

REVIEW

siRNA as a therapy for asthma

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RNAi is the process of sequence-specific, post-transcriptional/transcriptional gene silencing through siRNA. RNAi is a popular method of controlling gene expression and has potential in the development of drugs for several diseases, such as various types of cancer and viral infections. Gene therapy for asthma has already been developed and has demonstrated promising results in animal models. Recent progress in delivering siRNA to the respiratory system has also improved the therapeutic feasibility of RNAi for asthma. In this review, the mechanism, as well as the general strategies and problems associated with the application of RNAi in vivo, are discussed, focusing on the possibility of applying RNAi to alleviate airway inflammation in allergic asthma. Data from studies with siRNA against molecules involved in allergic responses are summarized, and the potential and limitations of RNAi as a novel therapeutic approach are discussed.

Keywords Allergy, asthma, off-target effect, RNAi, siRNA, therapy

Introduction

Asthma is a chronic airway inflammatory disease characterized by eosinophil infiltration and airway hyper-responsiveness (AHR). According to estimates from the WHO, 300 million individuals have asthma, while 255,000 individuals die from the disease annually, making asthma a global health problem [1]. The increasing incidence of asthma is associated with increased hygiene and decreased exposure to various microorganisms, an effect known as the 'hygiene hypothesis' that was first proposed by Strachan in 1989 [2], whereby microbial exposure in early life may prompt early immune maturation and prevent the development of allergic disease, including asthma.

The causes of asthma are complex and are likely to be a combination of environmental stimuli and susceptibility genes. Significant progress has been made in the past decade in the field of asthma genetics. Asthma susceptibility loci were reported in 1996 [3] and, subsequently, several genes and their polymorphisms were identified to modify the susceptibility and severity of asthma (reviewed in reference [4]). With an improved understanding of the interactions between environmental and genetic factors, additional preventional and/or therapeutic strategies for allergic diseases can be developed.

The majority of patients with asthma are affected by atopic (extrinsic) disease, with only a few patients experiencing non-atopic (intrinsic/non-IgE-mediated) disease. In atopic asthma, the individual is initially exposed to the allergens that activate T-helper (Th)2 cells and allergen-specific IgE synthesis, a process known as sensitization. Following sensitization and subsequent allergen exposure, allergen-bound IgE molecules activate mast cells, resulting in the release of histamine and other mediators, such as leukotrienes, prostaglandins and cytokines. These mediators contribute to increased vascular permeability, smooth muscle contraction and mucus production. Mast cells secrete chemokines and, along with epithelial cells, recruit other inflammatory cells, such as eosinophils and Th2 cells. Eosinophils release granular basic proteins and membrane-derived lipid mediators, which not only damage the bronchial epithelium, but also cause smooth muscle contraction and mucus secretion. In addition, eosinophils may also play a role in airway remodelling by releasing potent profibrotic cytokines, TGF β and other fibrogenic factors [5].

Asthma treatment currently consists mainly of inhaled corticosteroids, as well as both short- and long-acting β_2 -adrenoceptor agonists. However, these therapies are not always effective, do not cure asthma and are associated with side effects. In addition, several

therapeutic strategies aimed at modifying the immune system have been under development. Allergen-specific immunotherapy (SIT) induces immune tolerance, for example, by deleting T-cells or rendering them anergic and by inducing regulatory T-cells (Tregs) [6-9], and downregulates the production of allergen-specific IgE, while enhancing the production of IgA and IgG4. Subcutaneous immunotherapy is the only method of SIT with an FDA-approved formulation, and sublingual immunotherapy is an acceptable treatment for children.

Several other immunomodulatory methods that target effector cells or molecules involved in allergic responses by mAbs or inhibitor compounds have been reported. Omalizumab (Xolair), widely launched for the treatment of asthma, is a recombinant DNA-derived humanized IgG1 κ mAb that binds to the Fc (fragment crystallizable) region of the IgE molecule, preventing it from binding to IgE cell-surface receptors [10,11]. CAT-354 (MedImmune LLC), a human IL-13 mAb antagonist [12], and MEDI-528 (MedImmune LLC), an injectable humanized antibody specific for IL-9 [13], were undergoing phase II clinical trials for asthma at the time of publication.

In addition to 'traditional' drugs, gene therapy has been assessed in asthma, and has demonstrated promising results in mouse models. Several gene therapies, such as CpG oligodeoxynucleotides (ODNs), non-CpG ODNs, antisense oligonucleotides (AS-ONs), DNA vaccines and siRNA, all of which moderate airway inflammation by enhancing Th1 responses or by suppressing Th2 responses, have been used for asthma treatment. Recently, RNAi has been used as a strategy for gene-targeted therapy for cancer, viral infections and allergic diseases. This review presents the mechanism and gene targets of siRNA, and discusses the challenges associated with the clinical application of RNAi as a therapy for asthma.

Gene therapy for asthma

Gene therapy in asthma regulates the function of genes that affect immune responses in patients with asthma by augmenting Th1 responses, reducing the expression of Th2 cytokines, or suppressing Th2 effector cells. For example, unmethylated CpG ODNs have been used in the prevention [14] and suppression [15,16] of allergen-induced airway inflammation, as well as in the inhibition of airway remodeling [17,18], based on their ability to stimulate innate immunity mechanisms and to augment Th1 responses. DNA vaccines have been used as preventive or therapeutic agents against asthma by delivering a simple DNA plasmid to express antigen *in vivo* [19,20], or by inducing adaptive immunity to moderate allergic responses [14]. AS-ONs have also been broadly applied in the development of therapeutics for asthma or other allergic diseases [21-23]. Since RNAi was first identified in *Caenorhabditis elegans* in 1998 by Fire *et al* [24] and its existence was proven in mammalian cells in 2001 [25], studies and applications of siRNA have greatly advanced, thus rendering RNAi a potential therapeutic approach for several diseases.

The mechanism of RNAi

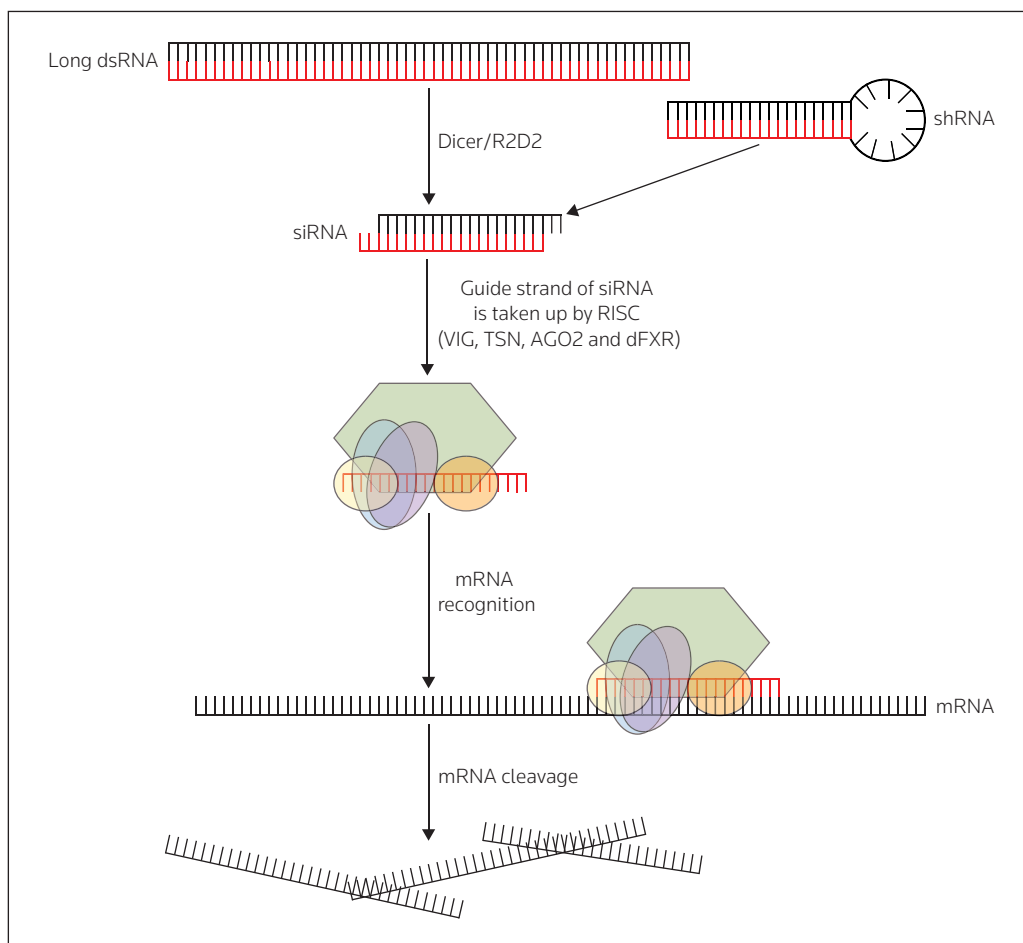
RNAi is similar to the earlier identified phenomena of post-transcriptional gene suppression in plants and quelling in *Neurospora crassa*. The classical suppressive mechanism of RNAi, which silences gene expression by the degradation of mRNA [26,27], has been fully characterized in *Drosophila* and is thought to have a similar mechanism of action in mammalian cells [25]. Long lengths of dsRNA are digested into siRNA (consisting of 21 to 23 dsRNA nucleotides with symmetric 2- to 3-bp overhangs) [28-31], the major effector molecule of RNAi by the R2D2/Dicer heterodimer [25,32]. The antisense strands of siRNAs are taken up by the RNA-induced silencing complex (RISC) and the RNA endonuclease activity of RISC is then directed to the homologous site of the target mRNA, resulting in mRNA cleavage [33-36] (Figure 1).

DNA targeting has also been reported as a suppressive mechanism of RNAi in mammalian cells: siRNAs induce DNA and histone H3 methylation, inhibiting gene transcription or the establishment of silenced chromatin [37,38]. The possible mechanisms of transcriptional regulation by siRNA were discussed and reviewed in 2005 by Kawasaki *et al* [39-41].

Targeting mRNA is a more efficient way of reducing gene expression at the protein level compared with direct blocking of protein activity by mAbs or inhibitor compounds. Prior to the discovery of RNAi, AS-ONs were the major anti-mRNA strategy to have been developed during the past 20 years. AS-ONs are synthetic ssDNA molecules that usually consist of 15 to 20 nucleotides, which are complementary to the target mRNA. The idea of using AS-ONs as a tool for the specific suppression of gene expression was first proposed more than 30 years ago [42]. The major mechanisms of action of AS-ONs include the activation of endonuclease RNase H to degrade mRNA [43], the inhibition of translation via steric blocking of ribosomal complex formation and the inhibition of RNA splicing [44]. However, in 2002, Bertrand *et al* compared the efficiency of siRNA and AS-ONs in cells and demonstrated that siRNAs were quantitatively more efficient at reducing gene expression and their effects were longer-lasting compared with AS-ONs [45]. The inefficiency of AS-ONs is probably a result of their sensitivity to endogenous nucleases [45,46]. In order to improve the *in vivo* efficiency of AS-ONs, several chemical modifications have been developed that increase nuclease resistance [46,47], affinity for RNA, thermal stability and efficacy, while decreasing AS-ON toxicity and non-specific immune stimulation *in vivo* [48].

RNAi in the treatment of allergic diseases/asthma

Although many genes have been identified to participate in the pathogenesis of asthma, not all of these genes are considered appropriate targets for gene therapy because of the redundancy in the activity of these genes in

Figure 1. The classical mechanism of action of RNAi.

Long dsRNAs are digested into the major effector molecule siRNA (consisting of 21 to 23 dsRNA nucleotides with symmetric 2- to 3-overhangs) [28-31], by the Dicer/R2D2 heterodimer [25,32]. The guide (antisense) strand of siRNA is unwound from the duplexed siRNA molecule and taken up by the RNA-induced silencing complex (**RISC**), comprised of the Vasa intronic gene (**VIG**), the Tudor staphylococcal nuclease (**TSN**), Argonaute 2 (**AGO2**) and fragile-X-related protein (**dFXR**) [33-36]. The guide strand of siRNA leads the activated RISC complex, which exhibits endonuclease activity, to the homologous site on the mRNA, leading to cleavage of the target mRNA.

shRNA short hairpin RNA

asthma pathology. The most promising targets include genes coding for cytokines (eg, IL-4, IL-5 and IL-13), chemokines (eg, eotaxin), cytokine and chemokine receptors (eg, IL-4 receptor [R] and IL-5R α , and CCR-3, respectively), growth factors and signal transduction mediators, as well as for transcription factors (eg, STAT6 and NF κ B) that are involved in airway inflammation. Evidence suggests that AS-ONs targeting factors involved in allergic responses could moderate allergic diseases (reviewed in references [21,23]). Such AS-ON-directed gene regulation can provide useful information for the choice of potential genes to be targeted by siRNAs in allergic diseases.

The therapeutic applications of RNAi are most well-studied in the fields of cancer and viral infections. siRNAs were also reported to inhibit activation of effector cells and Th2 responses in models of allergic disease *in vitro* [49-51]. A study by Trian *et al* demonstrated that siRNAs against protease-activated

receptor 2 (PAR-2) suppressed tryptase-induced free intracellular Ca²⁺ concentration in human airway smooth muscle cells, identifying PAR-2 as a potential therapeutic target in bronchial hyper-responsiveness [52]. The genes that have been assessed as siRNA targets for the treatment of allergic diseases in preclinical models are summarized in Table 1.

RNAi target genes for the treatment of asthma

Surface molecules

CD40

The expression of CD40, a member of the TNF receptor superfamily, is induced upon APC maturation and provides additional maturation signals to the APCs. The interaction between CD40 on APCs and CD154 (CD40 ligand) on T-cells activates T-cells; CD40 ligand crosslinking can increase Th2 cytokine expression and stimulate T-cell proliferation. Signals from cytokines (IL-4 and IL-13)

Table 1. Gene targets for RNAi in mouse models of allergic diseases.

Targets	Type of siRNA/route of administration	Experimental model	Reference
Transcription factors/signal transduction			
GATA-3	Lentiviral shRNA; intratracheal	Mouse model of allergic asthma	[65]
STAT6	Synthetic siRNA; intranasal	Mouse model of allergic asthma	[73]
SphK	Synthetic siRNA; intranasal	Mouse model of allergic asthma	[143]
Receptors			
NPRA	Synthetic siRNA nanoparticle; intranasal or transdermal	Mouse model of allergic asthma	[144]
Surface molecules			
CD40	Synthetic siRNA; intraperitoneal	Mouse model of allergic asthma	[56]
CD86	Synthetic siRNA; topical (ointment)	Mouse model of contact hypersensitivity/atopic dermatitis-like disease	[59]
Cytokines and chemokines			
IL-4	Lentiviral shRNA; intratracheal	Mouse model of allergic asthma	[Lee CC, Huang HY, Chiang BL: unpublished data]
IL-5	Lentiviral shRNA; intratracheal	Mouse model of allergic asthma	[90]
IL-13	Lentiviral shRNA; intratracheal	Mouse model of allergic asthma	[Lee CC, Huang HY, Chiang BL: unpublished data]
	Synthetic siRNA; intravenous	Mouse model of allergic asthma	[82]
Eotaxin	Local delivery; intratracheal	Mouse model of allergic asthma	[93]
MCP-1	Synthetic siRNA; intravenous	Mouse model of contact hypersensitivity/allergic contact dermatitis	[101]

GATA-3 GATA-binding protein-3, **MCP-1** monocyte chemoattractant protein-1, **NPRA** natriuretic peptide receptor A, **shRNA** short hairpin RNA, **SphK** sphingosine kinase

and ligation of CD40 are also crucial for the synthesis of IgE by B-cells [53]. In 2007, Linhart *et al* demonstrated that treatment with an anti-CD154 antibody at the time of sensitization prevented the development of allergy in a murine model of grass pollen allergy [54]. However, anti-CD154 antibody treatment in humans may cause thrombosis because CD154 is expressed on platelets [55]. Recently, Suzuki *et al* demonstrated that the administration of siRNA against CD40 reduced the nasal symptoms and eosinophilia associated with the mouse ovalbumin (OVA)-induced allergy model [56]. It was also demonstrated that the therapeutic effects of siRNA treatment were associated with impaired antigen-presenting function of dendritic cells and the generation of Treg cells.

CD86

CD86, one of the B7 family molecules, is expressed on the surface of APCs and provides a costimulatory signal for T-cell activation. Such a costimulatory signal, delivered through the interaction of CD86 with CD28, has been demonstrated to be required for allergen-induced IL-5 production in bronchial biopsy tissues derived from patients with atopic mild asthma, and for the production of IL-5 and IL-13 in PBMCs from the same patients [57]. In response to allergen, blocking the interaction between CD28 and CD86, but not CD80, inhibited the proliferation of airway T-cells obtained from patients with atopic asthma [57]. Consistent with data obtained from

the study by Larché *et al*, treatment with an anti-CD86 antibody effectively abolished the development of allergic asthma and AHR in a mouse asthma model [22,49]. Recently, Crosby *et al* reported that inhaled AS-ONs against CD86 could suppress airway inflammation and AHR in the mouse OVA-induced asthma model [58]. Ritprajak *et al* also demonstrated that local delivery of siRNA targeting CD86 efficiently inhibited contact hypersensitivity and atopic dermatitis-like disease in a mouse model [59].

Transcription factors

GATA-3

GATA-binding protein-3 (GATA-3) is a key transcription factor for Th2 polarization and initial Th2 cytokine expression [60,61]. The gene expression levels of GATA-3 are increased in the airways of individuals with atopic asthma [62], and airway inflammation was successfully inhibited in a dominant-negative GATA-3 mutant transgenic mouse model of asthma [63]. Consistent with findings from the study by Zhang *et al*, Finotto *et al* reported that local blockade of GATA-3 expression by AS-ONs reduced lung inflammation, including the infiltration of eosinophils, AHR and Th2 cytokine production in a mouse model of asthma [64].

In addition, lentiviruses expressing GATA-3-specific short hairpin RNA (shRNA), when administered locally, efficiently alleviated OVA-induced airway inflammation and hyper-

responsiveness in mice [65]. Sel *et al* also reported that downregulation of GATA-3 by a specific DNzyme efficiently prevented airway inflammation in a mouse model of acute and chronic experimental allergic asthma [66].

STAT6

STAT6 is activated by IL-4 [67] and IL-13 [68], and plays an important role in IgE production and AHR and inflammation [68,69]. STAT6 is also crucial for Th2 cell differentiation and, in a study of STAT6-deficient mice, Th2 responses could not be induced, thus blocking the generation of allergen-specific IgE production, eosinophilia and AHR [70,71]. In another study, STAT6-targeted RNAi reduced the production IL-4- or IL-13-induced eotaxin production efficiently [72,73]. Furthermore, Darcan-Nicolaisen *et al* demonstrated that siRNA against STAT6 inhibited allergic airway inflammation and AHR in a mouse model of OVA-induced asthma [73].

NFκB

There is both clinical [74,75] and experimental [76,77] evidence indicating that the activation of NFκB is enhanced in asthma. Mice lacking NFκB p50 exhibited a reduced eosinophilic response to allergen challenge as a result of the lack of IL-5 and eotaxin production [78]. NFκB p50 knockout mice were also deficient in the production of macrophage inflammatory protein (MIP)-1α and MIP-1β, required for T-cell recruitment to inflammatory sites. In OVA-sensitized mice, intravenous injection with NFκB p65 AS-ON reduced p65 protein levels and activation of NFκB in the lungs significantly, effects that were associated with reduced airway inflammation, as well as reduced AHR and OVA-specific IgE production [79].

Th2 cytokines and chemokines

IL-4 and IL-13

IL-4 and IL-13 are two key cytokines released from activated Th2 cells, and are associated with the development of asthma and other allergic diseases. IL-4 is required for the differentiation of naïve T-cells to Th2 cells. In addition to the role of IL-4 and IL-12 in the initial differentiation and expansion of antigen-specific Th2 cells, these cytokines are also important for the induction of class switching and IgE production in B-cells [53]. Consistent with these roles of IL-4, airway inflammation and antigen-specific IgE production could not be induced in IL-4-deficient mice [80]. In addition, Molet *et al* demonstrated that IL-4 AS-ONs decreased IL-4 expression and inhibited adoptively transferred late-phase allergic responses [81]. Recently, Lively *et al* demonstrated that intravenous delivery of chemically modified siRNA could reduce airway resistance by 60% in siRNA-treated mice [82]. Intratracheal administration of IL-4- or IL-13-specific siRNA has also been demonstrated to efficiently suppress AHR and eosinophilia in a mouse model of OVA-induced asthma [Lee CC, Huang HY, Chiang BL: unpublished data].

IL-5

IL-5 plays a critical role in the development of AHR and eosinophilia. IL-5 is important for growth and differentiation of eosinophils in the bone marrow

[83], as well as for eosinophil maturation [84], migration [85] and activation [86] in local tissues. Moreover, the levels of IL-5 correlate with clinical features [87] and IL-5 mRNA is upregulated in the bronchial mucosa of patients with allergen-provoked asthma [88]. Karras *et al* reported that intravenous delivery of IL-5 AS-ONs reduced eosinophilia and AHR in the mouse OVA-induced asthma model [89]. Similarly, local intratracheal delivery of IL-5 siRNA-expressing lentivirus suppressed lung eosinophilia and AHR [90].

Eotaxin

Eotaxin is the main chemoattractant for eosinophils. Eotaxin selectively binds to CCR3, which is highly expressed on eosinophils, basophils and mast cells, all of which are important cell types in the pathogenesis of asthma. In asthmatic airways, eotaxin production is increased in the bronchial lumen and mucosa [91]. In 2000, Fujisawa *et al* reported that chemokine-induced eosinophil degranulation was mediated exclusively through CCR3 [86]. In addition, eotaxin is more potent than other CCR3-binding chemokines, such as RANTES, MIP-3 and MIP-4, inducing eosinophil degranulation and leukotriene C₄ release [86]. Fortin *et al* demonstrated that eosinophilia and AHR were reduced in OVA-challenged rat by inhibiting CCR3 expression in the lung with CCR3-specific AS-ONs [92]. Recently, it was also demonstrated that eotaxin-specific shRNA could inhibit airway inflammation in the mouse OVA-induced asthma model [93].

Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1, chemokine [C-C motif] ligand 2 [CCL2]) is another chemokine that plays an essential role in the pathogenesis of allergic inflammation. MCP-1 attracts not only monocytes, but also T-cells [94,95] and NK cells [96], and induces histamine release from basophils [97] and mast cells [98]. MCP-1 also enhances Th2 polarization [99] and induces fibroblasts to produce TGFβ and procollagen [100]. In 2008, Ishimoto *et al* applied siRNA against MCP-1 in a mouse contact hypersensitivity model, resulting in the suppression of ear swelling and a decrease in the infiltration of monocytes/macrophages and T-cells [101]. Accordingly, siRNA against MCP-1 might represent a potential therapeutic modality for other allergic responses.

Factors affecting the efficacy of siRNA *in vivo*

siRNA has been proven to inhibit the expression of target genes *in vitro* efficiently and specifically. However, several problems are encountered when RNAi is applied *in vivo* [102]. siRNA molecules are small, unstable and easily excreted into the urine when administered in the bloodstream. Thus, the stability of siRNA *in vivo* is a major barrier to the application of RNAi therapeutically. Another major challenge is the specificity and efficiency of siRNA delivery *in vivo* [103,104]. Most mammalian cells do not express receptors for dsRNA (as present on *C. elegans* cells) and do not uptake siRNA [103]. Therefore, *in vivo* delivery of siRNA usually fails to

achieve significant and specific silencing effects [105,106]. Although DeVincenzo *et al* demonstrated that ALN-RSV01 (Alnylam Pharmaceuticals Inc/Kyowa Hakko Kirin Co Ltd/Cubist Pharmaceuticals Inc), an siRNA against the N-protein of the respiratory syncytial virus, was safe and well tolerated when delivered intranasally to humans [107], several problems must be overcome to improve the efficacy of siRNA *in vivo* to allow the therapeutic application of RNAi in other nonviral infectious diseases. The main strategies that are used to overcome the inefficiency of RNAi, as caused by the instability or incorrect and/or non-specific delivery of siRNA, are discussed in the following sections.

Stability of siRNA

Chemical modification

For synthetic siRNAs, chemical modifications and conjugates have been used to increase stability and nuclease resistance, as well as reduce non-specific effects and immune activation. These modifications include substitution of individual nucleotides with nucleotide analogs [46,108], phosphodiester modifications (phosphorothioate linkage and boranophosphonate linkage), 2'-sugar modifications (2'-*O*-methyl [2'-OMe] and 2'-deoxy-2'-fluoro [2'-F] modifications) and the addition of conjugates on one or both siRNA strands [105].

DNA vector-based siRNA

Using DNA vectors represents an alternative approach for the delivery of siRNA into cells. However, the delivery of synthesized or DNA vector-based siRNA results in only transient gene knockdown because of siRNA degradation and dilution as a result of cell proliferation [103]. Retroviral (Moloney murine leukemia virus and lentivirus [109]), adenoviral and adeno-associated viral (AAV) vectors [110,111] have therefore been used as siRNA delivery systems to overcome the limitations of synthesized or DNA vector-based siRNA.

Although a retroviral vector can deliver and insert the siRNA expression cassette into the genome of target cells, thus extending the duration of the siRNA silencing effect, the random insertion may cause mutation of other genes, resulting in a variety of side effects, such as the activation of oncogenes and the induction of tumors. Therefore, retroviral shRNA delivery (ie, viral delivery of the siRNA prior to being processed in the cell to its final form) can be used for local tumor therapy, but systemic administration should be avoided. Other non-integrative viruses, such as adenovirus and AAV, may be more viable as virus-based shRNA delivery systems for clinical application. However, nonviral delivery was used in the majority of clinical trials assessing siRNA in different diseases [112], therefore, at the time of publication, nonviral delivery systems, including synthesized and DNA vector-based siRNA, remained the most feasible therapeutic strategy.

The promoters that drive siRNA expression are the major factors determining the intensity and duration of gene silencing by siRNA-expressing vectors. RNA

polymerase (Pol) II and Pol III promoters could be used to express shRNA, siRNA or microRNA (miRNA). H1 and U6 are the two predominant RNA Pol III promoters used to drive shRNA expression in vector- or viral-based systems [113]. Recently, Takahashi *et al* compared the gene silencing kinetics of shRNA driven by U6, H1 or tRNA promoters, and demonstrated that the U6 promoter had a more potent and longer-lasting silencing effect compared with the other two promoters tested [114].

However, RNA Pol III promoters used for shRNA expression, such as H1, U6 and tRNA promoters, are ubiquitously expressed and are not time- or tissue-specific. Conversely, RNA Pol II promoters can be expressed in specific tissues or can be expressed conditionally [115]. Yuan *et al* designed shRNAs driven by the CMV promoter (RNA Pol II promoter), and were able to knockdown genes specifically and efficiently in mammalian cells [116]. Furthermore, in two studies in which the miRNA system was mimicked to establish a tissue-specific RNA Pol II promoter-driven siRNA approach, cell-type or tissue-specific gene silencing was achieved *in vivo* [117,118].

Routes of delivery of siRNA

One critical issue for the efficacy of RNAi therapy is how RNAi targets tissues. The routes of RNAi delivery *in vivo* are either systemic or local, depending on the tissues or cell types that are being targeted. Local delivery is usually used for targeting genes in the skin, muscles and joints, while systemic delivery efficiently targets the liver, lungs and tumors. However, both local and systemic delivery approaches are useful for lung and tumor targeting.

Ligand-targeted delivery

Ligands that recognize cell-specific receptors expressed by target cells can be conjugated to polymers and cationic lipids, facilitating specific cellular uptake of siRNA via receptor-mediated endocytosis. For example, the Arg-Gly-Asp motif peptide, a ligand for $\alpha_v\beta_3$ integrins that is expressed on activated endothelial cells and tumor cells, guides conjugated polymers containing siRNA fragments to specific target cells [119,120].

Antibody-targeted delivery

Cell-specific siRNA delivery usually depends on targeting surface markers by antibodies. Two major strategies used for cell-specific delivery of siRNA are based on ligand-recognition antibodies, which are fused with small basic nucleic acid-binding proteins and nanoimmunoliposomes. The use of antibodies to deliver siRNA to specific cell types has been reviewed by Liu [121].

Off-target siRNA effects and inappropriate immune activation

Although RNAi silences genes by sequence-specific methods, recent studies have indicated that siRNA can trigger off-target effects through miRNA-dependent or -independent mechanisms, resulting in unintentional immune activation in mammalian cells [122]. Current

efforts are aimed at improving the understanding of the causes of off-target siRNA effects and siRNA-triggered immunostimulation, in order that these undesirable effects can be avoided.

Causes of off-target siRNA effects

The off-target effects of siRNA can be mediated by sequence mismatches, or the induction of innate immune responses. Generally, non-target mRNA degradation is caused by partial complementarity between siRNA and mRNA. siRNA silencing is highly sequence-specific, although a single mismatch in the central region of siRNA can be tolerated. While several studies have demonstrated that even single nucleotide mismatches between siRNA and target mRNA significantly decrease the degradation of target mRNA, data from microarray analyses indicate that siRNA crossreacts with imperfectly matched mRNA targets [123]. The central region of the siRNA-mRNA binding pair is the most crucial for the maintenance of a silencing phenotype and, usually, changes in this site can distinguish between genes that differ by a single nucleotide. The degree and the position of the mismatch between the siRNA and the target mRNA determines the suppressive mechanism [124,125]. Four or more nucleotide mismatches can induce complete inactivation of the RNAi mechanism, preventing RISC from recognizing the target mRNA. However, partial or perfect matches between the siRNA seed region (positions 2 to 7, or 2 to 8) and the 3' untranslated region (UTR) of the target mRNA, but not the 5' UTR or the open reading frame, could activate the miRNA suppressive mechanism, blocking the translation of mRNA and causing non-specific gene silencing [126]. The interchange of the siRNA and miRNA suppressive mechanisms represents the major reason for the non-specific silencing of siRNA [123,127].

Long dsRNA induces immune responses through cytoplasmic receptors, such as retinoic acid inducible gene-I (RIG-I), melanoma differentiation-associated gene-5 (MDA-5), dsRNA-dependent protein kinase R (PKR) or 2'-5'-oligoadenylate synthase (2'-5'OAS). PKR bound to dsRNA or ssRNA phosphorylates the eukaryotic translation initiation factor 2 α (eIF2- α), leading to a strong binding of eIF2- α to eIF2B, and subsequent protein synthesis and apoptosis induction [128]. 2'-5'OAS is also activated by binding dsRNA that is longer than 15 bp. Upon RNA binding the conformation of 2'-5'OAS changes, the enzyme is activated and converts ATP to inorganic pyrophosphate and 2'-5'-linked oligoadenylates, which can promote the dimerization of RNase L. RNase L dimers degrade mRNA non-specifically. RIG-I and MDA-5 can induce the production of type I IFN, which, in turn, induces the expression of PKR and 2'-5'OAS, and causes non-specific gene silencing via the activation of IFN regulatory factor-3 and NF κ B [129].

The generation of inappropriate immune activation

It had been hypothesized that dsRNA longer than 30 bp induces antiviral responses in mammalian cells [130]. However, Sledz *et al* demonstrated that transfection

of siRNA upregulated IFN-mediated genes through dsRNA-dependent PKR expression [131]. In addition, Bridge *et al* demonstrated that siRNA vectors induced the production of IFN β [132]. Since this study, siRNA immunostimulation has been demonstrated to be mediated through TLR7/8 in a sequence-specific manner [133-135]. Moreover, Sioud *et al* reported that ss siRNA is more immunostimulatory than the siRNA duplex [136], while more recently, Pauls *et al* reported that siRNA induced the production of IL-6 and IL-8, but not IFN α , IFN β or IFN γ [137].

The toxicity of siRNA in vivo

Hutvagner *et al* demonstrated that 2'-OMe ONs inhibited RISC assembly in endogenous siRNA or miRNA *in vitro* and *in vivo* [138]. These results indicated that the RNAi machinery has a limited capacity. Grimm *et al* used an efficient ds AAV-8 vector to express 49 distinct shRNAs in the livers of adult mice [110]. It was demonstrated that 36 out of the 49 shRNA-expressing vectors caused liver damage, while 23 shRNAs caused animal morbidity and lethality that was associated with the downregulation of liver-derived miRNA. It was also demonstrated that this downregulation may cause a competition between shRNA and endogenous miRNA for binding to nuclear karyopherin exportin-5, a factor involved in transporting molecules out of the nucleus. Another molecule that is potentially involved in the saturation of the siRNA machinery is AGO2, which binds to siRNA, shRNA and miRNA [110].

Approaches to minimize the off-target effects of siRNA

Enhancing the efficiency of RNAi, while avoiding immunostimulation and off-target effects is the key point for the application of siRNA therapeutically. siRNA containing the 5'-UGUGU-3' motif, present on cationic lipid or polymer carriers, can induce TLR-mediated immune responses following intravenous or intraperitoneal administration [135]. However, Judge *et al* demonstrated that the delivery of siRNA containing the 5'-UGUGU-3' motif in the absence of a cationic lipid or polymer carrier did not induce IFN-mediated responses [135]. Because cationic lipid-siRNA complexes can enter immune cells more easily than naked siRNA, the delivery of siRNA containing immunostimulatory sequences with cationic lipid or polymer carrier should be avoided. In addition, Gondai *et al* reported that pppGn(2,3) associated with the 5' end of shRNA from the T7 RNA Pol abrogated the induction of IFN responses, which are triggered by siRNA synthesized using the T7 RNA Pol system [139].

Moreover, chemical modification at the 2'-ribose ring of RNA, such as 2'-OMe or 2'-F modifications, reduced the immunostimulatory capacity of siRNA [140,141]. However, the position and type (ie, purine or pyrimidine) of the siRNA nucleotide residues that are modified are critical for the stability and immunostimulatory properties associated with the siRNA. It has been demonstrated that 2'-OMe modification of siRNA sense-strand uridine or

uridine/adenosine residues blocked the induction of an immune response, but 2'-OMe or 2'-F modification in the pyrimidine residues did not abrogate the immunostimulatory properties of siRNA [142].

Conclusion

RNAi modulates gene expression by post-transcriptional and sequence-specific mechanisms. Potent therapeutic strategies based on RNAi have been designed for the treatment of cancer, viral infections [107] and allergic diseases [112]; however, significant challenges remain in the field of RNAi therapeutics. Biodistribution studies are needed to clarify RNAi specificity and penetrability at the target tissues. Although several studies have demonstrated that certain chemical modifications can enhance siRNA stability and efficacy, while avoiding the generation of off-target responses, further thorough studies of the RNAi mechanism of action, as well as of the mechanisms that trigger off-target effects and toxicity are warranted. Barriers for *in vivo* siRNA delivery can be overcome through an improved understanding of the RNAi mechanisms, thereby significantly increasing the likelihood of successful siRNA-based therapeutics against a broad spectrum of human diseases. Additional studies comparing siRNA-based and 'traditional' drugs as therapies for allergic diseases are needed, particularly in terms of efficacy and safety.

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