Review

RNA Interference Against Human Cancers: a Perspective

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Abstract:

RNA interference (RNAi) is a natural and highly conserved cellular process for targeting and specific cleavage of mRNA through small interfering RNAs (siRNA) of 21-23 nucleotides. RNAi approaches are now emerging as a novel coadjuvant therapy to correct abnormal expression of cancer oncogenes that contribute to sustained cell growth and chemotherapeutic resistance. Cancer cells undergoing RNAi-forced expression display novel genotype/phenotype as part of global gene expression changes, thereby increasing the activity of conventional chemotherapeutic drugs used in the treatment of cancers. Many delivery systems are in development, including virus vectors (retrovirus, adenovirus and adeno-associate virus), liposome complexes, nanoparticles and monoclonal antibodies, to carry the chemically synthesized 21-23 base-pair siRNAs or short hairpin RNA (shRNA) through the cell membrane into the cytoplasm and nucleus of the tumor cells. Here, we update the current uses of this approach in basic and clinical oncology studies.

Keywords: RNA. Oncogenes. Neoplasms. Drug therapy.

Introduction

RNA interference (RNAi) is a natural and conserved mechanism displayed by most eukaryotic cells to promote the targeting and degradation of foreign double-stranded RNA and RNA-mediated transcriptional silencing of endogenous genes.[1-2] Gene silencing mediated by RNAi is comparable to gene deletion (or knock-out) and as such this approach has been used to rapidly and effectively evaluate the function of thousands of human genes and searching causal relationship with targeted gene in biological processes in cellular and animal models. RNAi strategies are now emerging as a new class of therapeutics for many diseases, including cancer. Here, we will first describe the basic mechanism and the usefulness of RNAi applications in laboratory research. Next, we will discuss the results of recent studies demonstrating that RNAi strategies can efficiently inhibit the oncogenic activity of overexpressed genes and suppress the relevant cancer phenotypes, such as high proliferation rates, colony formation capability and migration or even to induce cell death by apoptosis.

RNAi: An Overview

In 1998, Fire and Mello[1] discovered an unexpectedly high activity of double-stranded RNA (dsRNA) molecules compared to single-stranded antisense RNA in producing the inhibition (or silencing) of gene expression after their exogenous introduction into the nematode *Caenorhabditis elegans*. This effect is now referred as RNAi. This natural process is highly active in worms, yeast, insects and plants, which depend on this mechanism for cellular defense against viral

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and transposon invasion.[3] This phenomenon was also observed in mammalians cells which produce endogenous microRNAs (miRNA) with the capability of guiding the cleavage of sequence-complementary mRNAs and transcriptional gene silencing.[2-4] These small non-protein-coding small RNAs are transcribed from DNA in the nucleus under environmental stress and in response to a very broad diversity of biological processes, including development, defense against viral infection and tumor suppression.[2-4] It is now becoming clear that several classes of evolutionary conserved DNA-encoding miRNA have common roles in controlling cell proliferation, cell death and early development.[2-4] There is also evidence that misregulation of RNA interference machinery plays a role in tumorigenesis and impacts cancer cells response to cytotoxic therapy.[4] More importantly, the physiological microRNA enzymatic machinery can recognize siRNAs against endogenous gene in the new experimental approach of targeted gene regulation which is now routinely explored in basic research and is expected to be used for therapeutic purposes in patients.[5-6]

The RNAi is a multi-step process in which a growing number of proteins are recruited into an ATP-dependent reaction.[2-4] RNAi silencing is initiated when long double-stranded RNA (dsRNA) molecules are processed into siRNA by the Dicer family of RNA

III ribonucleases. The cleavage generates 21-23 bp nucleotide fragments with symmetric 2-nt (preferably dT bases) 3'overhangs on each strand, preferably with one or two uridine residues in the 3'overhang on the sense strand.[2-4] These effectors siRNA bind to several proteins forming the RNA-inducing silencing complex (RISC complex) that contain helicase, exonuclease and endonuclease activities. The RISC complex promotes siRNA unwinding and cleave and separate sense and anti-sense RNA strands. Next, RISC incorporates sense strand and guides it to the antisense RNA strands to homologous target mRNA. An RNA with a perfect similarity promotes the cleavage, whereas a partial match to a target mRNA promotes silencing or repression of gene expression (Figure 1).

Regulatory microRNAs, such as the short hairpin precursors of ~70 nucleotides, are also processed by Dicer.[2-4] These short hairpin RNAs (shRNA) are first exported from nucleus to cytoplasm, and their linker sequence will be degraded by Dicer, making active single strands siRNA of 21-23bp nucleotides. The miRNA-protein complex (miRNP) binds to their target mRNA with near-perfect complementary, and can apparently act by decreasing target messenger RNA levels or by directly inhibiting translation.

RNAi has several important advantages over other
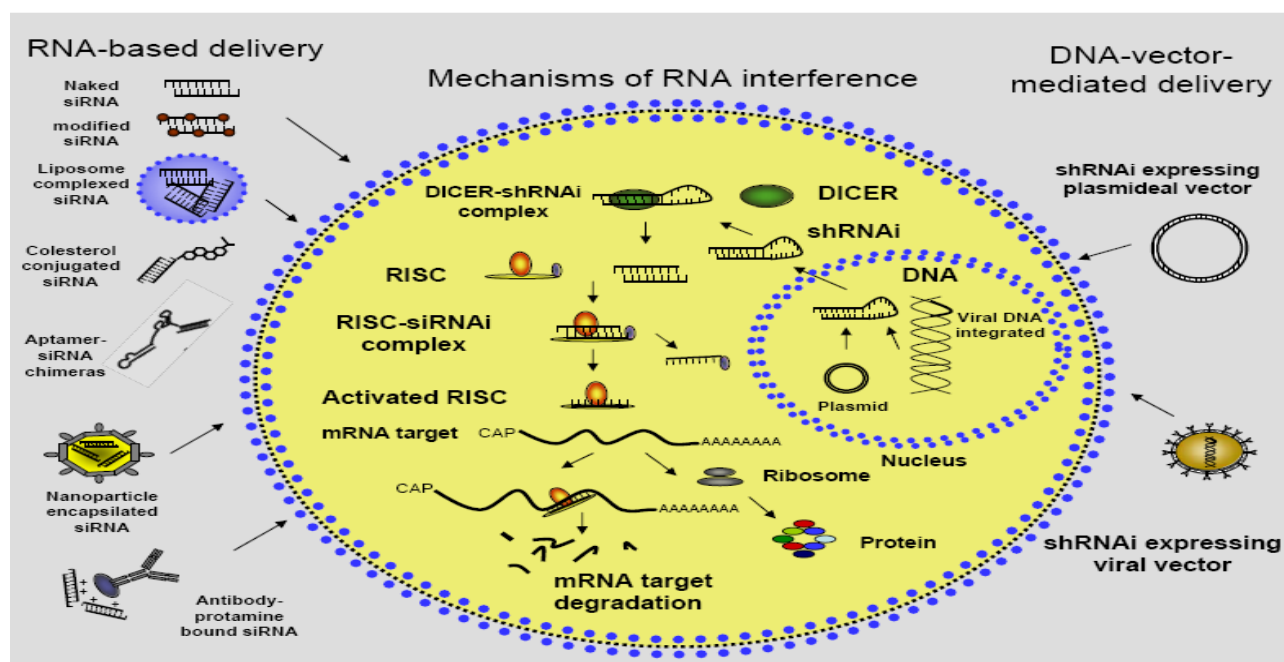
Figure 1 -Schematic representation of the key molecular events for RNAi mediated degradation of mRNAs (center) and the RNA-based siRNA drugs (left side) and DNA vector systems (right side) for delivery into the cells. The shRNA (transcripts synthesized by expression vector), microRNA (synthesized by nuclear DNA) and delivered by endosomal vesicles are processed to small interfering RNA (siRNA) duplexes of 21-23 bp by the enzyme Dicer, which displays RNAse-III-like activity. Next, a single strand of siRNA is released and incorporated into RNA-induced silencing complex (RISC). Activated RISC now guides single strand siRNA to a complementary target mRNA. Through pairing, mRNA is cleaved and the fragments are further degraded by RNAses.

methods for gene targeted inhibition such as antisense, catalytic RNA and DNA molecules and homologous recombination.[3,7] RNAi mechanism of action is gene–specific, allowing its application not only in deciphering gene function but also silencing any microbe's pathogenic genes inside of host cells. However, side effects (or off–target effect) are commonly generated when siRNA inappropriately silence non-specific genes that have. limited sequence similarity to the RNA targeted [3,7] Much of the off-target effects can be avoided by conducting a careful homology search in designing the first oligonucleotide to select sequences that are unique in the genome.[8]

knockdown target gene expression prompted the development and wild use of this technology as a tool to screen individual gene function in genetic model systems.[7-9] The usefulness for this rapid strategy for transient RNA silencing in cultured cells and small organisms has been demonstrated by the injection of the synthetic siRNA of 21-23 bp. Because of its predictive power, many companies now offer specific siRNA as well as shRNA–expressing plasmid and viral vector systems ready to use in various genetic models systems. Gene expression in *C. elegans* and *Drosophila* can be easily manipulated by soaking them in a solution containing synthetic siRNA, or by given them through the diet. Naked siRNAs and shRNA–expressing vector can be administrated in mice using a method called "tail vein injection" or hydrodynamic transfection method.[3,5-6] This method has been mostly applied for the delivery of RNAi constructs into liver parenchyma.[10] The synthetic siRNAs can be efficiently delivered into target cells by lipophilic agents or electroporation using a high voltage pulse (Figure 1). In some settings where a crucial threshold amount was necessary for maximum silencing of the overexpressed targeted gene, the lipophilic agents disrupted membrane and normal cell activity, causing organ damage.[11-13] Although these findings represent a significant obstacle to applying RNAi strategies, it is expected that introduction of specific chemical modifications may significantly diminish off-target effects.

Attenuated adenovirus, adeno–associate virus and lentivirus vectors have been developed for constructing gene targeting systems which integrate into the genome of living cells and specifically promote gene knockdown via constitutive expression of short hairpin RNA.[14] Most virus systems use RNA polymerase III (Pol III) promoters, such as U6, H1 or tRNA, known to synthesize small RNA for optimal production shRNA. The map of the lentivirus plasmid pLKO vector is depicted in Figure 2 with detailed explanation of how

to use it. The construction and use of lentivirus RNAi libraries have been of great help to carry out screening experiments intended to genome-wide loss-of-function in model organisms.[9,14] More importantly, RNAi approaches do not require that the entire gene sequence be known, thus, expressed sequence tags (ESTs) of unknown genes can be inhibited and linked to specific phenotype.

The co-infection of fertilized embryos with viral

Figure 2 –The construction in pLKO lentivirus plasmid vector for shRNA-mediated silencing in mammalian cells. At first step, oligonucleotides containing the sense and anti-sense sequences to target gene are designed and synthesized. These sequences are cloned according to restriction enzyme sites at the 3' and 5' termini of each sequence. The small interfering sequences are inserted into the short hairpin RNA sequence under the control of RNA-polymerase III of the U6 small nuclear RNA gene of opened pLKO plasmid. The vector contains the ampicillin and puromycin resistance genes for selection in bacteria and mammalian cells, respectively. The other elements in the vector are: hPGK, human phosphoglycerate kinase eukaryotic promoter; SIN/3' LTR, 3' self inactivating long terminal repeat; F1 ori, f1 origin of replication, pUC ori, pUC origin of replication; RSV/5'LTR, 5'long terminal repeat; Psi, RNA packaging sequence; and RRE, rev response element

vectors has provided to be a highly efficient method for mouse genome transgenesis.[15] Viral vectors are generally constructed incorporating a variety of design strategies.[14-16] For example, a drug-inducible system which allows the RNAi delivery coupled with the RNA polymerase II promoter could specifically knockdown gene expression up to 95%.[15] This system has been used for the creation of transgenic mice with the loss-of-function for p53 protein.[16] and the fibroblast growth factor receptor 2 protein.[17] In another study, the shRNA construction was prepared in a targeting vector to *rosa26* locus to allow Cre recombinase mediate integration of the exchange vector into mouse genome.[18] Using this strategy, it was possible to create a mouse strain in which the leptin receptor expression is stably silenced. More important, this mouse displayed a similar phenotype to those *db/db* mouse strains that do not express leptin receptor because of a punctual

mutation in the same gene.[18] In this sense, RNAi offers many modalities for genetically engineering animal models, in particular, for conditional gene knockdown when gene knock-out via homologous recombination is embryonic lethal.

Perspectives of Using RNAi Against Overexpressed Oncogenes

Cancer cells arise as result of the epigenetic and genetic changes at specific chromosomal locations known as amplicome and mutome.[19-20] A large number of cancer susceptibility genes have been characterized to promote carcinogenesis by different ways and classified according to their function in the cellular processes as oncogenes, tumor suppressor genes, genome stability genes and landscaper genes.[19-20] Somatic mutation, deletion, insertion and fusions of cancer susceptibility genes are particularly important in their activation/ inactivation. However, several signaling pathway were identified to be dysregulated in cancer cells because of the amplification, copy number gain and overexpression of the oncogenes. Thus, RNAi was proposed as a new anticancer strategy in order to reduce (or knockdown) oncogene over-expression. This strategy has been experimentally validated with success in many genes that control cellular proliferation, differentiation, invasion, metastasis and sensitivity to therapy (chemotherapy and radiation therapy) in various types of cancer cells. For example, RNAi approaches have been used for targeting and inhibiting the abnormal expression of epidermal growth factor receptor 1 and 2 (EGFR1/ ErbB1 and HER–2/ErbB2), protein kinase A (PKA), transforming growth factor, alpha and beta (TGF–α and TGF–β), insulin–like growth factor 1 receptor (IGFIR), polymerase (DNA–directed), epsilon 4 (p12 subunit) (P12), Mdm2 p53 binding protein homolog (mouse) (MDM2), breast cancer 1, early onset (BRCA1), B–cell CLL/lymphoma 2 (Bcl–2), estrogen receptor (ER), vascular endothelial growth factor VEGF, Multiple–drug resistant (MDR), ferritin (FTL), iron–responsive element (IRE), c–fos FBJ murine osteosarcoma viral oncogene homolog (C–fos), heat shock 27kDa protein 1 (HSP27), v–myc myelocytomatosis viral oncogene homolog (avian) (C–myc), RAF proto–oncogene serine/threonine–protein E6 and E7 Human papillomavirus proteins (HPV E6 and E7) and metallothionein (MT) genes. These studies are summarized in the excellent articles available in the literature.[21-29] In the majority of *in vitro* studies in culture

cells and *in vivo* studies with NUDE mice models, the suppression of a targeted oncogene by RNAi could change or diminish the relevant malignant phenotypes such as the high proliferation rates, colony formation capability, migration, or even to induce cell death by apoptosis.

There are now new and innovative genetic and bioinformatic approaches to study gene expression and the signaling pathways they regulate. These emerging high-throughput technologies have been applied in many settings to predict the impact of RNAi in cancer biology. By using DNA microarray platform, scientists can determine in a single experiment, the expression levels of hundreds or thousands of genes within a cell by measuring the amount of mRNA bound to each site on the array. Moreover, microarray experiments can be used to identify which genes are differentially expressed in the two different types of cancer patients or cell lines derived of a tumor, thereby creating specific disease profiles on the basis of their gene expression patterns.[30-31] We recently describe a model system for comprehensive comparison of molecular and biological features of cancer cells undergoing RNAi expression.[32-33] In this study, we demonstrated that knockdown of dermcidin oncogene by RNAi reduces *in vitro* breast cancer cell proliferation and *in vivo* tumor growth in Nude mice. The suppression of DCD expression could re-establish the sensitivity of these cells to cytotoxic drugs staurosporine, TNF-alpha hydrogen peroxide. Surprisingly, the transcription levels of many genes linked to proliferation were down-regulated, including the Myc transcription factor, EGFR and the Betacellulin (BTC) and Amphiregulin (AREG) growth factors. In addition, DCD knockdown promoted a significant decrease in the expression of the proteins disulfide isomerase-associated 3, 4 and 6 (PDIA), the heat shock 70 kDa protein (HSP-70), hypoxia-inducible gene 2 protein (HIG2), vascular endothelial growth factor A and B (VEGF-A and VEGF-B), which have functional roles on tumor oxidative stress resistance and induction of angiogenesis. It is remarkable that by changing the expression of an individual oncogene, we could reverse the biological network perturbations that empower cancer cells with selective growth and metabolic advantages and resistance to chemotherapeutics. It has become increasingly clear now that the neoplastic phenotypes are driven by the intraconnectivity and interconnectivity among the signaling pathways controlled by cancer causative genes.[34-35] There are many interesting examples of oncogenic driven-signaling networks associated with distinct cancer phenotypes, including oncogene addiction, senescence, angiogenesis, stemless, and drug resistance,

which could be arrested or neutralized by mean of RNAi against a phenotype-specific oncogene. Thus, RNAi-targeted therapy to the oncogenes Myc, RAS, VEGF and EGFR/ErbB1 and HER2/ErB2, by neutralizing the phenotypes they control, could increase the sensitivity of cancer cells to current chemotherapeutics.

The studies summarized here provide strong evidence that RNAi targeting of oncogenes can be a promise strategy to increase the sensitivity of cancer cells to current chemotherapeutics used to treat cancer patients. Nonetheless, it is not yet known if such results *in vitro* could be reproducible in a whole animal or patients because of current limitation and toxicity of the RNAi delivery system. Therefore, development of the safety and effectiveness of systemic delivery of RNAi into cancer cells are of the utmost importance to further exploring the potential of this high specific therapy in the clinic.

Acknowledgments

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