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RNA Interference for improving the Outcome of Islet Transplantation

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Abstract

Islet transplantation has the potential to cure type 1 diabetes. Despite recent therapeutic success, it is still not common because a large number of transplanted islets get damaged by multiple challenges including instant blood mediated inflammatory reaction, hypoxia/reperfusion injury, inflammatory cytokines, and immune rejection. RNA interference (RNAi) is an novel strategy to selectively degrade target mRNA. The use of RNAi technologies to downregulate the expression of harmful genes has the potential to improve the outcome of islet transplantation. The aim of this review is to gain a thorough understanding of biological obstacles to islet transplantation and discuss how to overcome these barriers using different RNAi technologies. This eventually will help improve islet survival and function post transplantation. Chemically synthesized small interfering RNA (siRNA), vector based short hairpin RNA (shRNA), and their critical design elements (such as sequences, promoters, backbone) are discussed. The application of combinatorial RNAi in islet transplantation is also discussed. Last but not the least, several delivery strategies for enhanced gene silencing are discussed, including chemical modification of siRNA, complex formation, bioconjugation, and viral vectors.

Keywords

RNA interference; Islet transplantation; siRNA; shRNA

I. Introduction

The first attempt to transplant pancreatic tissue was performed 29 years before the clinical introduction of insulin, when a young boy dying of diabetic ketoacidosis had three small pieces of sheep's pancreas implanted beneath his skin [1]. But the patient died after 3 days. In 1966, the first pancreatic allotransplantation was performed at the University of Minnesota [2]. Since then, more than 30,000 pancreas transplantations were carried out worldwide. In 2008, approximately 1300 patients were transplanted with pancreas in the United States with the majority of them being simultaneous pancreas-kidney transplantations [3]. Although there are some beneficial effects, pancreas transplantation

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remains an invasive procedure with significant mortality and morbidity [4]. Pancreas transplantation is also limited by the death of organs, and the need for non-specific immune suppression.

Insulin-producing β cells in the islets of Langerhans are the target of autoimmune aggression in type 1 diabetes, and non- β cells and the exocrine pancreas is unaffected. The islets constitute only a tiny fraction (1%) of the whole pancreas and it is functionally unnecessary to transplant the whole pancreas when only its endocrine tissue is required. Islet transplantation provides a less invasive alternative approach for the treatment of type 1 diabetes with reduced antigen load, relative simplicity, and low morbidity. However, since the initiation of clinical islet transplantation from early 1970s, most of these trials were failed, until the breakthrough at the University of Alberta in Edmonton, Canada. The “Edmonton protocol” was published in 2000, reporting that seven patients with type I diabetes became insulin independent after receiving islet transplantation with a prednisone-free protocol [5].

The Edmonton protocol has been replicated and further modified worldwide with many successes. In 2001, the National Center for Research Resources (NCRR) of the National Institutes of Health (NIH) established ten islet resource centers with the mission of (1) providing high quality human islets for both clinical and basic research use; (2) optimizing processes for islet isolation, purification, and storage; and (3) developing the methods for characterization of islets (<http://icr.coh.org>). In the same year, the Immune Tolerance Network (ITN) with the joint help from the Juvenile Diabetes Research Foundation (JDRF) and NIH began a trial at nine centers worldwide to perform transplants in 40 subjects to determine if the Edmonton results could be reproduced on a broad scale. The ITN aims at developing a core group of excellent clinical islet transplant centers for future testing of tolerance-based trials (<http://www.immunetolerance.org>). From 1999 to 2008, around 400 patients received allogeneic islet transplantation [6,7].

Despite these successes, only less than 10% of the recipients remain insulin independent for up to 5 years, and most recipients return to insulin because the islet function decreased over time [8]. After transplantation, Islet grafts face multiple challenges including innate and adaptive immune rejection, toxicity associated with immune suppressive agents, and insufficient islet revascularization [9,10]. Therefore, islets isolated from two to four donors are needed for single patient islet transplantation [11]. At present, even with expansion in the currently available organ donor pool, islet transplantation will only benefit about 0.5% of potential recipients [12].

Ex vivo genetic modification of islets before transplantation has the potential to overcome several problems associated with islet transplantation [10,11,13–17]. Growth factor gene expression has the potential to promote islet revascularization; while anti-apoptotic gene expression can reduce inflammation and immune rejection; and prevent islet cell apoptosis. This strategy can help to reduce the number of islets needed for each recipient and prolong the time that a recipient can maintain insulin independence after transplantation. The genetic modification of islets could be the over-expression of protective genes or inhibition of harmful gene expression [18]. Antisense oligonucleotides (ODNs), ribozymes, DNazymes, and RNA interference (RNAi) are major approaches which are currently being used to sequence specifically reduce or inhibit gene expression. Among these approaches, RNA interference (RNAi) is relatively new and evolutionally conserved biologic process that regulates gene expression using small interfering RNA (siRNA) mediated sequence specific, post-transcriptional gene silencing [19,20]. Since the discovery of RNAi by Fire and Milo in 1998, it has been widely used as a tool for basic research as well as a potential therapeutics [21–24]. The application of RNAi and other approaches have been extensively reviewed

[25]. siRNA molecules can recycle between different copies of mRNA, while antisense ODN can only block the translation of one mRNA before its degradation [26]. Therefore, siRNA is more effective than antisense ODN in reducing target gene expression [27]. Miyagishi *et al.* has demonstrated that the IC₅₀ for siRNA was about 100-fold lower than that of the antisense [28]. Similarly, Bertrand *et al.* has reported that siRNAs are more efficient in silencing target gene and its effect is lasting longer. In addition, RNAi could also be achieved through the delivery of shRNA, which is expressed endogenously and has longer gene silencing effect [27].

In this review, we will discuss the biological obstacles to islet transplantation and potential RNAi targets; principles and design element for RNAi; and delivery approaches.

II. Obstacles to islet transplantation

Since the success of Edmonton protocol, significant progress has been made in human islet transplantation. However, this protocol is still not widely used mainly due to the fact that islets from several donors are needed for a single recipient, since majority of the islets are damaged prior and post transplantation. The destruction of transplanted islets is a complex problem with many facets including enzyme and mechanical damages induced by the isolation process, hypoxia, inflammatory reaction, and immune rejection. Since many of these factors are inter-related, a thorough understanding of these molecular processes will help us to develop more effective therapeutic interventions.

A. Instant blood-mediated inflammatory reaction

Due to non-immunological and innate immunological factors, 50%–70% islets lose their viability immediately after transplantation by instant blood-mediated inflammatory reaction [29–31]. This phenomenon is also called “early graft loss” or “primary nonfunction.” The pathophysiology of blood-mediated inflammatory reaction involves platelet binding and activation, coagulation, complement activation, and infiltration of neutrophils, monocytes, and macrophages (Figure 1) [29]. Activation of coagulation could be triggered through both the intrinsic pathway by islet surface collagen residues and the extrinsic pathway by tissue factors or monocyte chemoattractive protein-1 (MCP-1) [32,33]. Complement is possibly activated through the alternative pathway [34,35]. Infiltration of these immune cells could be observed within 1 h after coagulation and complement activation [36]. There are multiple interactions between coagulation, complement, and inflammatory cells, which are responsible for the infiltration of inflammatory cells. Neutrophils and monocytes interact with activated platelets through P-selectin over-expressed on platelets [37]. Macrophages could directly be activated by tissue factor, fibrin, or fibrinogen [38]. Soluble C3a and C5a produced through complement activation are potent chemoattractants for neutrophils and macrophages [34]. The effects of blood-mediated inflammatory reaction are multiple. Firstly, both monocytes and macrophages are major phagocytes leading to the destruction of islet cells. Secondly, proinflammatory cytokines lead to islet cell apoptosis and necrosis. Last but not the least, the infiltrating neutrophils and macrophages will eventually induce subsequent adaptive immune response [39].

B. Inflammatory cytokine induced islet cell death

Mechanical and enzymatic stresses during the islet isolation, purification and transfection process result in the activation of resident islet macrophages, which release several pro-inflammatory cytokines including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ). In addition, infiltrating host immune cells (monocytes, neutrophils) also produce inflammatory cytokines [40]. These proinflammatory cytokines are responsible for the non-specific inflammation and the damage to transplanted islets during the early

stage of transplantation. The exact source and timing of proinflammatory cytokine production still remains elusive and greatly depends on the islet isolation, purification and transplantation protocols. For an example, Kupffer cells produce inflammatory cytokines, NO, and reactive oxygen species (ROS), which are toxic to intraportally transplanted Islets [41–43]. The release of cytokines by islet resident macrophages is known to occur during *in vitro* culture prior to transplantation [44]. The levels of IL-1 β and TNF- α increase soon after transplantation, and maximum level was reached at 3 h for IL-1 β and at 6 h for TNF- α . Depletion of kupffer cells significantly reduced the levels of IL-1 β and TNF- α , indicating the role of macrophage in producing inflammatory cytokines [45]. Another study also showed that IL-1 β mRNA was detectable in islet immediately after isolation and was increased after transplantation. IL-1 β mRNA increased by 9 fold on day 1, by 7 fold on day 3 after transplantation, and decreased to baseline level on day 7 [46]. It has also been reported that the expression of proinflammatory cytokines (including IL-1 β and TNF- α) were observed at 8 h after transplantation and declined after 24 h. In the same study, however, IFN- γ was not observed until 48 h post transplantation [47]. Ozasa *et al.* reported that IFN- γ mRNA was observed in allogeneic transplantation at 1, 3, 5, and 7 days after transplantation with a peak at day 5, however, it was almost absent in syngeneic transplantation[48]. Although the exact source and level of proinflammatory cytokines production varies among different transplantation protocols used, some common intracellular signal pathways are activated. These cytokines activate JNK/p38, NF- κ B, STAT-1, up-regulate the expression of several significant genes, and finally result in β cell death (Figure 2) [36–37].

IL-1 β —The binding of IL-1 β to its receptor IL-1R1 leads to the docking of the IL-1 receptor accessory protein (IL-1AcP) and followed by the recruitment of IL-1R1 activated kinase (IRAK) through an adaptor protein named myeloid differentiation factor 88 (MyD88). Recruitment of IRAK leads to the activation of TNF-receptor-associated factor-6 (TRAF6), resulting in the phosphorylation and degradation of I κ B. NF- κ B is then released from inhibitory I κ B, translocates into the nucleus, and induces the expression of multiple genes, including IL-1, IL-6, TNF- α , and iNOS [49,50].

IFN- γ —IFN- γ binds to IFN γ receptor 1 (IFN γ R1), which recruits IFN γ receptor 2 (IFN γ R2). IFN γ R1 and IFN γ R2 are associated with Janus tyrosine kinase 1 and 2 (JAK1/2) and results in the activation of JAK1 and JAK2. Then signal transducers and activators of transcription 1 (STAT1) is activated by JAK2 and then translocates to the nucleus, where it binds to the regulatory regions of different genes [51]. In addition, the ischemia induced STAT1 also regulates caspase gene expression through the activation of transcription factor IRF-1 [52].

TNF- α —Upon binding of TNF- α , TNF receptors form trimers and undergo conformational change, leading to the exposure of intracellular death domain. The death domain interacts with TNF- α associated death domain (TRADD), which serves as an adaptor to initiate three signal pathways, including the activation of NF- κ B, activation of MAPK pathway, and induction of apoptosis [53–55]. TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP) are associated with TRADD to recruit and activate kinase IKK, which in turn releases NF- κ B by degradation of I κ B [53]. TRAF2 also activates MAPK pathways and leads to the protein kinases such as c-jun N-terminal kinase (JNK) and p38 [56]. Apoptosis are activated through both MAPK pathway (direct activation of caspase 3) or FADD mediated activation of caspase 8, which in turn leads to the activation of effector caspase, such as caspase 3 [55].

Under *in vitro* conditions, IL-1 β induces functional impairment in the mouse and rat β cells, and prolonged exposure (6–9 days) to IL-1 β in combination with IFN- γ and TNF- α leads to

human, mouse, and rat β cell death [50]. The fate of β cells after exposure to inflammatory cytokines depends on the type of cytokines, time course and severity of perturbation to the key β cell gene networks [50,57]. Inflammatory cytokines upregulate or downregulate around 200 genes, which are either protective or deleterious to β cell survival and function [57,58]. These genes are related to metabolism, signal transduction, and transcription factors, suggesting that β cells are making attempt to adapt to the effect of cytokine exposure [57]. Several of these genes are putative targets of transcription factor NF- γ B [59]. Transcriptional activity of NF- γ B is related to the expression of pro-apoptotic genes in β cells [60]. The activation of NF- γ B is a necessary step for inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production [59]. The cytotoxic effect of cytokines is mediated through the production of radical NO as well as other free radicals, such as peroxynitrite and superoxide [46,61]. Increased iNOS gene expression leads to an early inflammatory process in islet transplantation, suggesting iNOS is an important mediator of graft inflammation and islet damage in the early stage of islet transplantation. Inflammatory cytokines initiate β cell apoptosis mainly through the extrinsic pathway and function through several molecules in the death receptor pathway, such as Fas (CD95), Fas-associated death domain (FADD), and the death-inducing signaling complex (DISC), leading to the activation and release of caspase 8, which in turn activate caspase 3 [62–64].

C. Hypoxia and ischemic reperfusion injury

In an intact pancreas more than 15% of the total blood flow goes to islets, which accounts for only 1% of the pancreatic tissue [65]. During isolation and purification process, the capillary network for blood supply is destroyed; therefore, oxygen and nutrients are solely supplied through diffusion. The oxygen tension in portal vein (where the islets are transplanted) is 8–10 mmHg, which is much lower than that in the intact pancreas ($PO_2 = 40$ mmHg) [66,67]. Although the angiogenesis initiates soon after transplantation, revascularization of islets may take up to 10–24 days [68]. Therefore, newly transplanted islets are under persistent hypoxic conditions prior to and post transplantation, until revascularization process is completed. The reperfusion following prolonged ischemia causes injury, commonly known as ischemia/reperfusion (IR) injury. For an example, IR injury in the liver activates Kupffer cells, which will produce inflammatory cytokines, ROS and NO, resulting in islet cell apoptosis [69]. Hypoxia induces the intrinsic pathway of apoptosis through the disruption of the mitochondrial integrity, induction of cytochrome C release, and the activation of caspase 9 and 3 [62–64]. Nutrient deprivation can also induce islet apoptosis through the intrinsic pathway [62].

D. Autoimmune recurrence and allogeneic immune rejection

In addition to hyperacute rejection, acute rejection, and chronic rejection experienced during the transplantation of other organs such as heart, lung, liver, and kidney, transplanted islets are under additional attacks due to autoimmune rejection against β cells in type I diabetic patients. In the following paragraph, we will briefly summarize the immunology of islet transplantation (Figure 3).

1. Autoimmune recurrence—Type I diabetes is caused by autoimmune mediated damage of islet β cells. It is characterized by the presence of β cell reactive T-cells, pro-inflammatory cytokines, and other molecules. Transplanted islets are also attacked by the same stresses that destroy host β cells. Among them, CD8+ T cells are one of the major players in destroying β cells. The infiltration of CD8+ T cells is observed in the pancreas of type 1 diabetic patients as well as transplanted pancreas [70,71]. In addition, activated CD4+ T cells are also involved in the killing of β cells. Reduced insulinitis is observed in the absence of CD4+ T cells [72]. The activation of CD8+ T cells is dependent on CD4+ T cells. CD8+ T cells kill target cells through direct contact and mediated by perforin and granzyme B.

Perforin can disturb target cell membrane integrity and facilitate the release of granzyme B into the cytoplasm of target cells, whereas granzyme B cleaves pro-apoptotic molecule Bid. Activated Bid sequesters anti-apoptotic Bcl-2 molecules and triggers β cell apoptosis through the intrinsic apoptotic pathway [73–75]. Fas is also upregulated in pancreatic β cells of type 1 diabetic patients and causes β cell apoptosis through FasL-Fas interaction [76,77].

2. Allogeneic immune rejection—Successful transplantation of allogeneic islets is difficult, because transplanted islets are recognized not only by the host immune system, but the adaptive immune rejections are also activated [78]. Failure of allogeneic islet transplantation is correlated with increased T cell reactivity or alloantibody titers [79,80]. Antigen presenting cells (APCs) process and present peptide fragments of various surface, secreted and shed protein from the donor. APCs from both donor and host (including dendritic cells, macrophages, passenger leukocytes, and mononuclear cells) are involved in the antigen presentation. After maturation, APCs provide three signals to fully activate T cells: signal 1, recognition of major histocompatibility complex (MHC) and the bound peptide by the T-cell receptor on the host T cells; signal 2, interaction of co-stimulation signals (such as CD40, CD80/86) with their corresponding receptors on host T cells; signal 3, soluble cytokines (IL-12, IL-10, IL-2) further stimulate the proliferation and differentiation of T cells [81,82]. All of these three signals are required; otherwise, it will result in the apoptosis, anergy, and differentiation of T cells. In addition, anti-donor antibodies are also developed in most allograft recipients after immunosuppressive medication is discontinued. The presence of alloantibodies usually lead to the graft failure of islets from donors with the recognized human leukocyte antigen (HLA) alldeterminants [79].

III. Potential targets for gene silencing

As a potent approach for inhibiting aberrant protein expression, RNAi can theoretically be used to silence any gene involved in the biological processes that lead to the damage of islets. However, due to the complexity of pathophysiology of islet transplantation, only some key mediators are potential targets for RNAi-based intervention. A wide variety of potential therapeutic targets are investigated in the past, which are discussed in the following paragraphs (Table 1).

A. Modulation of immune rejection

Immunosuppressive agents mediated non-specific immunosuppression is commonly used to reduce the immune rejection in islet transplantation, however, even the most islet-friendly immunosuppressive regimen are toxic to islets. For example, the treatment of islets with sirolimus (also known as rapamycin) or tacrolimus (also known as FK-506 or Fujimycin) causes islet apoptosis [83]. Immune modulation with other biological approaches could also provide alternative ways to reduce immune rejection [10].

1. Genetic modification of dendritic cells—Dendritic cells are one of the most important APCs, which play a critical role in the regulation of immune responses. The activation and maturation of T cells relies on the signals from APCs. T cells will undergo apoptosis, if any one of these signals is blocked. DCs are genetically modified to act as a suppressor of immune responses. These DCs are called tolerogenic DC (Tol-DC). Clinically, we could isolate DCs from the host and then administer them back to the host after making them tolerogenic. Inhibition of the genes associated with DC maturation is one of the promising approaches to generate Tol-DC. **NF- γ B** is an essential transcription factor for DC differentiation and maturation. It includes a group of proteins with similar structures. Inhibition of NF- γ B pathway has been shown to produce tolerogenic DC [84,85]. Silencing

of RelB, which is a primary NF- γ B protein, reduces the expression of MHC-II, CD80, CD86, and finally prevents DC maturation. RelB silenced DCs were able to inhibit antigen-specific alloreactive immune rejection, and decrease antigen specific T cell proliferation [86]. Significantly reduced allograft rejection was achieved after administration of RelB silenced donor DC [86]. Inhibition of the expression of p35, which is a subunit of **IL-12**, was an effective approach for antigen-specific immune modulation [87,88]. DCs treated with siRNA against p35 were able to induce potent Th2 deviation of antigen-specific response; reduce alloreactivity by effective inhibition of T-cell proliferation; shift allogeneic T cell polarization from Th1 to Th2. In another study, Tol-DC was generated through the treatment of antisense ODN to inhibit the expression of **CD80 or CD86**. Allograft survival was prolonged by administration of modified DCs, which could induce T-cell hyporesponsiveness and apoptosis of T-cells [89]. In addition, silencing of CD80 or CD86 with siRNA inhibited T cell responses in mixed lymphocyte reaction (MLR), generated T regulatory cells [90], and improved cardiac allograft survival [91]. Tol-DC could also be generated through the silencing of **CD40**, which is another critical co-stimulatory molecules [92]. Silencing of CD40 gene expression in DCs resulted in increased IL-4 production, and decreased IL-12 production and allostimulation activity. The Th2 cytokine production from allogeneic T-cells was stimulated [93]. **MyD88** is a key adaptor of toll-like receptor signaling [83]. It is critical for DC function and proinflammatory cytokine production. The silencing of MyD88 significantly improved the allograft survival [94]. Suppressor of cytokine signaling (**SOCS**) molecule is an intracellular inhibitor of Janus kinase. Silencing of SOCS gene in DC resulted in reduced allogeneic mixed leukocyte reactions. In addition, systemic administration of SOCS silenced DCs improved the survival of allograft in rat [95].

2. Reducing immunogenicity of donor islets—HLA is one of the primary causes of Th1/HLA class II antigen rejections in solid organ transplantation. Human islet also has an immunogenic potential, which depends on their HLA incompatibility with the recipient. The alloreactivity is correlated with acute rejection and graft failure. In HLA matching human islet transplantation, the alloreactivity is significantly reduced [96]. However, because of the limited sources of islet donor and extensive HLA polymorphisms, the availability of HLA-match donors is extremely limited. In addition, the fact that multiple donors being required to obtain a sufficient number of islets for transplantation makes the donor selection based on HLA more difficult. Therefore, HLA-unmatched islets are used to meet the clinical needs. These transplanted islets undergo aggressive immune rejections. Silencing of HLA expression provides a promising method to reduce the immunogenicity of HLA-unmatched donor islets [97]. Enhanced resistance to allo-reactive T cell toxicity was observed in human cells treated with lentivirus expressing shRNA against pan class I and allele-specific HLA [98]. Silencing of HLA in solid organ transplantation was also reported [99,100].

B. Preventing ischemic reperfusion injury and apoptosis

There are multiple challenges including ischemia/reperfusion, mechanical and enzymatic stresses during islet isolation and preservation processes. These factors can activate inflammation and intracellular stress signal pathways, and lead to the overexpression of pro-inflammatory mediators, such as proinflammatory cytokines, ROS, caspases, and transcription factors. The consequence is the loss of islet function and viability. In addition, further adaptive immune response against transplanted islets will also be triggered [101].

1. Block the apoptosis—During the isolation and preservation processes, islets are challenged with various stresses. After transplantation, there are multiple factors that could induce the islet cell death. The survival of transplanted islets could be improved by preventing islet β cells apoptosis [62]. **Caspases** are the most important mediators of apoptosis, which include initiator caspases (caspase 8 and 9), and effector caspases (caspase

3, 7). Inhibition of caspase activity with caspase inhibitors, or anti-apoptotic genes, has been reported to prevent apoptosis of islets and improve their function [62]. The silencing of caspase 3 and/or caspase 8 was able to reduce renal and liver ischemia/reperfusion injury, which is a significant problem in organ transplantation [102–104]. Silencing of caspase 8, which is a mediator of extrinsic apoptosis pathway, could minimize Fas or proinflammatory cytokines induced cell apoptosis. Silencing of caspase 3 (which is the converging point for both extrinsic and intrinsic apoptotic pathways) prevented β cell apoptosis triggered by both extrinsic and intrinsic stimulations. We have demonstrated that the silencing of caspase 3 protects islets from inflammatory cytokine induced apoptosis [105]. Delivery of caspase 3 siRNA to rat insulinoma (INS-1E) cells significantly reduced the number of apoptotic cells. *Ex vivo* transduction of human islets with adenoviral shRNA prior to transplantation reduced caspase 3 activity in islets and significantly improved islet function. All of the mice transplanted with adv-caspase 3 shRNA transduced islets achieved normoglycemia one day posttransplantation and maintained up to 17 days. In contrast, for mice transplanted with untreated islets, where normoglycemia was achieved in only 60% at day 1, and 80% at day 4 post transplantation. **Fas:** Fas/FasL interaction is another important mechanism for triggering β cell apoptosis [76]. Treating β cells with siRNA-Fas significantly reduced caspase 3 activity and inhibited Fas-mediated β cell apoptosis [106]. In other studies, Fas siRNA has been successfully used to reduce apoptosis in the liver [107,108] and heart transplantation [109]. Burkhardt *et al.* used siRNA to silence Fas expression in murine insulinoma cells. Their results showed that siRNA against Fas inhibited cytokine induced Fas mRNA expression and reduced cell surface Fas protein. However, due to the slow turnover of Fas protein, a complete inhibition was not observed until prolonged incubation with siRNA. In addition, Fas siRNA effectively reduced cytokine induced β cell apoptosis as determined by caspase 3 activity and TUNEL assay [106].

2. Prevent complement activation—Downregulation of complement 3 or complement C5a receptor with siRNA has the potential to prevent renal ischemia-reperfusion injury [102,110]. In another study, UW solution containing siRNA against complement 3 also showed the ability to protect donor organs in heart transplantation [109]. For islet transplantation, the complement pathway is activated during the ischemia-reperfusion injury and blood-mediated inflammatory reaction. siRNA targeting C3 and C5a receptors might be a potential target for improving the viability of transplanted islets.

3. Inhibit inflammatory pathway—As discussed in the previous section, proinflammatory cytokines activate several inflammatory signal pathways, upregulate multiple key genes, and finally results in islet cell death. The major mediators in this process are potential targets for preventing islet cell death. **iNOS gene:** iNOS gene expression on islets increased significantly after transplantation. The maximal iNOS gene expression was observed one day after islet transplantation and then declined progressively [46]. Increased iNOS gene expression led to an early inflammatory process in transplanted islets. Nearly, 50% of the cytokine-induced genes are NO dependent, emphasizing the role of this radical in the late effects of cytokines on insulin producing β cells [57]. Therefore, silencing of iNOS gene might reduce iNOS mediated inflammatory response and prevent islet cell death. McCabe *et al.* used lentiviral vector expressing shRNA to suppress IL-1 β -mediated induction of iNOS expression, resulting in significant protection against the cytotoxic effects of IL-1 β exposure [111,112]. We designed siRNA to inhibit rat and human iNOS gene expression [113,114]. Due to the difference between human and rat iNOS mRNA sequences, different iNOS siRNA were designed for efficient gene silencing. A dose and sequence dependent inhibition of iNOS gene expression and NO production was observed in rat β cell line (INS-1E cell) and human islets. iNOS gene silencing protected β cells from inflammatory cytokine-induced apoptosis and preserved their insulin secretion ability.

However, the effect of iNOS gene silencing on the apoptosis of islet was only moderate, as evidenced by 25–30% reduction in caspase 3 activity and in the percentage of apoptotic cells. Since an islet is a cluster of 200–1000 cells, the transfection efficiency of lipid/siRNA complexes on human islets was only 21–28%, in contrast to the high transfection efficiency (>90%) in β cell lines. Therefore, to achieve satisfactory protective effects on human islets, we need to improve the transfection efficiency, which could be achieved by the rationale design of vectors [105,115] or modifying the delivery approaches. This will be discussed in the later sections.

NF- γ B: Transcription factor NF- γ B plays an important role in the inflammation and ischemia-reperfusion injury. For example, several genes are putative targets of NF- γ B and their expression levels are changed after cytokine exposure [59]. Transcriptional activity of NF- γ B is related to the expression of pro-apoptotic genes in cytokine stimulated β cells [60]. Recombinant adeno-associated virus (AAV) encoding shRNA against NF- γ B RelA (p65) subunit was evaluated to reduce NF- γ B mediated inflammation [116]. The expressed shRNA against RelA significantly reduced p65 protein expression and suppressed IL-8 secretion in a cellular TNF- α induced inflammation model. In another study, siRNA against NF- γ B RelB significantly attenuated ischemia-reperfusion injury induced renal dysfunction and protected mice against lethal kidney ischemia [117].

IV. Method of gene silencing

A. Types of RNAi methods

The applications of RNAi generally utilize two types of molecules: chemically synthesized short interfering RNA (siRNA) or vector based short hairpin RNA (shRNA). Although siRNA and shRNA can be applied to achieve similar functional outcomes, they are intrinsically different molecules as discussed below.

1. Chemically synthesized siRNA—siRNA of 19–23 bp can be synthesized chemically in vitro. Advances in solid phase synthesis technique make it possible to produce siRNA with high purity and precise-controlled sequence. Both sense and antisense strands are synthesized separately and annealed to form double stranded siRNA duplex. After being delivered into cytoplasm, siRNA is directly incorporated into RNA-induced Silencing Complex (RISC) to have its function. Several rate-limiting steps for vector based shRNA are avoided in siRNA mediated gene silencing. Therefore, the gene silencing effect of siRNA is potent and can appear in a short time after transfection. This feature also makes the design of siRNA relatively simple, since the factors regarding short hairpin expression and process are not involved. However, the 2'-OH group in siRNA molecules makes them extremely unstable and susceptible to enzymatic degradation. Only transient gene silencing can be achieved with siRNA and it disappears within several days post transfection.

2. Vector-based shRNA—shRNA is an RNA sequence that makes a tight hairpin turn that can be used to silence gene expression via RNAi. Vector-based shRNA utilizes endogenous cellular machinery to function. After entering the cell nucleus, vector-based shRNA with stem loop structure is expressed to produce pri-shRNA in the nuclei, which is then processed into pre-shRNA by an enzyme named Drosha. Pre-shRNA is exported to the cytoplasm by exportin-5, where it is further processed by Dicer (an RNase III enzyme) to produce functional siRNA [118]. siRNA is then incorporated into RISC. Activated RISC with antisense strand is formed by cleavage of sense strand. Activated RISC recognizes target mRNA with a complementary sequence, and result in the cleavage of target mRNA (perfect complementarities) or translation inhibition (not perfect complementarities) [22].

B. Design elements of siRNA and shRNA

1. siRNA and shRNA sequences—siRNA sequence is the most important factor that determines the level and duration of gene silencing. Because of high sequence specificity, gene silencing efficiency changes dramatically with minor alteration in siRNA sequence. Mutation in the antisense strand usually makes the activated RISC unable to recognize target mRNA. There are several web-based tools available for predicting effective siRNA sequences and empirical rules are incorporated in the siRNA design software to optimize siRNA sequence [119–122]. These rules include: thermodynamic property [123], length of siRNA sequence [124], GC contents, RNA secondary structure, sequence region, single nucleotide polymorphism sites, avoiding repeats and low complex sequence, avoiding off-target effects on other genes or sequences [125,126]. siRNA sequences predicted by software, however, are not always very effective in silencing a target gene, and sometime the most potent siRNA sequence is missed. Practically, a potent siRNA can be selected through testing of several candidate siRNA sequences designed with bioinformatics tools.

Since shRNA works through the production of siRNA within the cells, there are plenty of common rules applicable to both siRNA and shRNA sequence design. Actually, people have used the siRNA designing software to predict shRNA sequence with success. Due to the similarity between siRNAs algorithm and that of shRNAs, it is not surprising to keep the gene silencing efficiency after converting siRNAs into shRNAs. However, some features are not required for siRNA but are essential for shRNA expression. For an example, strong GC preference at position 11 and the preference for AU but not GC at position 9 might be the different properties for shRNA sequences [123]. The incompatibility of siRNA design algorithm for shRNA design has also been reported [127]. It would be a good idea to design shRNA sequences using shRNA design algorithm. Similar to siRNA design, several shRNA sequences should be experimentally screened to find potent shRNA sequences. An obvious disadvantage of this approach is that the cloning of shRNA into plasmid is a time consuming process. Fortunately, pre-designed shRNA plasmids for most genes are now commercially available. In most cases, potent shRNA sequences for a particular gene could be found by screening several pre-designed shRNAs.

2. Expression cassette for shRNA—Although shRNA sequence design is critical for efficient gene silencing, it is equally important to have a proper shRNA expression cassette, which determines the magnitude, duration and specificity of shRNA expression. In addition, the structure of shRNA backbone also plays an important role in shRNA transcription, processing, and nuclear export. The effect of different promoters and shRNA structures has been extensively investigated to improve the level and duration of gene silencing.

a. Promoters: Promoters refer to DNA sequences that provide a binding site for RNA polymerase and transcription factors. DNA transcription starts after transcription factor binding to promoter sequences and recruitment of polymerase. The level and duration of shRNA expression depends on the properties of promoters used. Three types of promoters, such as constitutive promoter, inducible promoters, and islet specific promoter will be discussed here.

Constitutive promoters: There are three types of constitutive promoters used for shRNA expression, including polymerase III, polymerase II, and polymerase I promoters. U6 and H1 are the two most commonly used polymerase III (Pol III) promoters for shRNA expression. This is because Pol III promoters transcribe small, highly structured RNAs without poly (A) and 5'-cap in mammalian cells [128]. However, Pol II promoters such as CMV [129,130], U1 promoter [131], and E1b promoter [132] are also used for shRNA expression. Particularly, CMV promoter can be used for co-expression of a cDNA and a

shRNA with a single promoter or to drive the expression of a shRNA embedded in a miRNA structure [130]. Usually, a poly (A) signal is needed for the termination of CMV promoter expression. A modified CMV promoter can also be terminated by poly(T) termination signal [129]. To increase the activity of U6 or H1 promoter, hybrid promoters composed of CMV enhancer and U6 or H1 promoter were constructed for shRNA expression [133–135]. Polymerase I promoter has also been reported for shRNA expression [136], however, the use of pol I promoter is still not well investigated.

Inducible promoter: High level constitutive expression of shRNA may saturate cellular machinery and cause cell toxicity. In addition, it is undesirable to constitutively silence genes that are essential for cell viability. Therefore, inducible promoters that can silence genes in a tunable pattern were developed. Different conditional RNAi strategies have been reviewed elsewhere [137]. Here, we list some examples to discuss the concept of using inducible promoters for gene silencing. Czuderna and coworkers [138] constructed tetracycline-inducible U6 or 7SK promoters by introducing a tetracycline operator (tet O) sequence between TATA box and transcription start site. shRNA expression was induced by adding tetracycline which could dissociate the repressors from the tet O. Similarly, tetracycline inducible H1 promoter was also constructed to silence β -catenin [139]. An IPTG-H1 promoter has been developed by placing a lac operator between TATA box and transcription start site [140]. Reversible gene silencing was achieved with above expression systems and the gene silencing effect will disappear within 3–4 day after removing the inducers. An alternative way for inducible gene silencing is Cre-LoxP recombination system, which usually results in an irreversible gene silencing. In this system, a Lox-flanked stuffer sequence with termination sequence (five thymidines) is inserted between H1 promoter and transcription start site. After expression of Cre protein, the stuffer sequence is removed due to the recombination of two loxP sites and thus activate the promoter [141]. Hypoxia inducible promoter may have additional advantages for its application in islets, which are usually under the hypoxic condition after isolation. The use of hypoxia inducible promoter could restrict the gene or shRNA expression within hypoxic islets. Therefore, it could avoid unwanted gene expression and silencing on normoxic tissue or organs and prevent potential toxic side effects. Lee *et al.* constructed pRTP801-Luc or pRTP801-VEGF plasmids bearing a hypoxia inducible promoter. After transfection into rat islets, transgene expression was higher in hypoxic islets than those in normoxic condition [142,143]. Although there is still little report about the use of hypoxia inducible expression cassette for gene silencing in islets, we believe that its application in islet gene silencing will increase in the future.

Islet-specific promoter: Several islet-specific promoters have been tested to drive the specific transgene expression in islets. Among them, rat insulin promoters are being extensively investigated [106,144,145]. To improve the performance of rat insulin 2 gene promoter in isolated pancreatic islets, an expression cassette including 1.5 kb of the porcine INS 5' UTR and the 3' UTR of the bovine growth hormone gene was constructed, which showed robust and specific gene expression on islet β cells [146]. Chai *et al.* have compared a series of rat insulin promoter sequences with various lengths. The highest promoter activities was observed in a modified RIP3.1 promoter including the non-coding regions in exon1, intron1, and part of exon2 of the rat insulin gene 1 as well as the insulin gene promoter region. This promoter showed 5-fold higher activity in INS-1E cells than a full-length RIP promoter or a CMV promoter. In addition, RIP3.1 promoter demonstrated β cell specific gene expression and glucose responsive gene expression [144]. Recently, the promoter for pri-miR-375 has been indentified, which could selectively express pri-miR-375 in pancreatic islets [147]. Since miRNA based shRNAs have been successfully used for shRNA expression, the finding of this promoter provided an option for islet specific shRNA expression.

b. shRNA backbone structure: High level of shRNA expression does not necessarily lead to efficient gene silencing, because nuclear export, dicer enzyme processing and other rate limiting steps are also critical for efficient gene silencing. These processes are greatly influenced by the shRNA structure, which includes the stem length, loop sequence, the sequence of flanking regions. A typical shRNA is composed of a 19–21bp sense sequence and a 19–21bp antisense sequence linked with 6–9bp loop sequence. A termination sequence of 5–6 thymidines is usually included at the end of stem-loop structure, except for shRNA driven by a pol II promoter. Various shRNA structures have been studied to improve gene silencing efficiency. Siolas *et al.* found that synthetic 29-mer shRNAs were more potent than 19-mer shRNAs, while the loops sequence did not have any effect [148]. In contrast, Jeanson-Leh *et al.* reported that increase in the stem length from 19-mer to 29-mer did not show any improvement in gene silencing [149]. It was also reported [123] that gene silencing of shRNAs with 19-mer stem was better than those of shRNA with 29-mer stem in the context of a 9-bp loop.

An emerging trend in shRNA design is to incorporate siRNA into a miRNA precursor backbone so that it can be processed efficiently by intracellular miRNA machinery. miR30-based shRNA has been reported to be more efficient than conventional shRNA [150–152]. Boden *et al.* designed a shRNA against HIV-1 transactivator protein TAT with a miR-30 backbone, which showed 80% more potency in silencing target gene than a conventional shRNA [150]. In another study, Li *et al.* compared 14 pairs of conventional shRNA and mir30-based shRNA against luciferase gene, and 10 pairs of conventional shRNA and mir30-based shRNA against the mouse tyrosine gene. Results indicated that in 11 out of 14 pairs of shRNA against luciferase and all of those for mouse tyrosine gene, a better gene silencing was observed in conventional shRNA rather than in miR30-based shRNA [123]. Boudreau *et al.* have also demonstrated that optimized shRNA are more efficient than miR30-based shRNA in silencing of several genes, including green fluorescent protein (GFP), spinocerebellar ataxias, and Huntington disease [153]. In our own studies, we also found that miR30-based shRNA against human iNOS genes was less potent compared with conventional shRNA [115]. These discrepancies indicate that current understanding of shRNA design is still limited and further exploration is necessary to develop a satisfactory rule for shRNA design. The use of miRNA backbone for shRNA expression also has the potential to reduce toxicity [154] and to have tissue or cell type specific gene silencing [155]. In addition, we could also construct pri-miRNA cluster, in which multiple miRNA based shRNA is driven by a single promoter to form a single transcript [156,157].

Recently, several other shRNA structures have been investigated for different applications. In one study, a tRNA-shRNAs chimeric expression cassette was constructed, in which a tRNA promoter was used to drive the expression of shRNA linked to the 3' acceptor stem of tRNA [158]. This system could achieve similar or even high gene silencing than shRNAs expressed from U6 or H1 promoters [158–160]. Since exportin-t, but not exportin-5, was used for the nuclear export of tRNA-shRNAs, it could reduce toxicity related to exportin-5 saturation [159,161]. Tandem siRNAs: Zheng *et al.* designed a dual promoter system (pDual) by inserting siRNA sequence between two promoters (including mouse U6 and human H1 promoters) with opposing directions [162].

3. Off-target effects—Since the first report by Jackson *et al.* in 2003 [163], many studies have shown that both siRNA and shRNA could cause off-target effects. From a therapeutic standpoint, the off-target effects should be avoided to prevent unintended effects. Therefore, understanding the mechanisms of off-target effects and developing strategies to enhance RNAi specificity is critical for the application of RNAi. There are two types of off-target effects: specific off-target effects, which are caused by the partial sequence complementarity between mRNA and siRNA, and non-specific off-target effects, which are the outcome of

overall translation inhibition due to the activation of immune system and cellular toxicity. The mechanism of off-target effects was reviewed extensively elsewhere [164,165].

Specific off-target effects: Silencing of non-target genes is possible if there is as little as 8-nt homology at the seed region sequence [166]. The off-target effects may also be observed when mRNA shared 7-nt homology with the guide siRNA sequence [167]. However, not all the mRNA with this level of homology will be silenced and it depends on the location of complementary region within siRNA and the mRNA. Seed region is the nucleotides 2–7 at the 5' end of siRNA. Complementarity between the siRNA seed region and the 3' UTR of mRNA is an important predictor for off-target effects [166]. Several approaches have been developed to avoid the specific off-target effects. It could be prevented by designing siRNA sequences to minimize the complementarity between seed region of siRNA and the 3' UTR of mRNA [168]. One or two base mismatch between siRNA and target mRNA are usually tolerated without losing gene silencing efficiency [169]. This allows us to modify the siRNA sequence to minimize the complementarity with 3'UTRs of the untargeted mRNAs. However, the modification of siRNA sequence is associated with the risk of losing gene silencing efficiency. Since the passenger strand is not involved in the recognition with target mRNA, modifications of passenger strand is preferred and more tolerant. For example, breaking a passenger strand into two segments significantly reduced off-target effects [170]. RNA-DNA chimeras [171] have also been developed to avoid off-target effects.

Nonspecific off-target effects: The innate immune systems could be activated by several elements including dsRNA, plasmid or viral vectors, cationic delivery carriers. The activation of immune system causes the inhibition of global gene expression and false positive gene silencing effect. There are multiple receptors involved in the activation of immune system. They are cytoplasmic receptors, including dsRNA-dependent protein kinase R (PKR), retinoic acid-inducible gene-1 (RIG-1), and melanoma differentiation-associated gene-5 (MDA-5) [172–174] and endosomal toll-like receptors (TLRs) [175,176]. For an example, introduction of long dsRNA into cells leads to the activation of PKR, and results in the activation of innate immune system and type-I IFN production [174]. Although the use of shorter siRNAs could minimize the activation of immune response, sequence dependent activation of TLR7 and 8 could not be eliminated [177]. For vector based shRNA, siRNA expressed inside the nucleus and exported into the cytoplasm. The use of vectors based shRNA could thus avoid the activation of TLR3, which recognize dsRNA. However, the plasmid vectors will activate TLR9, unless the unmethylated CpG motifs were removed from the vectors [178,179]. High levels of siRNA and shRNA could also compete for intracellular miRNA/shRNA process machinery. The saturation of exportin-5 and RISC component, Argonaute-2 will cause cell toxicity [180]. This can be minimized by designing of proper expression cassettes to carefully control shRNA expression levels and avoid the saturation of miRNA machinery.

V. Combinatorial RNAi

Due to the complexity of molecular mechanism of islet cell death, it is almost impossible to effectively prevent islet cell apoptosis by silencing the expression of a single gene or over-expression of a single protective gene. Therefore, combinatorial RNAi strategies which could synergistically work on multiple targets are required to achieve satisfactory outcomes. This could be realized by simultaneous knockdown of different genes involved in multiple biological processes or signal pathways. Alternatively, we could also simultaneously silence certain harmful genes and at the same time over-express protective genes. Different strategies for combinatorial RNAi will be discussed (Figure 4).

A. siRNA Pool

The use of several siRNAs target different regions of a single gene is more efficient than the use of a single siRNA sequence in reducing gene silencing. Cheng *et al.* tested a pool of two siRNA sequences against TGF- β 1 gene in HSC-T6 cells [181]. In that study, the siRNA pool showed better inhibition of TGF- β 1 protein expression as determined by western blot analysis. In addition, an enhanced inhibition of the expression of downstream genes including type α 1 (I) collagen and alpha-smooth muscle actin (α -SMA) were also observed. In another study, Chen *et al.* used siRNA pool targeting different regions of human hepatitis B surface antigen. The treatment of this siRNA pool showed efficient inhibition of hepatitis B surface antigens production at HBV-producing HepG2.2.15 cells [182]. Alternatively, co-silencing of multiple genes with siRNAs has also been used to achieve synergistic effect. For an example, siRNA against three different genes including TNF- α , complement 3 (C3) and Fas were tested to reduce ischemia/reperfusion injury to donor organs. The siRNAs used in this study, were supposed to silence several different genes involved in apoptosis, complement activation, and inflammations [109]. In other studies, combination of siRNAs targeting complement 3 and caspase 3 [102] or siRNAs targeting caspase 3 and caspase 8 [103] have been used to prevent renal ischemic injury. siRNA pool targeting mRNAs of multiple genes will be suitable for improving the islet viability because of the involvement of multiple processes in islet damage.

B. Co-expression of multiple shRNAs

There are several scenarios for co-expression of multiple shRNAs from a single vector: (1) multiple shRNAs with the same sequence, (2) multiple shRNAs against different regions of the same gene, or (3) multiple shRNAs against different genes. For the first one, the purpose is to increase the intracellular shRNA levels. However, it is rarely used because the shRNAs levels could be controlled more conveniently by selecting proper promoters. The use of multiple shRNAs against different regions of a single gene is believed to be more efficient in reducing target gene expression levels, possibly due to the synergistic effect. The advantage of using multiple shRNAs against different genes is the possibility to block multiple mediators in the signal pathways. Co-expression of multiple shRNAs were widely studied as an antiviral treatment modality [183]. Although the disease targets are different, the shRNAs expression strategies could be applied to genetic modification of islets. Here, some strategies for co-expression shRNAs will be reviewed.

Gonzalez *et al.* constructed a vector where multiple shRNAs against HLA class genes were under the control of a common U6 promoter. A dose dependent increase in RNAi efficiency was achieved though raising shRNA copy numbers up to six [184]. Several other studies demonstrated the feasibility of co-expressing multiple shRNAs by utilizing miRNA backbone. In these studies, strong promoters (such as a CMV promoter or a ubiquitin C promoter) were used to control the expression concatemerized multiple miRNA (miR30 or miR155) based shRNAs [156,185–187]. However, the increase in shRNA copy number does not necessary improve gene silencing efficiency. Further investigation will help us to understand the optimal hairpin numbers, positions, backbone sequences required for efficient RNAi.

Long hairpin RNA (lhRNA) [188,189] or extended hairpin RNAs (e-shRNAs) [190,191], also showed the potential to co-express several siRNAs. For an example, Liu *et al.* constructed a vector with e-shRNAs containing two siRNAs and found that a stem length of 43 bp is the minimal requirement. The 66 bp was the minimal length needed to produce three siRNAs [191]. Instead of expressing multiple shRNAs, a single hairpin with multiple siRNAs was expressed in this type of construct. Different levels of gene silencing were observed among these siRNAs and were dependent on their position to the stem loop. The

siRNA most close to the loop showed comparable gene silencing to single shRNA, however, other siRNAs usually showed less gene silencing efficiency [189,191]. In addition, three siRNA units is the upper limit for one construct and the gene silencing efficiency was significantly reduced when four siRNA units were included within one construct[191].

Alternatively, it is also possible to express shRNAs from two or more independent promoters. For example, two shRNAs against different isoforms of glycogen synthase kinase 3 gene were under the control of separate U6 promoters and achieved efficient inhibition of target gene with additive effect [192]. A similar study showed the use of separate U6 promoters to control the expression of shRNAs targeting different Smads [193]. Co-expression of multiple miRNA based shRNAs against different gene has also been reported [194].

C. Co-expression of shRNAs and cDNAs

We have recently demonstrated the beneficial effects of co-expressing multiple genes from a single vector for improving the outcome of islet transplantation. The combination of growth factor and anti-apoptotic genes showed synergistic effect in protecting transplanted islets [16,17]. A vector could co-express both siRNA and cDNA will be particularly interesting [195]. Not only is the inhibition of harmful genes but also overexpression of protective genes is needed to protect islets. We constructed vectors for co-expression of shRNAs against iNOS genes and a VEGF cDNA. In this vector, VEGF was designed to promote the revascularization of transplanted islets. The shRNA against iNOS were designed to reduce the expression of iNOS genes and minimize iNOS mediated islet cell death. In this study, we also investigated the use different expression cassettes for co-expression of shRNA and cDNA from a single vector. The plasmid vector contained two expression cassettes, where VEGF was under the control of a strong CMV promoter and shRNA was driven by U6, H1, or CMV promoters. Alternatively, it was also possible to express cDNA and shRNA from a single expression cassette. In this case, we used CMV promoter to drive the expression of miRNA based shRNA and VEGF cDNA was inserted between the CMV promoter and shRNA (Figure 5). This strategy was reported previously by Qiu *et al.*, where a miRNA based shRNA was placed in the 3'-UTR of a red fluorescent protein (RFP) for co-expression of both shRNA and RFP [196]. Similar study was also reported by Shin *et al.*, where a GFP gene was inserted between the Tet response element promoter and miR30-based shRNA. This construct allows the co-expression of both GFP and shRNA [194]. In our study, both monocistronic and bicistronic systems showed high VEGF expression and efficient reduction of iNOS gene expression. These vectors could be further modified by replacing VEGF cDNA with another cDNA such as hepatocyte growth factor (HGF), which will enhance angiogenesis and promote β cell proliferation. The shRNA against iNOS genes could be replaced various other shRNA targeting pro-apoptotic and inflammatory genes.

VI. Delivery strategies for enhanced gene silencing

Although good progress has been made since the discovery of RNAi technique, the application of RNAi for therapeutic purposes is still limited, because of several biological barriers for siRNA delivery [197]. Systemic delivery of siRNA is required to block host against islet graft immune rejection. However, siRNA face several biological barriers after systemic administration [197], which usually result in the degradation of siRNA. Another potential issue for systemic siRNA delivery is the lack of its targeting ability, which requires the use of targeted delivery system to avoid gene silencing on other tissues or organs. In addition, the systemic administration will possibly induce the immune reaction not only by the siRNA but also by the delivery system, especially, when a viral vector is used. Alternatively, *ex vivo* delivery is an ideal approach for genetic modification of islets prior to transplantation. Since after isolation, islets are usually preserved in storage solutions before

transplantation, we can transfer siRNA into islets prior to transplantation. This *ex vivo* siRNA delivery approach can also be used to generate immune tolerant dendritic cell (Tol-DC).

The design of proper delivery systems is critical for the clinical application of RNAi technology. Both non-viral and viral vectors have been widely used. The main advantage of viral vectors is their high transduction efficiency. However, the use of viral vectors is undermined due to safety issues and also the construction of individual viral vector is a time consuming process. Alternatively, non-viral approaches, which could avoid the problems for viral vectors, have become a promising approach for gene silencing. In this section, we will discuss siRNA delivery systems applicable to islet transplantation (Figure 6). Firstly, we will discuss the possibility of using free siRNA and chemically modified siRNA. Secondly, we will visit the cationic lipids or polymers for siRNA delivery. Thirdly, the use of bioconjugation will be discussed, which could enhance siRNA delivery into the cells and increase siRNA stability. Finally, we will summarize some viral vectors used for gene silencing including adenovirus and lentivirus (Table II).

A. Free siRNA

Free siRNAs are difficult to be transfected into the cells mainly because of their poor cell membrane permeability and sensitive to nuclease mediated degradation. However, it is still possible to deliver free siRNAs to islets with some physical approaches, such as hydrodynamic injection [198], in situ perfusion [199], and microporation [200]. Zheng *et al.* protected donor organs for heart transplantation with siRNA-containing solution [109]. In that study, heart graft from BALB/C mice were preserved in solution containing siRNAs against several genes involved in ischemia/reperfusion injury. This treatment resulted in efficient silencing of target genes in the grafts and lead to improved graft function. Due to the similarity among different organ transplantations, this approach should be equally applicable for islet transplantation.

However, native siRNA is degraded rapidly due to nuclease attack, therefore, chemical modification of siRNA has been extensively studied to (1) minimize nuclease degradation; (2) avoid innate immune system activation; and (3) reduce off-target effects [201,202]. Since most siRNAs were produced through chemical synthesis, it is technically possible to incorporate various modifications into siRNA backbones (Figure 7). These modifications could be applied to phosphodiester or ribose. Non-bridging oxygen in the phosphodiester could be replaced with sulfur (phosphorothioate), boron (boranophosphate), or methyl (methylphosphonate) groups. The 2'-position of the ribose could also be modified to improve the stability of siRNA. The modification of ribose includes 2'-O-methyl (2'OMe), 2'-fluoro(2'-F), 2'-O-fluoro- β -D-arabinonucleotide (FANA), 2'-O-(2-methoxyethyl) (MOE), and locked nucleic acid (LNA), which contains a methylene bridge connecting the 2'-O with the 4'-C of the ribose ring. Single or combination of various types of modifications could be used depending on the aim of modification, which will be briefly discussed below.

Enhancing serum stability—unmodified naked siRNAs are extremely unstable with a serum half-life of less than 5 min [203,204], which restricts their clinical application. Thus, chemical modification of siRNAs has been extensively studied to improve the resistance of siRNAs to nuclease degradation. Theoretically, all above mentioned modifications can be applied to increase the serum stability of siRNA. The modification strategies should be carefully designed, since extensive modification of siRNAs usually leads to decreased potency [205–207]. Alternatively, selective modification of siRNAs at minimal position has been demonstrated to significantly improve the serum stability, while keeping the gene

silencing potency. The 2'-O-Me or 2'-F-RNA modification have been applied to the termini of the strands or a pyrimidine nucleotide which are vulnerable position for nuclease cleavage [22,205,208].

Reducing innate immune system activation—As we discussed previously, siRNAs will activate the innate immune system and result in non-specific off-target effects. Chemical modifications can also be used to avoid or minimize siRNA-mediated activation of immune system. For an example, 2'-O-Me modification of siRNA was able to minimize the immune system activation [209]. Since the 2'-O-Me group is a competitive inhibitor of TLR7, minimal number of 2'-modification is sufficient to inhibit the TLR-7 mediated immune stimulation [210]

Reducing off-target effects—Chemical modification could be applied to reduce specific off-target effects through minimizing the unwanted participation of siRNA in miRNA pathways. For an example, modification of the +2 position with 2'-O-Me on the antisense strand effectively reduced seed region mediated off-target effects [166]. The seed region could also be replaced entirely with DNA residues to reduce off-target effects while preserving the gene silencing potency [171]

B. Complex formation

Both cationic liposomes and polymers are widely used to form complexes with siRNA or plasmid shRNA through electrostatic interaction between positive charged carriers and negatively charged nucleic acids. Complex formation helps to condense siRNA or plasmid DNA and thus facilitate their cellular uptake.

Cationic lipids—Since the introduction of lipofectin at late 1980s [211], many cationic liposomes have been tested for delivery of plasmids, and recently for the delivery of siRNA. There are several commercial cationic lipid formulations for siRNA delivery, including lipofectamine 2000, oligofectamine, lipofectamine, RNAifect, and Eugene HD. We have tested lipofectamine to transfect a plasmid encoding an enhanced green fluorescent protein gene, pCMS-eGFP into intact human islets. However, only low transfection efficiency was observed, this is due to the fact that human islets are a cluster of around 1000 non-dividing cells [14]. In our further studies, a novel lipid with methylsulfonic acid in the cationic head group region to enhance lipid/DNA interaction was synthesized, which showed higher transfection efficiency than those of lipofectamine in intact human islets [212]. In contrast to plasmids, siRNA is efficiently transfected into human islets as well as INS-1E rat β cell line. In this study, we used lipofectamine 2000 to transfect fluorescein-labeled siRNA. The transfection efficiency was determined by flow cytometry analysis (Figure 8). There was increase in transfection efficiency with increase in siRNA concentration, with maximum transfection efficiency of 95.9% and 28.3% on rat β cell line and intact human islets, respectively [113]. This demonstrated the feasibility of *in vitro* genetically modifying human islets with siRNA/lipid complex.

Cationic polymers—In addition to cationic lipids, cationic polymers are also being used for plasmid or siRNA delivery. Although we could achieve decent *in vitro* transfection efficiency by optimizing transfection conditions, the cellular toxicity of cationic carrier is a major concern for its therapeutic applications. Furthermore, the poor intracellular dissociation of siRNA/polycation complex may reduce the intracellular bioavailability of siRNA. Recently, several biodegradable carriers have been developed to reduce toxicity and improve transfection efficiency. Here, we focus on the some recent progresses, rather than a comprehensive review of the use of cationic polymer for siRNA delivery which was extensively discussed elsewhere [213].

Reducible biodegradable carriers are designed based on the different redox potential between intra and extra cellular environment. The disulfide bonds in these carriers are usually stable enough to keep the integrity of the carrier and protect siRNA before cellular uptake. After cellular uptake, the disulfide bonds are cleaved, in response to the reducing intracellular environment. This will result in the degradation of carriers and release of siRNA. Reducible polymers with disulfide bonds have been investigated for delivery of both plasmid and siRNA [214–220]. The advantage of using reducible polymers is obvious, which not only reduced carriers associated toxicity, but also enhanced dissociation of siRNA from polycation/siRNA complex and thus improved gene silencing efficiency. The performance of these carriers could be further improved by incorporation of endosomal escaping moieties, such as histidine [218], and protonatable pendants [219]. Since the endosomal release of siRNA is a significant barrier for siRNA and gene delivery, those components which could facilitate “endosomal escape” will greatly enhance the gene silencing efficiency.

Poly (amino ester) (PAE) is another type of biodegradable polymers which has been extensively studied for gene delivery. Green *et al.* generated a library of PAE and determined parameters such as polymer type, polymer weight, DNA loading, and other biophysical properties. The leading PAE carrier found in their studies were better than jetPEI and lipofectamine2000 [221]. PAE has also been successfully used for siRNA delivery into lung cancer cells [222], hepatoma cells and primary hepatocytes [223], and fibroblasts [224]. Besides, several other biodegradable polymers can also be used for siRNA delivery, including polyphosphoesters [225–227], poly(2-aminoethyl ethylene phosphate) [228], poly(ethylene glycol)-peptide copolymers [229] and ketalized polyethylenimine [230].

C. siRNA bioconjugation

Bioconjugation of siRNA with lipids, polymers or other molecules will help to enhance its cellular uptake, systemic stability, and targeted delivery to specific cells. Technically, siRNA could be modified at the 5' or 3' terminus of sense strand or antisense strand. Because of the pivotal role of antisense strand in gene silencing, the modification of sense strand is preferred. Both cleavable and non-cleavable bonds can be used for siRNA conjugation. Acid-sensitive or reducible disulfide bonds are the most commonly used, because of the enhanced release of siRNA from conjugation inside the cell. It could avoid potential negative effects of bioconjugation and provide more amount of free siRNA available for gene silencing.

The molecules used for conjugation with siRNA determine the properties of siRNA conjugate. Attachment of lipids usually increases the hydrophobicity of siRNA, which are highly hydrophilic macromolecules. Lipophilic molecules such as cholesterol [231], bile acid and long chain fatty acid [232,233], and vitamin E [234] could be conjugated to siRNA. After systemic injection, siRNA-lipid conjugate binds with lipoproteins depending on the degree of hydrophobicity. The interaction between siRNA-lipid conjugate and lipoproteins, lipoprotein receptors and transmembrane proteins has a great influence on the biodistribution and cellular uptake of siRNA after intravenous injection [232]. For an example, siRNA bound to low density lipoprotein (LDL) is mainly delivered to the liver, while a broader distribution was observed for siRNA binding to high density lipoprotein (HDL).

Conjugation of siRNA with cell-penetrating peptides (CPPs) such as TAT [235,236], penetratin [236] and Transportan [237], has also been studied for siRNA delivery. siRNA-peptide conjugates show a cellular uptake as efficient as that achieved by cationic lipids and resulted in efficient silencing of target genes. However, conjugation of siRNA with peptides

does not improve the *in vivo* stability of siRNA [236]. In addition, innate immune response could be activated after intratracheal administration of pentratrin-siRNA conjugate [236], may be due to the immunogenicity of peptide.

To increase the systemic stability of siRNA, poly(ethylene glycol) (PEG) has been conjugated to siRNA by several research groups. In this type of conjugation, siRNA can be linked with PEG through disulfide bond or beta-thio-propionate linkage. Polyelectrolyte complex micelles (PEC micelles) were formed between PEG-siRNA and cationic peptide (KALA) [238] or cationic polymers, such as polyethylenimine (PEI) and poly(L-lysine) (PLL) during the application of siRNA-PEG conjugate. A core was formed between negatively charged siRNA and polycation through condensation, while PEGs are surrounding the core to form a hydrophilic shell. This special core-shell structure can effectively protect siRNA against enzymatic degradation; prevent self-aggregation between PEC micelles, and increase the circulation time after systemic administration. This delivery system can efficiently silence VEGF gene expression in PC-3 cells *in vitro* [238,239]. After intravenous injection, enhanced distribution of PEC micelles in tumor was observed, which resulted in significant reduction in VEGF expression in tumor and suppressed tumor growth [240]. siRNA-PEG PEC can be further improved by conjugation of lactose moiety at the distal end of PEG (siRNA-PEG-Lac). This will confer targeting ability to this carrier. The cellular uptake of siRNA in hepatoma cells was enhanced via receptor mediated endocytosis [241,242]. Delivery of siRNA against RecQL1 gene with lac-PECs inhibited multicellular HuH-7 spheroids growth for up to 21 days, while almost no effect was observed with oligofectAMINE/siRNA lipoplexes. It is probably due to the facilitated cellular uptake of Lac-PECs into the spheroids cells [242]. In addition to the enhanced delivery, siRNA are also conjugated with probes or dyes for bioimaging [243]. Multiple component siRNA conjugate was prepared which includes iron oxide core coated with crosslinked dextran coat, cy5.5 dye, as well as siRNA. After *in vitro* incubation, this conjugate was efficiently taken up by murine islets. The cellular uptake of siRNA was observed by magnetic imaging of iron core and near-infrared imaging. This study showed the possibility to integrate gene silencing and imaging components into a single conjugate to create a multiple function siRNA conjugates.

D. Viral vectors

As naturally evolved vehicles, viral vectors utilize the virus infection mechanism for efficient gene transfer into host cells. Although safety concerns retarded the clinical use of viral vectors, their application in genetic modification of islets has the potential to improve the outcome of islet transplantation. This is mainly because of their high transduction efficiency compared with non-viral vectors. Here, we will discuss the properties of some commonly used viral vectors and their application in islet transplantation.

1. Adenovirus—Adenovirus is non-enveloped DNA virus, which could infect both dividing and non-dividing cells. Recombinant adenovirus derived from adenovirus serotype 5 is commonly used for gene delivery. E1 region, which is essential for virus replication, is usually deleted to make replication-deficient adenovirus. In addition, adenoviral genome will not integrate to the targeting cells; therefore, a transient gene expression is achieved by adenoviral vector. The advantages of adenovirus include capacities to carry large DNA insert, easy to produce high titer virus, and its ability to infect non-dividing cells with high efficiency and high gene expression level.

We and others have used replication deficient adenoviral (Adv) vectors for *ex vivo* transduction of genes to islets to reduce the immune attack, prevent apoptosis, and promote the revascularization of transplanted islets [13,16,17,62,105,115,244]. Bain *et al.* first

reported the feasibility of using Adv vector for gene silencing in islets and β cell lines [245]. Adv vectors expressing siRNA against GLUT2 gene was used in their study to transduce rat islets. Maximum reduction in GLUT2 gene expression was achieved 3 days post transduction with > 90% at both mRNA and protein levels. After then, Adv vectors have been used to silencing different genes in islets to study the function of genes, such as synaptotagmin 9 [246], pyruvate carboxylase [247], tissue factor [248], and proislet amyloid polypeptide [249]. The maximum silencing efficiency was varied from 35% to 75% and achieved 3–4 days post-transduction. Gene silencing efficiency is determined by several factors: (1) the properties (turnover time, abundance) of target gene mRNA and protein; (2) the design of siRNA targeting sequence as well as expression cassette.

Since caspase 3 played an important role in islet β cell apoptosis induced by different factors, the inhibition of caspase activities was a potential approach to improve the graft survival [62]. Recently, we determined the level and duration of caspase-3 gene silencing after transfection of caspase-3 siRNA into INS-1E cells and demonstrated transient gene silencing which did not last beyond 3 days. In an attempt to enhance the level and duration of caspase-3 gene silencing (Figure 9A), we then constructed Adv vectors expressing shRNA against caspase 3 (Adv-caspase-3-shRNA) and determined their gene silencing efficiency in human islets. This adenoviral vector showed efficient caspase-3 gene silencing, which became more significant on day 5 (Figure 9B). Further, Adv-caspase-3-shRNA mediated caspase-3 gene silencing was able to counteract inflammatory cytokine induced islet cell apoptosis *in vitro* (data not shown). Taking care of these encouraging *in vitro* results, we then transduced human islets with this adenoviral vector prior to transplantation under the kidney capsules of diabetic NOD-SCID mice. There was some improvement in islet survival and function after transplantation [105].

Adv vectors have several particular advantages for gene silencing in human islets. (1) Thorough understanding of the structure of Adv DNA may provide much flexibility in genetically engineer Adv vectors to make them more proper for shRNA delivery and expression. For example, it is convenient to construct shRNA expression vectors with different promoters and backbones with commercially available Adv shuttle vectors for optimal gene silencing; (2) high expression levels achieved by Adv vectors will help to increase gene silencing efficiency and help to get more beneficial effect. (3) Adv vectors has the capacity to carry a large insert, which will make it possible to express multiple shRNAs; or to co-express an shRNA to silence harmful genes and a cDNA to protect islets [115]. The disadvantage of Adv vector is the transient expression profile, which is not suitable for long-term gene silencing. Since the majority of islet cell death occurs within several days after transplantation, the use of Adv to silence genes involved in this process is appropriate.

2. Lentivirus—Lentivirus is a type of retroviral vector, which reverse transcribes into DNA, be actively transported into the nucleus, and integrate into the genome of target cells. Therefore, stable gene expression or silencing could be achieved by using lentiviral vector [250]. Unlike other retrovirus such as moloney murine leukemia virus, which could only transduce dividing cells, lentivirus can transduce both dividing and non-dividing cells. Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped lentiviral vectors is the most commonly used vector, which could transduce a broad range of cell types [251]. Lymphocytic choriomeningitis virus (LCMV)-pseudotyped is more efficient in transducing human islet insulin-secreting beta cells and shows less toxicity compared with VSV-G pseudotyped lentivirus [252]. Lentivirus were successfully used for genetic modification of islets with various genes, including reporter genes (GFP, Luciferase) [253,254], antioxidative genes [255], antiapoptotic genes (cFLIP) [253], anti-inflammatory and immunoregulatory genes (CTLA4, TGF- β , interleukin-1 receptor antagonist, and IL-4) [256–258]. Unlike Adv vectors which give high level transient gene expression, a prolonged

gene expression is usually achieved after transduction islets of lentiviral vectors. For example, after transduction of islets with lentiviral vectors encoding a luciferase gene, bioluminescence is persistent for above 140 days post transplantation. However, the bioluminescence of islets transduced with Adv vector encoding a luciferase gene decrease from thousand fold at the beginning to ten fold over background at 60 days posttransplantation [254]. An optimized *ex vivo* transduction procedure should neither induce significant toxicity nor affect the insulin release function of islets [255]. In addition, lentiviral vectors induced immune system activation was also minimal [258]. Under optimized conditions, lentivirus could transduce around 10% to 30% of islet cells in an intact islet, which are mainly the cells at the periphery [253,259,260]. This is still enough to have significant effect on the viability, probably due to the “barrier” effect, that is shield the core of islets by protecting the periphery of islets [253].

Several other approaches have also been investigated to improve the transduction efficiency. Callewaert *et al.* demonstrated increased transduction efficiency from around 11.2% to 80.0% by dissociation of islets to single cells prior to transduction and re-aggregation of islets before transplantation [260]. However, improvement in the transfection efficiency did not translate into the improved function of islets. More cells are needed to achieve normoglycemia compared with intact islets. Barbu *et al.* tested the possibility to transduce islets by whole pancreas perfusion. This transduction procedure could transduce 30% of the cells, while keeping the structural integrity of islets. However, the cells within the core of islets still cannot be effectively transduced [261].

Lentiviral vector has also been used for shRNA delivery for gene silencing [262]. Because of the long term expression achieved by lentiviral vectors, it has been used to stably silence harmful genes to prevent the death of islet β cells. For example, transduction of lentiviral vectors carrying shRNA against iNOS gene, significantly reduced IL-1 β induced iNOS expression in islet β cells and protect β cells from IL-1 β induced cell death [111]. Since the silencing effect achieved by lentiviral vector is long term, it is suitable for delivery of shRNA against genes that need to be continuously silenced. Otherwise, an inducible or switchable expression cassette should be used to control the timing of gene silencing and thus avoid potential side effects.

VII. Concluding Remarks

Theoretically, any gene of interest could be silenced or effectively inhibited, which is unachievable by conventional small molecule chemicals. In the past few years, RNAi technology has moved rapidly from bench to bedside at unprecedented speed. Currently, there are more than ten ongoing and completed clinical trials using siRNA as therapeutics (<http://clinicaltrials.gov/>). It is interesting to note that one of these trials sponsored by Quark Pharmaceuticals was related to kidney transplantation, where a siRNA named I5NP was tested for the prophylaxis of delayed graft function in kidney transplantation patients. Although recent progresses have already demonstrated its great potential, the application of RNAi technology for improving the outcome of islet transplantation is still a fledgling area. Further understanding of the biological perspective of the islets transplantation is essential, as it may help to discover effective RNAi target genes. A combination of multiple gene silencing or simultaneously gene silencing and gene over-expression is likely to have improved beneficial effects through synergistic effects. Meanwhile, the advances in RNAi technology will enable us to design more potent siRNA or shRNAs, while minimizing the side effects caused by off-target effects and immune stimulation. Last but not the least, the development of safe and efficient delivery systems is the key for successful gene silencing.

List of abbreviations

2'-F	2'-fluoro
2'OMe	2'-O-methyl
α-SMA	alpha-smooth muscle actin
AAV	Recombinant adeno-associated virus
Adv	adenoviral
APCs	Antigen presenting cells
C3	Complement 3
CPPs	Cell-penetrating peptides
DISC	Death-inducing signaling complex
e-shRNAs	Extended hairpin RNAs
FADD	Fas-associated death domain
FANA	2'-O-fluoro- β -D-arabinonucleotide
GFP	Green fluorescent protein
HDL	High density lipoprotein
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
IFN-γ	Interferon- γ
IFNγR1	IFN γ receptor 1
IKK	I κ B kinase
IL-1AcP	IL-1 receptor accessory protein
IL-1β	Interleukin-1 β
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R1 activated kinase
ITN	Immune Tolerance Network
JAK1/2	Janus tyrosine kinase 1 and 2
JDRF	Juvenile Diabetes Research Foundation
JNK	c-jun N-terminal kinase
LCMV	Lymphocytic choriomeningitis virus
LDL	Low density lipoprotein
lhRNA	Long hairpin RNA
LNA	Locked nucleic acid
MCP-1	Monocyte chemoattractive protein-1
MDA-5	Melanoma differentiation-associated gene-5
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction

MOE	2'-O-(2-methoxyethyl)
MyD88	Myeloid differentiation factor 88
NCRR	National Center for Research Resources
NIH	National Institutes of Health
NO	Nitric oxide
PEC micelles	Polyelectrolyte complex micelles
PEG	Poly (ethylene glycol)
PEI	Polyethylenimine
PKR	dsRNA-dependent protein kinase R
PLL	Poly (L-lysine)
RFP	Red fluorescent protein
RIG-1	Retinoic acid-inducible gene-1
RISC	RNA-induced Silencing Complex
ROS	Reactive oxygen species
shRNA	Short hairpin RNA
SOCS	Suppressor of cytokine signaling
STAT1	Signal transducers and activators of transcription 1
tet O	Tetracycline operator
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
Tol-DC	Tolerogenic DC
TRADD	TNF- α associated death domain
TRAF2	TNF-receptor-associated factor-2
TRAF6	TNF-receptor-associated factor-6
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VSV-G	Vesicular stomatitis virus glycoprotein

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Figure 1.
Instant blood-mediated inflammatory reaction.

Figure 2.
Extrinsic and intrinsic pathways for islet cell apoptosis.

Figure 3.

Autoimmune recurrence in type 1 diabetes and activation of T-cells in host immune rejection against transplanted islets. (A) After transplantation, β cell reactive CD8⁺, CD4⁺ T-cells, pro-inflammatory cytokines, and other molecules present in the host attack and destroy transplanted islet β cells. (B) Activation of T-cells in host immune response to transplanted islets. Antigen presenting cells (APCs) take and process antigens from donor and present antigens to host T cells to activate host immune response. Dendritic cells, macrophages, passenger leukocytes, and mononuclear cells are major APCs involved in the antigen presentation. Three signals are needed to fully activate T cells: signal 1, recognition of major histocompatibility complex (MHC) and the bound peptide by the T-cell receptor on the host T cells; signal 2, interaction of co-stimulation signals (such as CD40, CD80/86) with their corresponding receptors on host T cells; signal 3, soluble cytokines (IL-12, IL-10, IL-2) further stimulate the proliferation and differentiation of T cells. These interacting cell surface molecules are also RNAi targets for reduced immune rejection.

Figure 4. Combinatorial RNAi strategies for islet genetic modification. **(A)** siRNA pool targeting different sites of a single gene or targeting multiple genes. **(B)** Co-expression of multiple shRNAs, **(I)** multiple shRNAs under separate promoters, **(II)** miRNA cluster mimics, **(III)** long hairpin RNA. **(C)** Co-expression of shRNAs and cDNAs, **(I)** from a single miRNA backbone, **(II)** from separate promoters.

Figure 5. Bipartite Vectors for co-expression of VEGF cDNA and shRNA against iNOS gene. Silencing of iNOS gene expression with a bipartite vector: (a) with two separate promoters, (b) with shRNA embedded in miRNA backbone and controlled under a single promoter. (c) VEGF expression levels of bipartite vectors. Modified from [115]

Figure 6.

Delivery strategies for gene silencing. Cationic liposomes and polymers are commonly used as transfection reagents for siRNA and shRNA. To avoid the use of cationic carriers, siRNA can also be conjugated to polymers such as poly(ethylene glycol) carrying targeting ligand(s). For enhanced and/or prolonged gene silencing, shRNA is often cloned into a viral (adenovirus, lentivirus, or adeno associate virus) vector. After administration, these delivery systems enter the cells mainly through endocytosis. Vector based shRNA is further translocated into cell nucleus, where shRNA is expressed to produce pri-shRNA, which is further processed into pre-shRNA by an enzyme named Drosha. Pre-shRNA is exported to the cytoplasm by exportin-5, where it is processed by Dicer (an RNase III enzyme) to produce functional siRNA. For siRNA bioconjugation or siRNA complex, the escape and release of free siRNA from endosome into cytoplasm is an essential step. Finally, siRNAs will be incorporated into RNA-induced silencing complex (RISC) and guide the recognition and degradation of target mRNA.

Figure 7. Common modification of introduced to siRNAs. **(A)** Modification of non-bridging oxygen internucleotide linkage. **(B)** Modification of the sugar unit.

Figure 8.

Transfection efficiency of siRNA into intact human islets. (A) Fluorescence is seen throughout islet surfaces with some concentrations on edges and interior surface. (B) Following transfection with FITC-labeled siRNA human islets were analyzed by flow cytometry after dispersing into single cells by trypsinization. Compared to the background fluorescence in control group, 21.5 & 28.3% of islet cells incorporated siRNA at 100 & 400 nM concentrations, respectively. Reprinted from [113].

Figure 9.

Silencing of caspase-3 gene with siRNA and adenoviral shRNA. (a) Effect of caspase-3 siRNA on caspase-3/7 activity in INS-1E cells. After transfection of INS-1E cells with siRNA/Lipofectamine 2000 complexes, the cells were incubated with the cytokine cocktail for additional 16 h. Short term transient caspase 3 were achieved which lasted up to 3 days transfection. (b) Adv-H1-caspase-3-shRNA silencing effect on caspase 3/7 activity in human islets. Upon transduction, islets were incubated in the fresh medium without cytokines, then were collected at the indicated days for determining caspase 3/7 activity. The silencing effect lasted over 5 days post transduction. Modified from [105].

Table 1

Some potential RNAi targets for improving islet transplantation

Obstacles	Solutions	RNAi target genes
Immune rejection		
Immune tolerant dendritic cells *		NF- κ B [84–87,117]
		IL-12 [87,88]
		CD80 or CD86 [89–91]
		CD40 [92,93]
		MyD-88 [94,263]
		SOCS[95]
Reduce islet immunogenicity [#]		Human leukocyte antigen [97–100]
Ischemia/reperfusion injury & Apoptosis		
Block apoptosis cascades [#]		Caspase 3, 8 [102–105]
		Fas [106–109]
Prevent complement activation [#]		C3, C5 α receptor [102,109,110]
Inhibit inflammatory pathways [#]		iNOS [111–114]
		TNF- α [109]
		NF- κ B [116,117]

* Targets in the host;

[#] targets in the transplanted islets

Table 2

Delivery strategies for enhance RNAi

Free siRNA	
Physical delivery	Hydrodynamic injection, In situ perfusion, Microporation
Chemical modification	Modifying phosphodiester backbone: Phosphorothioate RNA, boranophosphate RNA, methylphosphonate RNA Modifying ribose: 2'-O-methyl (2'OMe), 2'-fluoro(2'-F), 2'-O-fluoro-β-D-arabinonucleotide (FANA), 2'-O-(2-methoxyethyl)(MOE), locked nucleic acid
Complex formation	
cationic polymers	Polyethylenimine, Poly(L-lysine), Chitosan, Cyclodextrin, Poly(disulfide amine), Poly(beta-amino ester)
cationic lipids	Lipofectamine-2000, Fugene HD, RNAifect, Solid neleic acid lipid particle (SNALP)
Bioconjugation	
siRNA-lipid	Cholesterol, Bile acid and long chain fatty acid, Vitamine E
siRNA-peptide	Cell penetrating peptides: TAT, penetratin, Transportan
Other conjugates	PEG-siRNA, Targeting ligand-PEG-siRNA, Imaging probe(dye)-siRNA
Viral vectors	
Adenoviral vectors	AdEasy Adenoviral Vector System (Stratagene); Knockout Adenoviral RNAi System (Clontech); BLOCK-iT Adenoviral RNAi Expression System (Invitrogen); AdenoQuick cloning system (OD260)
Lentiviral Vectors	BLOCK-iT Lentiviral RNAi Expression System (Invitrogen); Lenti-X shRNA Expression System (clontech); SMARTvector 2.0 Lentiviral shRNA technology (Dharmacon); shRNA libraries TRC lentiviral shRNA library(openbiosystems).