Therapeutic potential of RNAi in metabolic diseases

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Over the past years RNA interference (RNAi) has exploded as a new approach to manipulate gene expression in mammalian systems. More recently, RNAi has acquired interest as a potential therapeutic strategy. This review focuses on the potential therapeutic use of RNAi for metabolic diseases, the current understanding of RNAi biology, and how RNAi has been utilized to study the role of different genes in the pathogenesis of diabetes and obesity. Also reviewed are the in vivo proof-of-principle experiments that provide the preclinical justification for the development of RNAi-based therapeutics for diabetes and the key challenges that currently limit its application in the clinical setting.

INTRODUCTION

RNA interference (RNAi) is an ancient mechanism of gene regulation, found in eukaryotes as diverse as yeast and mammals, and probably plays a central role in controlling gene expression in all eukaryotes (1). Using small interfering RNA (siRNA) molecules, RNAi can selectively silence essentially any gene in the genome. Once in a cell, a short double-stranded RNA (dsRNA) molecule is cleaved by an RNase called Dicer (2) into 21- to 23-nucleotide guide RNA duplexes called siRNAs that become bound to the RNA-induced silencing complex (RISC) (3,4). Within the RISC, one of the two strands of the siRNA is chosen as the antisense strand via cleavage of the passenger strand (5–7), so that they can target complementary sequences in messenger RNAs (mRNAs) involved in a disease (8). After pairing with an siRNA strand, the targeted mRNA is cleaved and undergoes degradation thereby interrupting the synthesis of the disease-causing protein (9). The RISC complex is naturally stable within the cell, enabling siRNAs to cut multiple mRNA molecules consecutively and, therefore, suppressing protein synthesis in a potent and targeted way.

RNAi was described by the journal *Science* as the "Breakthrough of the Year" in 2002 having the potential to become a powerful therapeutic drug. RNAi-based therapeutics has potentially significant advantages over traditional approaches to treating diseases, including broad applicability, therapeutic precision, and selectivity avoiding side effects. This widespread applicability, coupled with relative ease of synthesis and low cost of production make siRNAs an attractive new class of small-molecule drugs. RNAi-based drugs are designed to destroy the target RNA and therefore stop the associated undesirable protein production required for disease progression.

Several recent studies using highly sensitive microarray analyses have shown that siRNAs can have off-target effects by silencing unintended genes (10,11). These off-target effects can be minimized by modifying the siRNAs to prevent incorporation of the sense strand into RISC and by choosing sequences with minimal complementarities to known genes in the database (12). Finding siRNAs that are active at low concentrations should help to abrogate some of these problems.

For RNA-based therapies, manufacture has been seen as a solvable problem, while delivery and stability have been the most significant obstacles. There are two strategies for delivering siRNAs in vivo (13). One is to stably express siRNA precursors, such as short hairpin RNAs (shRNAs), from viral vector using gene therapy; the other is to deliver synthetic siRNAs by complexing or covalently linking the duplex RNA with lipids and/or delivery proteins. To solve the problem of cell penetration, most researchers have either complexed the RNA with a lipid or modified the RNA's phosphate backbone to minimize its charge. Despite the questions and unsolved problems, several companies are moving ahead to develop RNAi therapy for many diseases including diabetes.

RNAi IN VIVO TARGETING LIVER

Liver is the prime organ target for systemically delivered siRNA, which tends to concentrate in this organ whether delivered hydrodynamically or with cholesterol or lipid carriers (14–20). Initial studies showing the activity of RNAi in vivo involved delivery of siRNA in mouse liver by using the hydrodynamic method, this consists of a rapid injection of a large volume of aqueous solution into the mouse tail vein, which creates a high pressure in the vascular circulation, leading to an extensive delivery of siRNA into hepatocytes (14–18). However, the sudden volume load induces right-sided heart failure, and the resulting high venous pressures permit the siRNAs to enter into the cells (21). This procedure allows high efficiency of siRNA uptake and potent siRNA activity in hepatocytes, but is not clinically viable because of the potential damage of the liver and other organs and, therefore, is limited

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only to research on liver function and metabolism or liver infectious diseases such as hepatitis (15–17).

As one step toward the liver-targeting delivery, liver delivery of chemically modified oligonucleotides with cholesterol conjugates was tested, as described in recent publications (19,20). Soutschek et al. (19) developed chemically modified siRNAs to silence an endogenous gene encoding apolipoprotein B (apoB). apoB is a molecule involved in the metabolism of cholesterol, and the concentrations of this protein in human blood samples correlate with those of cholesterol, and higher levels of both compounds are associated with an increased risk of coronary heart disease (22–24). The siRNAs synthesized by this group contained selective stabilizing modifications and were joined to a cholesterol group that was chemically linked to the terminal hydroxyl group of the sense-strand RNA (19). Intravenous injections of the siRNA-cholesterol conjugates in mice resulted in uptake into several tissues, including the liver, jejunum, heart, kidneys, lungs, and fat tissue and efficiently reduced the levels of apoB mRNA by more than 50% in the liver and by 70% in the jejunum. This reduction resulted in a lowering of the levels of blood cholesterol comparable to that observed in mice in which the apoB gene had been deleted (23). These results demonstrated that siRNA can be delivered systemically targeting the liver and suggest that RNAi has the potential to become a new therapeutic for the treatment of metabolic diseases.

RNAi AND METABOLIC DISEASES

Targeting Liver In Vivo

Insulin resistance is a major hallmark in the development of type II diabetes, which is characterized by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in muscle. Thus, the regulation of hepatic gluconeogenesis is an important process in the adjustment of the blood glucose level, and pathological changes in the glucose production of

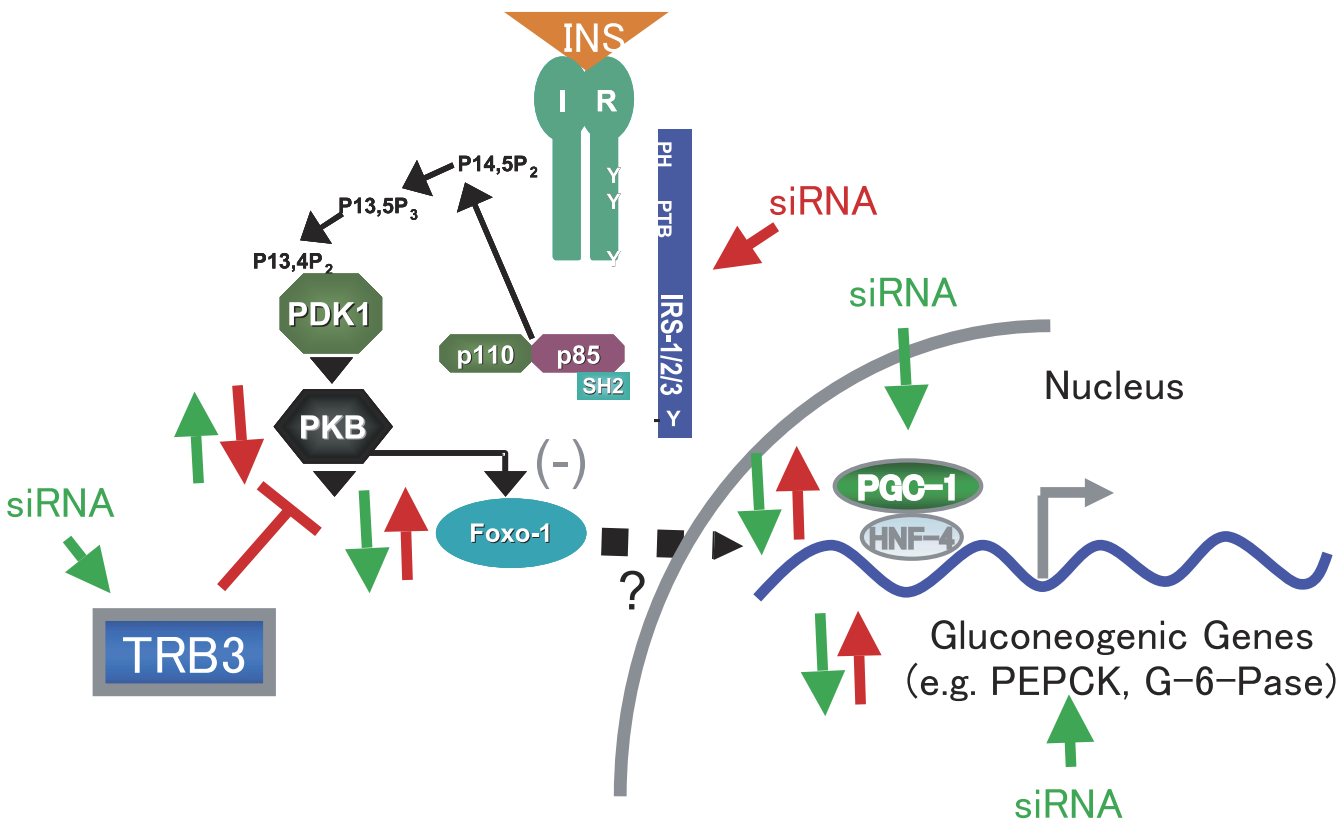

Figure 1. Insulin signaling pathway regulating gluconeogenic genes in liver. Insulin receptor (IR) kinase activation leads to the phosphorylation of insulin receptor substrate (IRS) proteins resulting in the activation of the lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase) and the 3-phosphatidylinositol-dependent kinase-1 (PDK1). PDK1 phosphorylates the protein kinase B (PKB/Akt), which in turn phosphorylates the transcription factor Foxo1. Phosphorylation of cytoplasmic Foxo1 triggers the release of nuclear Foxo1 from its co-activator, whereupon it is exported from the nucleus, thus terminating the transcription of genes encoding gluconeogenic enzymes through the transcription factor hepatocyte nuclear factor 4 (HNF-4) and the peroxisome proliferator-activated (PPAR) γ-co-activator 1 (PGC-1). The mammalian tribbles homolog TRB-3 binds PKB and thereby prevents its phosphorylation and activation. Recent studies utilized RNA interference (RNAi) technology to target key genes involved in the regulation of gluconeogenesis, such as IRS-1, IRS-2, PGC-1, TRB-3, and phosphoenolpyruvate carboxykinase (PEPCK). Green arrows indicate positive regulation of insulin signaling and glucose homeostasis. Red arrows indicate negative regulation of insulin signaling and glucose homeostasis. siRNA, small interfering RNA.

the liver are a central characteristic in type II diabetes (25). The liver's central role in the control of glucose homeostasis is subject to complex regulation by substrates, insulin, and other hormones. Pharmacological intervention in signaling events that regulate the expression of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and the catalytic subunit glucose-6-phosphatase (G-6-Pase), as well as glycogen synthesis and fatty acid oxidation, in liver has been long regarded as a potential strategy for the treatment of metabolic aberrations associated with this disease (Figure 1). However, availability of drugable targets has obstructed drug discovery in this area, making some of these targets uniquely suitable for an RNAi approach. A few studies utilized RNAi technology to target key genes involved in the regulation of gluconeogenesis (Figure 1) and provided in vivo proof-of-principle for the development of RNAi-based therapeutics for diabetes.

Several studies have demonstrated the use of RNAi to target genes involved in insulin action in liver (26). One group developed an adenovirus-mediated RNAi technique that utilizes shRNAs to substantially and stably knock down insulin receptor substrates, IRS-1 and IRS-2, expression specifically in the livers of mice to better understand the roles of these proteins in hepatic insulin action (26). By this technique, they have taken advantage of the relative tissue specificity of adenovirus for liver and the genetic specificity of shRNA-mediated RNAi to create liver-specific down-regulation of IRS-1 and IRS-2. By knocking down IRS-1 and IRS-2 separately and together in liver, they showed that IRS-1 signaling may be more closely linked to the regulation of genes involved in glucose homeostasis, whereas IRS-2 signaling may have specific roles in the regulation of hepatic lipid metabolism. Moreover, the concomitant knockdown of IRS-1 and IRS-2 in liver resulted in fasting hyperglycemia, fasting hyperinsulinemia, insulin resistance, glucose intolerance, and dyslipidemia in mice. Thus, the differential modulation of hepatic IRS expression and signaling may represent a key component of the molecular pathophysiology that underlies both type II diabetes mellitus and the metabolic syndrome. Moreover, adenovirus-mediated RNAi represents a promising procedure to further understand the hepatic gene function and in vivo physiology.

A different study showed the adenoviral delivery of peroxisome proliferator-activated (PPAR) γ-co-activator 1 (PGC-1) siRNA to the liver (27). This nuclear hormone receptor co-activator has been implicated in the onset of type II diabetes. Hepatic PGC-1 expression is elevated in mouse models of this disease, where it promotes constitutive activation of gluconeogenesis and fatty acid oxidation (28). Treatment with PGC-1-siRNA caused reduction of gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G-6-Pase), fasting hypoglycemia, and hepatic insulin sensitivity in mice, reflecting in part the reduced expression of the mammalian tribbles homolog TRB-3, a fasting-inducible inhibitor of the serine-threonine kinase protein kinase B (Akt/PKB) (29) (Figure 1). In addition, knockdown of hepatic TRB-3 expression improved glucose tolerance in mice (27). These results suggested a potential role for TRB-3 and PGC-1 in the treatment of type II diabetes. Using this technique, a different group validated in vivo the protein kinase Jun kinase-1 in an animal model of insulin resistance as a target for metabolic diseases and demonstrated the successful liver target validation via adenoviral vector-based RNAi (30).

Recently, a therapeutic, vector-based RNAi approach was used to induce posttranscriptional gene silencing of hepatic PEPCK using nonviral gene delivery (31). PEPCK is the rate-controlling enzyme in gluconeogenesis, and altered rates of gluconeogenesis are responsible for increased hepatic glucose output and sustained hyperglycemia (32). Treatment of diabetic mice with PEPCK siRNA caused a 50% decrease in hepatic PEPCK protein content and was sufficient to lower blood glucose and to improve glucose tolerance. These data reinforce the significance of PEPCK in sustaining diabetes-induced hyperglycemia and validate liver-specific intervention at the level of PEPCK for diabetes gene therapy.

Targeting Central Nervous System (CNS) for Obesity

RNAi holds promise for the development of novel therapeutic strategies for disorders that are yet difficult to treat and might be beneficial for the treatment of diseases, such as obesity, neuropathic pain, and depression. After the in vitro success in down-regulating gene expression in neurons, chemically synthesized siRNAs were tested for their in vivo gene knockdown ability in the brain. In the first such attempt, siRNA directed against the gene expressing agouti-related protein (AGRP) was locally injected into the hypothalamic arcuate nucleus of adult mice (33). AGRP gene expression is elevated by fasting, and in obesity, due to leptin insufficiency (34,35), while reversal of obese phenotypes by adrenalectomy reverses the elevation of AGRP mRNA (36). Injections of synthetic analogs of AGRP, and of AGRP itself, also stimulate food intake and body weight (37,38). Together with the observation that transgenic overexpression of the AGRP gene leads to hyperphagia and obesity (34), these data suggest that antagonism of AGRP may reduce food intake and body weight, thus potentially serving as a therapy for obesity. In addition, AGRP contributes to the energy homeostasis via inverse agonism at the melanocortin receptors expressed in several hypothalamic nuclei (39,40). Therefore, local injection of siRNA enabled investigating the physiological role of AGRP in the hypothalamic arcuate nucleus. AGRP mRNA levels were down-regulated up to 50% within 24 h of siRNA injection and led to a marked increase in the overall metabolic rate (33). Interestingly, an increased metabolism was not observed in AGRP-knockout mice (41). Thus, reducing AGRP expression in adult mice may have identified a metabolic role for AGRP not apparent from complete ablation of the gene, suggesting that agents antagonizing the effect of AGRP may be useful to treat obesity without producing unacceptable loss of appetite. Improvements in delivery

systems, especially in conjunction with viral-based gene transfer protocols (42), could result in greater and even longer-term reduction of target gene expression, suggesting that RNAi may prove to be a useful tool to assess physiological functions of mammalian genes in vivo, especially those expressed in the brain.

Another attempt to use siRNA in the brain demonstrated 30% reduction in the mRNA as well as protein levels of the α_{2A} adrenoreceptor (α_{2A}-AR), following local siRNA injections in the brainstem of neonatal rats (43). The α_{2A}-ARs have a critical role in regulating neurotransmitter release from sympathetic nerves and from adrenergic neurons in the central nervous system, as well as in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (44). Interestingly, in transgenic mice, a direct increased expression of these receptors can lead to impaired insulin secretion and glucose intolerance (45). Remarkably, this knockdown was also transient and reported only with repeated injections of siRNA for up to 3 days. These studies clearly advocated the need for alternative means of siRNA administration in order to prolong the gene knockdown effect. Moreover, local injections of siRNA restrict its entry to just a small number of cells that are in close proximity to the injection site. Therefore, when using such strategies, it is critically essential to specifically target those neuronal populations that are significant for manifestation of the loss-of-function phenotype in vivo.

Targeting Pancreas

Type I diabetes originates from autoimmune/inflammatory destruction of insulin-producing β cells in the pancreatic islet (46). Islet transplantation offers a potential cure for type I diabetes, and its conceptual advantages include the simplicity of the procedure with minimal complications, an automated process for high-yield islet isolation, and the potential to correct hyperglycemia before irreversible damage to organs occurs (47). However, its success has been limited, due to loss of cells by apoptosis stimulated by the procurement, ischemia, and the isolation process itself. Islet yield is diminished by apoptosis in response to mechanical stimuli from the isolation process, donor brain death, cytokine induction from stresses related to hemodynamic instability, and the procurement process, including cold perfusion and storage. Therefore, any process that would inhibit apoptosis or related insults should increase the yield of viable islets available for transplantation from one donor pancreas. Two different studies have described the successful introduction of siRNA directly into pancreatic islet cells, both during in situ perfusion and from intravenous tail vein injection (48,49). In one of the studies, the insulin 2 (Ins2) gene was targeted with siRNA, and mice received siRNA via hydrodynamic tail vein injection. In vivo delivery of siRNA to pancreatic islets revealed a 33% reduction in Ins2 mRNA levels. These studies provided the first steps for the use of RNAi technology in pancreatic islets and may provide a solution to maintain viable cells for transplantation.

Targeting Muscle and Adipose Tissues

Delivering siRNA in vivo to animal tissues such as muscle and adipose tissue is complicated, and it might involve using physical, chemical, or biological approaches and in some cases a combination of them. In the case of skeletal muscle, it can be accessible by local siRNA administration. Two recent studies with nonformulated siRNA delivered by direct injection into mouse muscle followed by electroporation demonstrated a significant gene silencing (50,51). In addition, a local hydrodynamic approach, in which a sufficient volume of siRNA was rapidly injected into a distal vein of a limb was tested for siRNA delivery in muscles of animal models and demonstrated a knockdown of both reporter and endogenous gene (52). However, targeting muscle and adipose tissue through systemic delivery of siRNA seems to be challenging, and no success has been reported in the literature. The most difficult issue might be the stability of siRNA in the extracellular and intracellular environments after systemic administration. Several problems need to be solved to deliver active siRNA in these target tissues, such as the excretion of siRNA through urine, its instability in the serum environment, the nonspecific distribution of siRNA throughout the body decreasing the local concentration in these tissues, and the overcoming of the blood vessel endothelial wall and tissue barriers. When siRNA finally reaches the target cells, cellular uptake and intracellular RNAi activity require efficient endocytosis and, indeed, this might be the most important issue for difficult-to-transfect cell types like muscle cells and adipocytes.

RNAi THERAPEUTICS FOR DIABETES

Despite several questions and unsolved issues related to the use of siRNA, different companies such as Sirna Therapeutics, Inc. (San Francisco, CA, USA), Alnylam Pharmaceuticals (Cambridge, MA, USA), Acuity Pharmaceuticals (Philadelphia, PA, USA), Atugen AG (Berlin, Germany), Benitec Australia Limited (Dulwich Hill, NSW, Australia), CytRx Corporation (Los Angeles, CA, USA), and Devgen (Gent, Belgium) are moving ahead to develop RNAi therapy for diabetes and diabetes complications (53).

Sirna Therapeutics, Inc., an RNAi therapeutics company, has selected a systemically delivered, chemically modified siRNA compound as its candidate for advancement to human clinical testing against the hepatitis C virus (54). The selection of this clinical candidate reflects two major advancements. The first advancement is the design, chemical modification, and synthesis of a stable and potent siRNA compound, and the second advancement is the development of a nanoparticle delivery technology capable of efficient and specific delivery of the siRNA compound to hepatocytes. This delivery technology enables Sirna Therapeutics to address other liver-associated disease indications such as diabetes. This group also claimed that they had promising results for the application of RNAi technology

in the treatment of diabetes using systemically delivered siRNA to reduce phosphatase 1B (PTP-1B) in liver, a validated target in diabetes associated with insulin resistance (55).

CytRx Corporation has initiated a program with University of Massachusetts Medical School in 2003 to develop drug compounds for use in the treatment of obesity and type II diabetes and to use RNAi technology for the development of novel products for the prevention, treatment, and cure of these diseases. The new unit initially focused on using a genomic- and proteomic-based drug discovery approach that leverages RNAi to swiftly screen and identify key drug targets and pathways in obesity and type II diabetes. CytRx has recently determined to advance a siRNA against RIP140, a novel target for metabolic diseases, into animal studies. Suppression of RIP140 by gene deletion or siRNA results in the acceleration of fat burning in animals and fat cells, respectively (56,57).

Other companies like Alnylan Pharmaceuticals developed chemically modified siRNAs to deliver systemically (58), opening the possibility of RNAi therapeutics for a range of different diseases, such as metabolic and cardiovascular diseases.

CONCLUSION

In the past few years, RNAi has arrived as a novel and essential biological process, as well as a powerful experimental tool and a potential therapeutic strategy. RNAi can be used to silence endogenous genes involved in the cause or pathway of metabolic diseases and holds considerable promise as a therapeutic approach to silence disease-causing genes, particularly those that encode so-called "non-drugable" targets. In addition, the high potency, specificity, and chemical structure of siRNAs may eliminate the toxicity and adverse events commonly seen with small molecule and other oligonucleotide approaches. Several studies have demonstrated efficient in vivo delivery of siRNAs and therapeutic benefit in mice. Ongoing and future preclinical studies in animal models will hopefully help optimize RNAi therapeutics for applications in humans. Although development of RNAi-based therapeutics for diabetes is in its infancy, early clinical studies are soon to begin assessing the use of this new class of therapeutics to tackle metabolic diseases, including diabetes and obesity.

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COMPETING INTERESTS STATEMENT

The author declares no competing interests.

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