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Noncoding RNA for Cancer Gene Therapy

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Abstract

Gene therapy is a prospective strategy to modulate gene expression level in specific cells to treat human inherited diseases, cancers, and acquired disorders. A subset of noncoding RNAs, microRNAs (miRNAs) and small interference RNAs (siRNAs), compose an important class of widely used effectors for gene therapy, especially in cancer treatment. Functioning through the RNA interference (RNAi) mechanism, miRNA and siRNA show potent ability in silencing oncogenic factors for cancer gene therapy. For a better understanding of this field, we reviewed the mechanism and biological function, the principles of design and synthesis, and the delivery strategies of noncoding RNAs with clinical potentials in cancer gene therapy.

Keywords

Noncoding RNA; MicroRNA; Small interference RNA; RNA interference; Cancer; Gene therapy

1 Introduction

Gene therapy is a method of modulating gene expression level by introducing exogenous genetic materials into specific cells to treat human diseases including cancers. The most frequently used genetic material in gene therapy is DNA and RNA. DNA molecules used in

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gene therapy are usually disease-related genes (or gene fragments) to achieve gain-of-function effects, as well as antisense oligonucleotides for loss-of-function results (Zamecnik and Stephenson 1978). In the case of RNA, a subset of noncoding RNAs, microRNA (miRNA) and small interference RNA (siRNA), are emerging as popular effectors for gene therapy (Table 1). They are RNAs of small molecular weight, without protein-coding potentials. They have been shown to have potent biological functions accomplished by the mechanism of RNA interference (RNAi), which is a process triggered by double-stranded RNA molecules (Fire et al. 1998; Elbashir et al. 2001) and has been proved to be a powerful approach for reducing expression of mRNAs encoding pathogenic factors.

1.1 Mechanism and Function of Noncoding RNAs

miRNAs are endogenous small noncoding RNAs of ~18–25 nucleotides in length that regulate gene expression in a sequence-specific manner via the degradation of target mRNAs or the inhibition of protein translation. Most miRNA genes are transcribed by RNA Pol II to produce primary miRNA transcripts (pri-miRNAs) that contain a 5′ cap and a 3′ poly(A) tail (Lee et al. 2004; Cai et al. 2004). Pri-miRNAs harbor local hairpin structures and flanking sequences, which are subsequently cleaved within the nucleus by Drosha and DGCR8/Pasha (Denli et al. 2004; Gregory et al. 2004; Lee et al. 2003), to generate ~70-nt hairpin precursors known as pre-miRNAs. Next, the pre-miRNA is exported into the cytoplasm by Exportin-5 and further cleaved into a mature ~22-nt miRNA:miRNA* duplex by an RNase III enzyme Dicer, and its partners TRBP (TAR-RNA binding protein)/Loquacious and PACT (protein activator of PKR) in human cells (Hutvagner et al. 2001; Ketting et al. 2001). Subsequently, an RNA-induced silencing complex called RISC is assembled with the protein Argonaute (Ago) 2 (Gregory et al. 2005; Maniataki and Mourelatos 2005). The miRNA strand is selectively incorporated into the RISC complex (Schwarz et al. 2003; Du and Zamore 2005) and guides the complex specifically to its mRNA targets through complementary base-pairing interactions between the seed sequence (base 2–8 in the 5′ end of the mature miRNA) and the binding site within target mRNAs. Through this mechanism, they exert their silencing functions by either mRNA degradation or translation inhibition (Fig. 1).

miRNAs exhibit a wide range of physiological functions, especially in cancer biology. Some miRNAs act as oncogenes or tumor suppressors. For example, oncogene miR-21 is upregulated in cancer cells, promotes cell growth, and suppresses apoptosis (Chan et al. 2005; Krichevsky and Gabriely 2009). Tumor suppressor let-7 family is usually downregulated or deleted in multiple cancer types, and restoration of let-7 expression leads to regression of tumors (Kumar et al. 2008; Johnson et al. 2007; Esquela-Kerscher et al. 2008; Takamizawa et al. 2004; Yang et al. 2008). miR-200 family is well known to be associated with cancer cell metastasis and apoptosis (Park et al. 2008; Gregory et al. 2008; Schickel et al. 2010). Based on the knowledge, efforts to overexpress tumor suppressor miRNAs and inhibit oncogene miRNAs to treat cancers have achieved positive results. Slack et al. delivered exogenous let-7 to established mouse models of non-small cell lung cancer and significantly reduces tumor burden (Trang et al. 2010). Naldini et al. functionally knocked down miR-223 expression by introducing decoy miRNA targets into mouse models

(Gentner et al. 2009). Techniques for successful administration of miRNAs in vivo make it possible for miRNAs to act as good candidates for cancer therapy.

Much like miRNA, siRNA and shRNA are also potent mediators of sequence-specific gene silencing by RNAi mechanism. siRNA is a double-stranded small RNA of ~21 nt in length. It is exogenously synthesized as oligonucleotides (hereafter named as siRNA), or generated by 25–27-nt short hairpin RNA (shRNA) expressed from a DNA vector. The transcript of shRNA forms a stem-loop structure which can be further processed by Dicer to give rise to double-stranded ~21-nt siRNA. By either way, the functional guide strand of siRNA is assembled into RISC for target silencing by RNAi mechanism (Fig. 1). The interactions of siRNA and their targets are based on full complementarity of base pairing, which is different from miRNAs. The biological functions of siRNAs are mainly dependent on their target genes, because siRNAs can be flexibly designed and synthesized to target and modulate the function of any transcript theoretically. To date, siRNA has been extensively used in cancer gene therapy by targeting oncogenes such as *BCL-2* (in chronic myeloid leukemia), tyrosine kinase receptor *EphA2* gene (in ovarian cancer cells) (Landen et al. 2005), and *Ews–Fli1* gene fusion (in Ewing sarcoma cells) (Hu-Lieskovan et al. 2005).

1.2 Design and Synthesis of Noncoding RNAs for Cancer Gene Therapy

For gene therapy, the efficacy of exogenous genetic materials is largely determined by the compatibility with endogenous cellular machinery to perform their functions, as well as the delivery methods. However, multiple side effects of miRNA and siRNA in cancer gene therapy have been reported, including off-target effects, induction of immune system responses (Robbins et al. 2009), and saturation of endogenous RNAi pathway components (Khan et al. 2009; Grimm et al. 2006). The side effects sometimes can cause severe clinical outputs, thus limit the application of noncoding RNAs in gene therapy. To maximize the efficacy and minimize the side effects, it is necessary to follow some rules when designing noncoding RNAs for cancer gene therapy. Generally, it is important to consider the targeting sequences, the length, and the chemical modification of 3′ and 5′ ends of noncoding RNAs.

Synthetic miRNAs are usually present in the form of pri- or pre-miRNAs. Their targeting sequences (i.e., seed sequences) are determined by the nature of a specific miRNA. For synthetic siRNA and expressed shRNA, the targeting sequences are fully complementary to and determined by target mRNA sequences. The public TRC portal launched by the Broad Institute (The RNAi Consortium, http://www.broadinstitute.org/rnai/public/), as well as some commercial siRNA manufacturers, have developed online tools to help design specific and potent targeting sequences of siRNA based on the consideration of mRNA target sequence, secondary structures, siRNA stability, and minimizing sequence-dependent off-target effects. In addition, when designing targeting sequences for an interest gene, one should always pay attention to avoid the immunostimulatory effect of the synthetic sequences. It was reported that transfection of siRNA elicited interferon (IFN) responses (Sledz et al. 2003). A strategy to minimize this side effect is to avoid immunostimulatory sequences in siRNA design, e.g., 5′-GUCCUUCAA-3′ and 5′-UGUGU-3′ (Hornung et al. 2005; Judge et al. 2005).

In addition to the targeting sequence, the length and the modification of 3′ and 5′ ends of noncoding RNAs for cancer gene therapy also need to be paid attention to. It was reported that 27-bp double-stranded RNAs can be up to 100 times more potent than 21-mer siRNAs due to more efficient processing by Dicer, and incorporation of DNA nucleotides into siRNA also enhanced Dicer processing (Kim et al. 2004, 2005). To reduce interferon production in target cells, avoiding 5′ triphosphates of siRNA by chemical synthesis needs to be considered (Kim et al. 2004). Similarly, 2-nt 3′ overhangs alleviate interferon induction effect by resembling endogenous products processed by Dicer (e.g., mature miRNA) (Marques et al. 2006). In addition, 2′-*O*-methyl modification of siRNA increases the stability and retains targeting specificity, but reduces interferon production (Judge et al. 2006; Morrissey et al. 2005). Conjugating cholesterol to the sense strand of the siRNA duplex is another common modification that manifested to be a successful strategy to enhance systemic delivery efficiency by promoting liver uptake of siRNA (Soutschek et al. 2004).

1.3 Delivery of Noncoding RNAs for Cancer Gene Therapy

Besides the chemical structures (RNA sequence and end modification), in vivo delivery method is another critical determinant affecting the efficacy of noncoding RNAs for cancer gene therapy. The obstacles for in vivo delivery include protecting from endogenous nuclease digestion, evading immune detection, and promoting extravasation from blood vessels to target tissues and cells. To overcome these obstacles, a variety of in vivo delivery methods for noncoding RNAs have been developed (Table 2).

Noncoding RNA molecules could be simply delivered in a naked form at a relatively high dosage; for example, a dose of 50 mg/kg with the inhibitor of oncomir miR-10b (antagomiR-10b) was injected via tail vein, and it successfully suppressed the metastasis of mouse breast cancer by silencing endogenous miR-10b (Ma et al. 2010). To protect noncoding RNA from degradation and enhance the delivery efficiency, a variety of synthetic vectors have been developed, such as lipid-based carriers (Li and Szoka 2007), polymersomes (Lee et al. 2005), cell-penetrating peptides (Martin and Rice 2007), and inorganic nanoparticles (Sokolova and Epple 2008). Using nanoliposomes 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), Calin et al. demonstrated successful delivery of both miR-520d-3p and *EphA2*-targeting siRNA to mouse model and found that the dual therapy was more potent in antitumor efficiency than either monotherapy alone due to simultaneously targeting both *EphA2* and *EphB2* oncogenes (Nishimura et al. 2013). Viral vectors are also widely used to express noncoding RNAs in vivo. Commonly used viral vectors for this purpose include retrovirus, lentivirus, adenovirus, and adeno-associated virus. Using an adeno-associated virus vector, systemic administration of miR-26a in a mouse model of liver cancer resulted in retarded growth and apoptosis induction of cancer cells (Kota et al. 2009). With an adenovirus vector, Slack and colleagues successfully delivered exogenous let-7 to established mouse models of non-small cell lung cancer and significantly reduces tumor burden (Trang et al. 2010). Naldini and colleagues presented technologies to functionally knock down miRNA expression by introducing decoy miRNA targets via lentiviral vectors into mouse models (Gentner et al. 2009). In addition, novel methods for in vivo delivery of noncoding RNAs are developing very fast. Recent study by

Slack et al. reported that a novel construct, attachment of peptide nucleic acid anti-miRs to a peptide with a low pH-induced transmembrane structure (pHLIP), could transport an anti-miR-155 across plasma membranes under acidic conditions and reduced tumor growth. This method could selectively target the anti-miR to the acidic tumor microenvironment, evade systemic clearance by the liver, and facilitate cell entry via a non-endocytic pathway (Cheng et al. 2015). The discovery that a small molecule enoxacin (Penetrex) could enhance the activity of the RNAi pathway may also help to increase the efficacy of in vivo delivery of miRNA and siRNA (Shan et al. 2008).

For research purpose only, the technology of transgenic animal represents a liable method that is frequently employed to study in vivo function of expressed noncoding RNAs in cancer treatment. Inducible expression of miR-21 in a conditional transgenic mouse model revealed the oncogenic role of this miRNA in inducing pre-B-cell lymphoma and supports the efforts to treat human cancers through pharmacological inactivation of miRNAs such as miR-21 (Medina et al. 2010). The transgenic method provides valuable research data and applicable experience for related clinical trials.

2 Conclusion

miRNAs and siRNAs represent an extensively used class of noncoding effectors for cancer gene therapy. They both utilize RNAi mechanism to perform their biological functions in cancer treatment. The efficiency of miRNAs and siRNAs depends on multiple factors such as targeting sequence, end modification, and systemic delivery method. The understanding of the interaction between noncoding RNAs and their targets has been applied to clinical trials. To date, the targeting siRNAs for *BCL-2* (e.g., Chronic myeloid leukemia), *VEGF* (solid tumors), and *PLK1* (e.g., liver tumor) are undergoing or have completed clinical trials (from *ClinicalTrials.gov*). With progress in these studies, noncoding RNAs are believed to contribute a lot more to the field of cancer gene therapy.

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Abbreviations

miRNA	MicroRNA
siRNA	Small interference RNA
RNAi	RNA interference
Ago2	Argonaute 2
RISC	RNA-induced silencing complex
TRBP	TAR-RNA binding protein

PACT	Protein activator of PKR
shRNA	Short hairpin RNA
TRC	The RNAi Consortium
DOPC	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine

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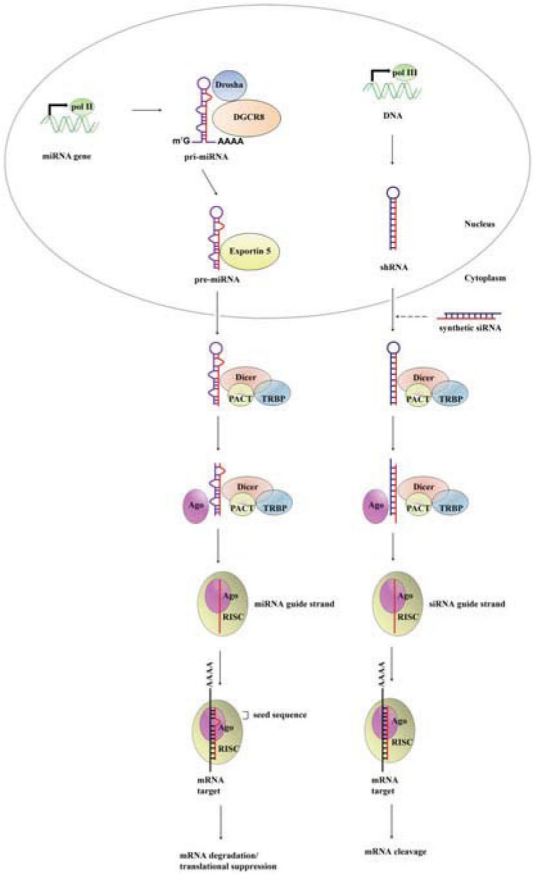

Fig. 1.

The biogenesis process and RNAi mechanism of miRNA and siRNA/shRNA. Most miRNA genes are transcribed by RNA polymerase II (pol II) to produce primary miRNA transcripts (pri-miRNAs) that contain a 5′ cap and a 3′ poly(A) tail. Pri-miRNAs are subsequently cleaved within the nucleus by Drosha and DGCR8/Pasha to generate ~70-nt hairpin precursors known as pre-miRNAs. The pre-miRNA is exported into the cytoplasm by Exportin-5 and further cleaved into a mature ~22-nt miRNA:miRNA* duplex by Dicer, and its partners TRBP and PACT. Subsequently, an RNA-induced silencing complex called RISC is assembled with the protein Argonaute (Ago). The miRNA strand (guide strand) is selectively incorporated into the RISC complex and guides the complex to its mRNA targets through complementary base-pairing interactions between the seed sequence (base 2–8 in the 5′ end of the mature miRNA) and the binding site within target mRNAs. The target mRNA is silenced by either mRNA degradation or translation inhibition. Similarly, shRNA with stem-loop structure is transcribed by RNA polymerase III (pol III). In the cytoplasm, shRNA and synthetic siRNA are subject to the processing by Dicer and its partners TRBP and PACT to give rise to double-stranded ~21-nt siRNA. The guide strand of siRNA is assembled into RISC for target cleavage and gene silencing by RNAi mechanism. miRNA, microRNA; siRNA, small interference RNA; shRNA, short hairpin RNA; RISC, RNA-induced silencing complex

Table 1

Characteristics of noncoding RNAs for cancer gene therapy

	microRNA	**siRNA**	**shRNA**
Length	Primary (various), precursor (~70 nt), mature (18–25 nt)	~21 nt	25–27 nt
RNA structure	Primary (contains a hairpin), precursor (hairpin), mature (double-stranded)	Double-stranded	Hairpin
Targeting specificity	Intrinsically determined by seed sequence	Artificially designed	Artificially designed
Production	Chemical synthesis, viral expression, transgene expression	Chemical synthesis	Viral expression
Delivery method	Viral, non-viral and transgene	Non-viral vectors	Viral vectors

Table 2

Delivery methods of noncoding RNAs for cancer gene therapy

Method	RNA species delivered	Advantages	Disadvantages
Non-viral vectors			
Naked delivery	miRNA, siRNA	No carriers needed	High dosage required
Lipid-based carriers	miRNA, siRNA	Robust, effective, and selective delivery	Sophisticated preparation needed
Polymersomes	siRNA	Robust, effective, and selective delivery	Sophisticated preparation needed
Cell-penetrating peptides	miRNA (e.g., pHLIP)	Effective and selective delivery	Expensive, sophisticated preparation
Inorganic nanoparticles	siRNA	Easy preparation	Limited efficiency, sometimes toxic
Viral vectors	miRNA, shRNA	Effective delivery, stable expression	Biosafety risk, immunogenic
Transgene	miRNA	Stable expression, non-immunogenic	Research purpose only