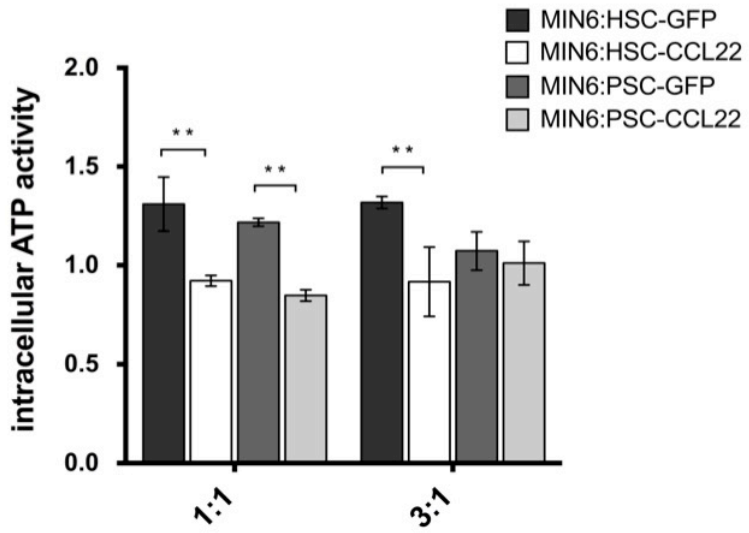

Figure 4. 13. Intracellular ATP activity of MIN6:transfected SC heterospheroids. 15 islets were picked in 120 µl medium and 60 µl reagent was added on each well. All experiments were performed in triplicate. **p<0.01. *p<0.05 statistical significance compared to homospheroid as control. Mean±SD.

4.1.5 Recruitment of Tregs to CCL22 expressing pseudoislets

We obtained more viable and functional pseudoislets with 300 cells as initial cell number. Since we also needed sufficient amount of CCL22 expression by SCs in heterospheroids for Treg recruitment, we selected 1:1 cell ratios (MIN6 to SCs) for the *in vivo* experiments. 400 pseudoislets were implanted into abdominal external oblique muscle of recipient mouse. 10 day after transplantation, tissues from recipient mice were isolated and analyzed using flowcytometer. Percentage of $FoxP3^+$ cells in the $CD4^+$ cell population indicates presence of Tregs in a graft. We found more than 4 fold higher Tregs in MIN6:HSC-CCL22 heterospheroids compared to MIN6 homospheroids. In addition, we found over 2 fold-increase in the recruitment of Tregs with MIN6:SCs-GFP heterospheroids compared to that of MIN6 homospheroids (Figure 4.14). Recruitment of Tregs to the implantation site by only GFP transfected SCs indicated that SCs had also potential to recruit some Tregs towards their vicinity by their nature. Implanted heterospheroids containing MIN6 cells and transfected SC cells secreting CCL22, promoted significant recruitment of Tregs towards the implantation site (Figure 4.14 and Figure 4.15).

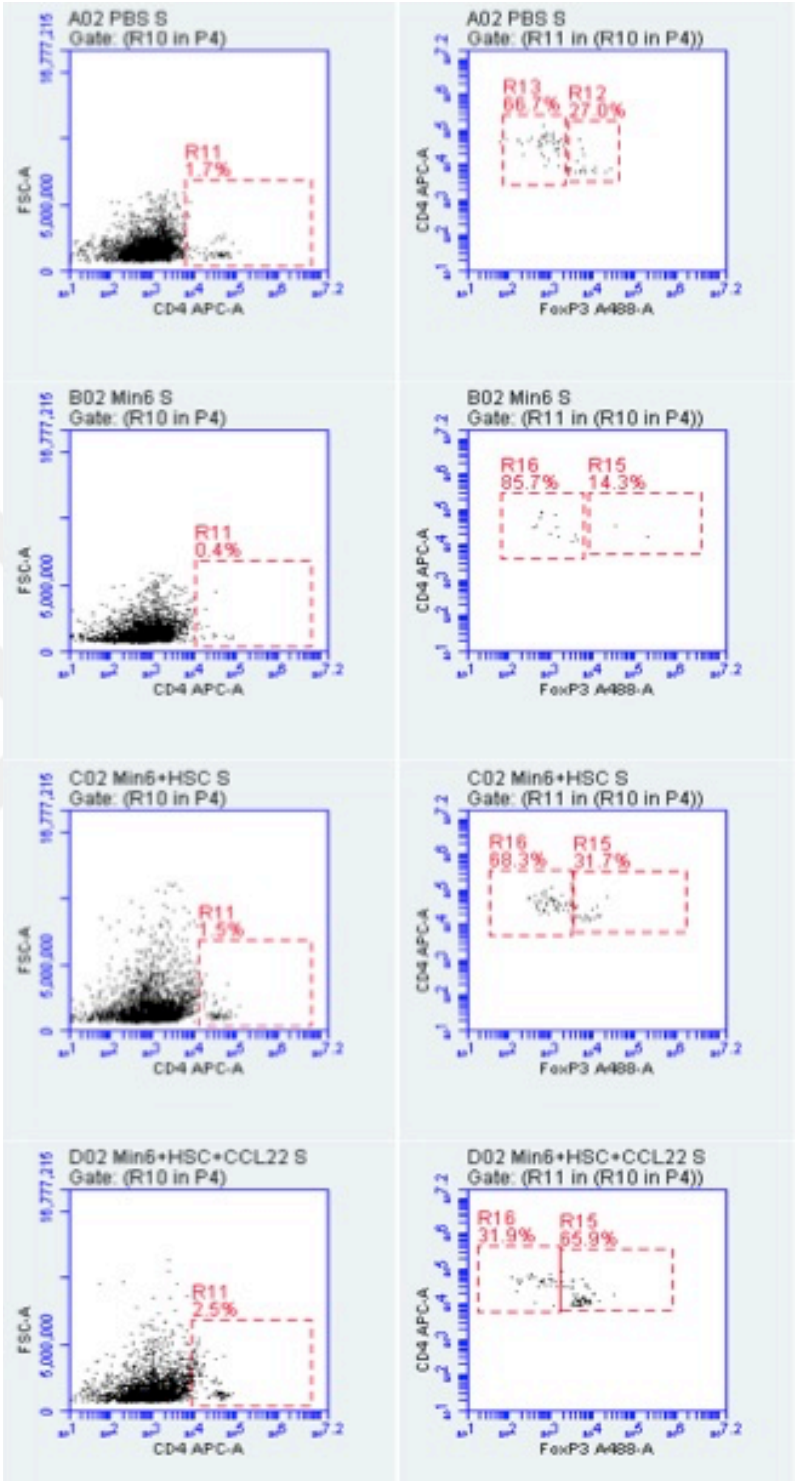

Figure 4. 14. Recruitment of Tregs *in vivo*. Pseudoislets (~400) were implanted into abdominal external oblique muscle of male $CD1^{+}$ mouse. Tissue was retrieved 10 days post transplantation and recruitments of Tregs were proved by flow cytometer for CD4+CD25+FoxP3+ cells.

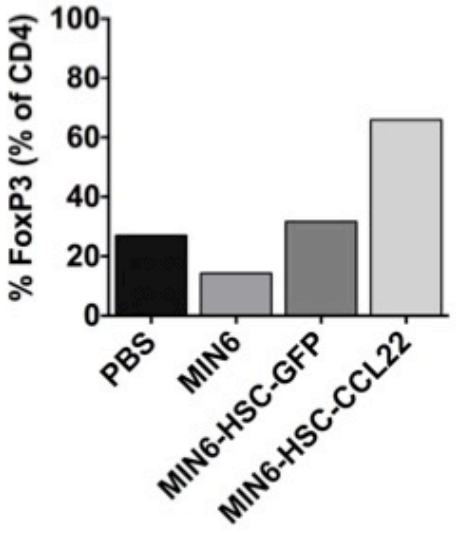

Figure 4. 15. Recruitment of Tregs *in vivo*. Percentage of FoxP3^{+} cells in the CD4+ cell population indicates presence of Tregs in graft.

4.2 Discussion

Type I diabetes is an organ specific disease caused by autoimmune destruction of insulin secreting pancreatic β cell. Although transplantation of pancreatic islet is a promising approach for treatment of type I diabetes, it is limited by factors such as lack of suitable donors and usage of systemic immunosuppressive drugs to prevent graft rejection[58]. Therefore, there is an increasing unmet need for transplantable islets, which are mostly isolated from brain-dead patients [60-61]. However, viability and functionality of these pancreatic islets are unfavorably affected by brain death [62]. Thus, researches have focused on creating pseudoislets that mimic naïve islets for treatment of type 1 diabetes. In this study, one of our aims was to address challenges of acquiring high islet numbers and repeated islet isolation by forming pseudoislets through aggregation of MIN6 cells.

HSCs can regulate immune system through expanding Tregs and inhibiting T cell responses [23-24, 112]. It has been also showed that cotransplantation of islets with HSCs have protected islet grafts from rejection by forming immune barrier such as inhibition of T cell or recruiting Tregs[25, 113]. However, there are a few studies showing effects PSCs on pancreatic β cells. According to previous studies, co-culture of PSCs with mouse islet or RIN5F rat pancreatic β cells impairs their function by reducing insulin expression and promoting apoptosis [31-32]. In this study, for the first time, we have prepared pseudoislets comprising of SCs and MIN6 cells as an alternative model system. We focused on the immunomodulatory potential of both transfected PSCs and HSCs for creation of local immune-privileged environment and to avoid usage of immunosuppressive drugs for transplantation of islets. Hence, we have chosen 1:1 and 3:1 MIN6 to SC cell ratio to

ensure sufficient amount of CCL22 secretion by transfected SCs and to obtain an immunologically privileged microenvironment. We compared effects of both HSCs and PSCs on the viability and functionality of MIN6 cells in spheroids. We have showed that smaller pseudoislets had higher viability than larger pseudoislets in all cell ratios (Figure 4.5). Viability of MIN6 homospheroids was 92±4% with 300 cells as initial cell number, whereas it decreased to 83±10% with 1000 cells as initial cell number. As shown previously, small islets were favorable for successful transplantation due to minimized hypoxia whereas larger islets suffered from poor nutrient transport [114-115]. For this reason, we suggested suitable initial cell number as 300 cells for heterospheroid formation and continued further experiments with small islets.

In addition, we have showed that PSCs reduced viability of pseudoislets whereas HSCs slightly increased (Figure 4.5C). With 300 initial cells, we obtained viability as 92±4% for 1:1 and 91±6% for 3:1 (MIN6 to HSC ratio); however, it was calculated as 86±6% for 1:1 and 83±4% for 3:1 (MIN6 to PSC ratio). On the contrary, we found significantly higher intracellular ATP level in MIN6:PSCs heterospheroids (Figure 4.7A). Intracellular ATP activity indicates metabolic activity and hence the presence of viable cells; however, we obtained larger pseudoislets with PSCs compared to HSCs. Thus, this large size of heterospheroids was the reason for high ATP level. In addition, we found increased apoptotic activity in all heterospheroids compared to homospheroids (Figure 4.7B). Previous studies demonstrated that PSCs would compromise insulin secretion and viability of islets. [31-32]. In the present study, we did not observe significant reduction of insulin secretion from heterospheroids (MIN6:HSCs and MIN6:PSCs)

compared to homospheroids although there were slight decreases in stimulation index of MIN6:PSC heterospheroids (Figure 4.8). We concluded that MIN6:HSCs heterospheroids are morphologically more similar to MIN6 homospheroids and they are more viable and functional than MIN6:PSCs heterospheroids.

Secretion of CCL22 from cancer cells enable them to escape from immune system. Recently, CCL22-based recruitment of Tregs to islet graft site has been considered to protect β cells from autoimmune attack and long-term protection of islet allografts from rejection was achieved by implantation of CCL22 plasmid *in vivo*. This strategy prevented autoimmune disease through recruiting Tregs and other immune cells like iNKT cells and pDCs to graft site as CCR4 receptor involved in Treg migration are found also on the membrane of other immune cell subsets, which probably modulate local autoimmunity[16-18]. In the present study, SCs were transfected by retroviral vector to express CCL22 protein and we tested effects of transfected SCs and secretion of CCL22 on viability of heterospheroids. We obtained similar effects of transfected SCs on morphology and viability of heterospheroids compared to untransfected SCs (Figure 4.5, 4.6, 4.11, and 4.12). Average viability of MIN6:HSC-CCL22 heterospheroids was found slightly higher than MIN6:PSC-CCL22 heterospheroids (MIN:HSC-CCL22; 96±2% for 1:1 and 95±1% for 3:1 and MIN6:PSC-CCL22; 93±4% for 1:1 and 90±4% for 3:1). Viability of MIN6 homospheroids was calculated as 95±2% which is similar to MIN6:HSC-CCL22. Moreover, we found similar intracellular ATP level in all groups (Figure 4.13).

Consequently, we indicated that transfection of SCs and CCL22 secretion from pseudoislets did not adversely affect morphology and viability of heterospheroids. In addition, we showed HSCs are more promising compared to PSC for heterospheroid formation. Thus, we formed MIN6:HSC heterospheroids and implanted 400 pseudoislets to external abdominal oblique muscle of $CD1^+$ mouse to test our model system in vivo and prove recruitment of Tregs to graft site. We showed increased number of Tregs with the presence of SCs in pseudoislets and even higher percentage of Tregs with the expression of CCL22 from SCs (Figure 4.14.) We obtained lower Tregs with homospheroids to control ones, which were only PBS implanted. Other $CD4^+$ cells could have attacked to MIN6 pseudoislets; thus, it shows that our pseudoislet model expressing CCL22 was able to minimize immune attack after transplantation.

Although harnessing of CCL22 chemokine or co-culture of pancreatic islets with SCs for islet transplantation have been reported, as far as our knowledge, this is the first study that integrates CCL22 to the heterospheroids comprising MIN6 cells and transfected SCs, which express CCL22 protein to provide graft tolerance. Here we combined two novel immunomodulation approaches (usage of SCs and CCL22 chemokine) into one promising model system. With this system, we aimed to overcome not only requirement of immunosuppressive drugs prevent graft rejection but also repeated islet isolation and donor limitation. In addition, manipulating immune cells trafficking by CCL22 gene therapy and harnessing SCs may be a novel therapeutic approach for not only diabetes but also for other type of autoimmune diseases and organ/tissue transplantation.

For further experiments, implantation of these pseudoislets into a diabetic mouse model can be tested to show immunomodulation effects and promotion of vascularization. Activation and migration of other immune cells to implantation site can be investigated in a diabetic mouse model. Thus, prevention of graft rejection can be achieved with this system.

Chapter 5

Conclusion

In summary, we have tested effects of different types of stellate cells on viability and functionality of MIN6 cells when placed together in heterospheroid. We designed a local-immune privileged environment to prevent graft rejection, recruiting Tregs to graft site. Stellate cells were transfected to express a chemokine, CCL22, which can recruit Tregs. This chemokine is also secreted from cancer cells to evade immune destruction therefore attracted us to utilize it in our system. Viable and functional pseudoislets prepared with MIN6 and stellate cells were formed using the hanging drop method. We have observed that transduction of HSCs to express CCL22 did not have a detrimental effect on the viability and functionality of pseudoislets and also we showed that our model system enhance recruitment of Tregs to the implantation site. Stellate cells have also excellent angiogenesis promotion capabilities thereby making this strategy even more promising for clinical translation to provide a way to transplant islets without the use of immunosuppressive drugs. Moreover, manipulating the migration of Tregs and solving the vascularization issues may also be a novel therapeutic approach for other autoimmune diseases and tissue/organ transplantation.

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