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Personalized Cancer Approach: Using RNA Interference Technology

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Abstract Normal cellular survival is dependent on the cooperative expression of genes' signaling through a broad array of DNA patterns. Cancer, however, has an Achilles' heel. Its altered cellular survival is dependent on a limited subset of signals through mutated DNA, possibly as few as three. Identification and control of these signals through the use of RNA interference (RNAi) technology may provide a unique clinical opportunity for the management of cancer that employs genomic-proteomic profiling to provide a molecular characterization of the cancer, leading to targeted therapy customized to an individual cancer signal. Such an approach has been described as "personalized therapy." The present review identifies unique developing technology that employs RNAi as a method to target, and therefore block, signaling from mutated DNA and describes a clinical pathway toward its development in cancer therapy.

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Introduction

Recent advances in proteogenomics have led to the creation of network models that outline signaling interactions on which cancer cells are dependent for survival and that can be distinguished from non-malignant cell signals. It is our premise that "personalized" targets will significantly guide the future management of cancer. Knowledge of a "personalized," molecularly unique target of cancer will thus open an ever-widening door to the application of targeted therapy. One specific way to control gene expression involves RNA interference (RNAi) technology, a natural process through which expression of a targeted gene or a set of targeted genes is dampened with high specificity and selectivity. At present traditional cancer treatment aims to shut down the metabolic pathways of cancer cells through the use of chemotherapy and radiation therapy, a treatment strategy associated with severe side-effects. We propose that through the compilation of a patient genome database that recognizes the level of connectivity of genomic-proteomic nodes, a more effective anti-tumor therapy based on genomic targets could be identified [1–4]. These highly connected targets, or nodes, capitalize on the cancer cells' weakness, exposing a vulnerability [2, 5, 6]. Disordered cancer circuitry, paradoxically, is pathway-dependent as the cells are reliant on a limited number of redirected pathways [7]. The same re-wired pathways that give cancer cells their prolific attributes may also provide the means by which they and they alone can be made susceptible to attack [7].

Through genomic-proteomic profiling, we hope to discern high-frequency commonalities (targets) among cancer patients related to the spread, survival, and self-renewal of cancer cells [2, 3, 8]. It has been shown that tumor tissue overexpresses these targets when compared to normal

tissue as the result of re-wired pathways. There are data to support a targeted RNAi knockdown strategy [9–12], and published studies further strengthen the foundations for a more personalized approach to cancer study and treatment.

Standard treatment for advanced cancer

In 2009, 570,280 people died of cancer in the United States [13]. The social and economic cost to our society is enormous. Chemotherapy remains the primary systemic treatment for patients with advanced cancer. Despite an increased understanding of the mechanisms of action of chemotherapy drugs and better documentation of single and combined therapy pharmacokinetics, the use of these agents continues to be largely empirical. The average survival of patients diagnosed with advanced cancer has remained less than 1 year despite billions of dollars invested for new therapy development over the last 50 years. Insofar as most of our chemotherapy regimens are based on cytokinetic principles primarily effective against highly proliferative cells, sensitivity within a heterogeneic tumor cell population, containing a high proportion of cells in G_0, is limited, and specificity is curtailed, as reflected in a narrow therapeutic window. Anti-cancer chemotherapeutics are traditionally classified by mechanistic categories: classic and non-classic alkylating agents, antimetabolites, intercalating agents, topoisomerase I inhibitors, topoisomerase II inhibitors, and antimicrotubule agents. The therapeutic rationale for the use of these agents to treat advanced cancer continues to be based on four key principles. First, the assumption that unlike "normal" cells the growth of tumor cells is exponential and, therefore, follows a cell kill kinetics when exposed to phase- or cycle-specific cancer drugs (now significantly modified using Gompertzian modeling). Second, by assuming a tumor-specific inherent sensitivity, therapeutics are empirically chosen based on the body organ from which the cancer arose (i.e., lung, prostate, breast, etc.). Third, by combining two or more drugs, inherent and acquired resistance to each of the component agents would be decreased. Fourth, that a combination of chemotherapy agents with different toxicities would allow maximum dosing of each of the component drugs without excessive toxicity. These principles were adapted for cancer management more than 50 years ago as a result of successful screening with sensitive bacteria to antibacterial drugs [14, 15]. Unfortunately, we have learned that cancer is far more complex than bacteria. In addition, most traditional chemotherapeutics have compromised efficacy because of the narrow window between therapeutic effectiveness and toxic dose.

Although small-molecule-based targeted therapies (imatinib in gastrointestinal stromal tumor (GIST), Tarceva in non-small-cell lung cancer, and cetuximab in colorectal cancer) have produced short-term clinical benefits and quality-of-life improvements, these targeted biotherapeutic approaches have not substantially improved cancer cure rates [16]. While the use of small-molecule- or antibody-based therapy has marked the start of a new management approach for cancer therapy, response and survival rates have not been dramatically altered, leaving the oncology community with few quality treatment options for advanced cancer.

RNAi and preclinical development in cancer

A naturally occurring process of gene regulation and defense, RNAi suppresses the expression of foreign genes and functions in embryonic development [17, 18] whereby small sequences of intrinsic antisense RNA, microRNA or extrinsic double-stranded RNA (dsRNA) (i.e., viral) trigger repression of targeted-gene expression. The synthetic small interfering RNA (siRNA) molecules introduced into cells for which an expressed gene is targeted trigger the target gene messenger RNA (mRNA) degradation or sequestration for translational repression. The RNAi process starts with loading of double-stranded siRNA onto the ATP-dependent RNase III enzyme Dicer to trim the siRNA to 22 nucleotides segments and to form the initiation complex. With the assistance of Dicer, double-stranded siRNA is loaded onto an Argonaute protein containing an "RNA interfering silencing complex" (RISC) where one strand of the siRNA (the passenger strand) departs with the assistance of adenosine triphosphate (ATP)-dependent helicase (Fig. 1) [11]. Loaded RISCs then seek out target mRNA through sequence matching for cleavage, degradation, and sequestration. Endonucleolytic cleavage of the target mRNA occurs at a single site at the center of the target mRNA-siRNA antisense strand duplex [10] and is mediated by Argonaute 2 protein (Ago2) [19].

Over a decade of research aimed at optimizing antisense oligonucleotide (ASO) technology has paved the way for the development and the applications of RNAi technology [20]. The activity and specificity of RNAi is several orders of magnitude greater than ASO technology [10, 21–23]. For example, delivery of the negatively charged nucleic acids across the cellular membrane barrier is the main difficulty for ASO technology; lessons learned in overcoming this obstacle in transfecting cells with ASO were applied to RNAi [24]. Nucleic acids and nucleic acid stabilization modifications have been developed to address the delivery issues and are now commercially available. Potential pitfalls learned through the use of ASO technology have established criteria for the essential control experiments needed to validate preliminary data generated

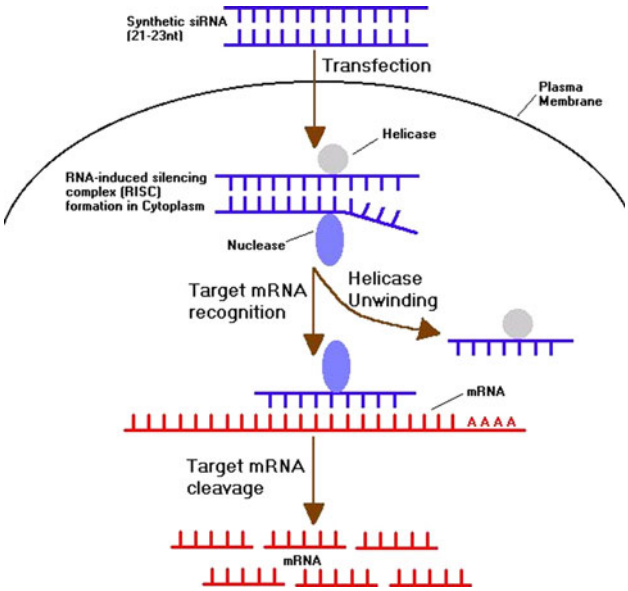

Fig. 1 Putative mechanism of ribonucleic acid (RNA) interference (RNAi) in mammalian cells. RNA may be generated through cleavage by the mammalian homolog of the prokaryotic enzyme Dicer, which cleaves long, double-stranded RNA molecules into fragments of 21–23 bp. It is also possible that synthetic RNAi can be introduced through transfection. RNAi molecules that interact with helicase and nuclease to form a complex called the "RNA-induced silencing complex" (RISC). Helicase uses adenosine-5′-triphosphate (ATP) to unwind RNAi, promoting the antisense strand to bind to its target mRNA. Nuclease in the RISC cleaves the mRNA, which is quickly degraded by other RNAs

with RNAi technology [25]. Biodistribution studies using ASO technology have provided guidance on potential tissue-targeting requirements for RNAi [26–28].

Anti-tumor effects of RNAi

The well-recognized genetic and phenotypic dissimilarities between cancer cells and normal cells make the use of RNAi, with its high degree of specificity, appealing and thus RNAi been used in several cancer-related studies. Martinez et al. [29] used the *p53* mutant-specific RNAi molecule to knock down the single nucleotide mutant expression and thus restore the wild-type *p53* function in heterozygous tumor cells. Furthermore, an antitumor effect is achieved by *ras* mutant knockdown that diminished the oncogenic phenotype [30]. RNA silencing targeting mutant *ras* reduced mutant expression by 90% without significantly altering the wild-type expression that was demonstrated both in vitro and in vivo [31, 32]. An RNAi molecule delivered by retroviral particles specifically inhibited the mutant *K-ras*$^{v-12}$ allele in the human pancreatic carcinoma cell line CAPAN-1 without affecting wild-type K-ras expression, leading to the loss of anchorage-independent growth phenotype and tumorigenicity. The growth of human colon carcinoma cell line SW480 was reduced approximately 70% as the result of mutant *K-ras* knockdown. Additional examples were reported by targeting various types of cancer oncogenes, including oncogenic fusions (e.g., *bcr-abl* in myelogenous leukemia) [33]; virally expressed genes (e.g., HPV *E6/E7* in cervical cancer cells) [34]; cancer-related overexpressed genes such as *HER-2/neu* [35, 36]; multidrug resistance (MDR) genes [37]; telomerase [38]; oncogenes such as PDX-1 [39], SRC-3 [40], mesothelin [41], ZIP4 [42], and the antiapoptotic bcl-2 gene [43], with which all resulted in cancer growth inhibition as demonstrated in vitro and in vivo. RNAi targeting of critical signaling components was also able to inhibit tumor cell growth in vivo[30, 39, 44–47] (i.e., metastasis [48–50], angiogenesis [51, 52], and chemoresistance) [9, 53].

Mediators of RNAi

The action of RNAi can be mediated either through the chemically synthesized small interfering RNA (siRNA) or the short hairpin RNA (shRNA) transcribed from an DNA expression vector. DNA-based shRNA and RNA-based siRNA are intrinsically different types of molecules, thus their delivery into cells, pathways utilized for RNA interference, and potential off-target effects are also different. Although both types of molecules are capable of delivering target-specific gene knockdown, each type of molecule has its comparative advantages and disadvantages. Recently, we developed a class of bi-shRNA that is able to take advantage of multiple natural RNAi mechanisms for more effective and thorough target-gene knockdown. All three classes of RNAi molecules and their respective mechanism of action are described.

siRNA and mechanism of action

Ideal siRNA is synthetic double-stranded RNA of between 19and 23 nucleotides with a 2 nucleotide 3′ overhang for activity enhancement [54]. Synthetic modifications can be made to include 2'-O-methyl modification, locked nucleic acid (LNA), or DNA for stability and activity [55–58]. Alternatively, Dicer substrate siRNA, with a stem-loop structure resembling the pre-microRNA structure, shows significant dose advantages over conventional siRNA [59].

The life cycle of siRNA inside transfected cells is diagrammatically illustrated in Fig. 2. Upon delivery, siRNA steadily increases its accumulation in cells for 4 h before reaching plateau, indicating a pharmacodynamically saturable, or turnover, process [60]. Ohrt et al. labeled siRNA

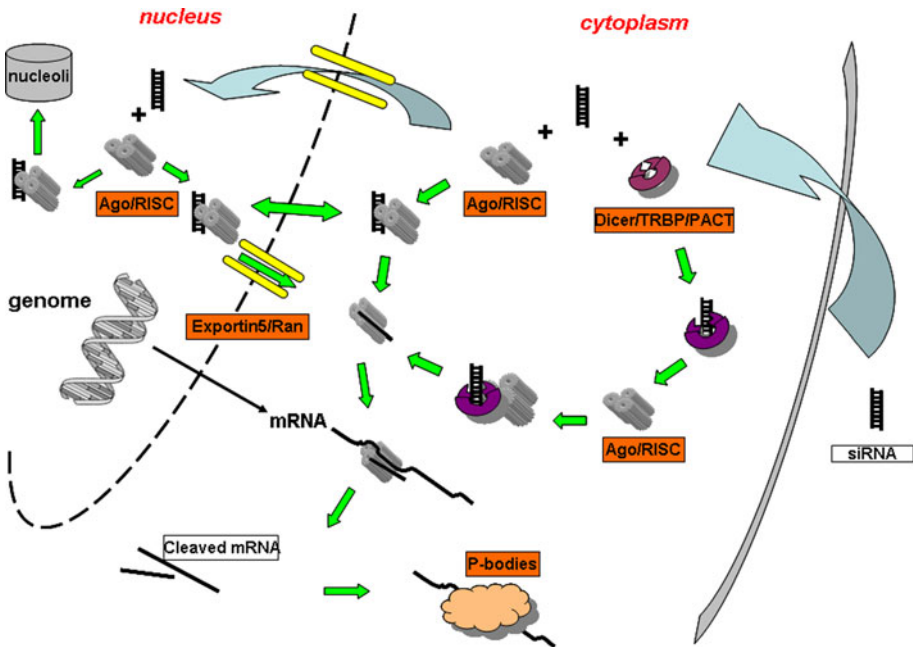

Fig. 2 Schematic of the small interfering RNA (siRNA)-mediated RNA interference pathway. After entry into the cytoplasm, siRNA is either loaded onto RISC directly or uses a Dicer-mediated process. After RISC loading, the passenger strand departs, thereby initiating the RNA interference process via target mRNA cleavage and degradation. Small interfering RNA loaded RISCs are also found to be associated with the nucleolus region and may be shuttled in and out of the nucleus through a yet-unidentified process

with fluorescent dye at the 3′ end of either strand of siRNA and found an accumulation of siRNA evenly distributed throughout the cytoplasm [61]. At 48 h postinjection, 99% of siRNA appeared to have been degraded, with only 1% fluorescence remaining in the cell. The spatial and temporal distribution of siRNA within the cell is in accordance with the observed kinetics of siRNA-mediated RNA interference activity, which peaks around 24 h postdelivery and diminishes within 48 h. Berezhna et al. observed nuclear localization of siRNA targeted against small nuclear RNA and cytoplasmic localization of siRNA targeted against viral mRNA, suggesting selective localization and compartmentalization of siRNA based on its intended target [62].

After siRNA enters into the cells and survives degradation within, it is loaded onto a pre-loading complex that consists of ATP-dependent helicase and the endonuclease Dicer. Dicer is a multi-domain RNase III-related endonuclease responsible for processing dsRNA to siRNAs [63]. In mammalian cells, only one Dicer gene has been identified to date [64], and it is an integral component of the RNA-interference pathway. RNase III activity of Dicer processes pre-microRNA (miRNA) and dsRNA to mature miRNA and siRNA with defined size and structure, respectively, and it transfers the processed products to the RISC [65, 66]. The distinction between the RNA-interfering pathway for siRNA and that for miRNA may not be as obvious in mammalian cells as in Drosophila, for which one Dicer each for miRNA and for siRNA have been identified. Recent crystallography studies have shown Dicer to be a molecular ruler by with preferential binding to the 5′ phosphate of dsRNA with 2 nt 3′ over-hang measures that processes dsRNAs into 22 nucleotide siRNAs [67, 68]. The double-stranded Tat-RNA-binding protein (TRBP) and/or the double-stranded RNA-activated protein kinase (protein kinase R; PKR) activating protein (PACT) interact with mammalian Dicer to facilitate miRNA processing and RNA interference. Significant inhibition of the short hairpin RNA (shRNA), not siRNA, mediated gene-silencing effect was observed in cultured cells with TRBP and PACT knockdown, suggesting that TRBP and PACT function primarily at the step of miRNA maturation [69]. Although TRBP and PACT are structurally related, they exert opposite regulatory activities on PKR, and they directly interact with each other to stimulate Dicer cleavage of dsRNA or shRNA to siRNA [69]. Embryonic stem (ES) cells with Dicer knockout can effectively load processed siRNA onto RISC and carry out RNA interference as efficiently as $Dicer^{+}$ ES cells [63], a finding that strongly indicates a perfectly processed siRNA can be loaded onto RISC for RNAi without the assistance of the TRBP/PACT/Dicer complex. The TRBP/PACT/Dicer complex; however, is required to process shRNA, long dsRNA, or Dicer substrate siRNA to the appropriate size and form for their loading onto RISC. The TRBP/PACT/Dicer complex, although not necessary for siRNA activity, facilitates more efficient loading of siRNA onto RISC, which provides a considerable advantage as evident by a 10fold lower concentration of Dicer substrate siRNA required for activity when compared to its conventional siRNA counterpart [59].

Upon association of duplex siRNA with holo-RISC, which is mainly composed of one of the Argonaute family of proteins, Dicer and TRBP, the resulting complex is identified as the RISC-loading complex (RLC) [70]. In the RLC, Dicer hands over the dsRNA to Ago protein by a

presently undetermined process whereby the two strands of the duplex are separated, resulting in the departure of the passenger strand [71–73]. The RNase H-like slicer activity of Ago-2 cleaves the passenger (sense) strand, providing thermodynamically favorable conditions for passenger strand departure. This process is referred to as the "cleavage-dependent pathway" [74]. Passenger strand departure can also be accomplished via a cleavage-independent bypass pathway in which the passenger strand with mismatches is induced to unwind and depart by ATP-dependent helicase activity [71, 74, 75]. The RISC with single-stranded guide strand siRNA is then able to execute multiple rounds of RNAi through sequence recognition. Adenosine triphosphate is not required for shRNA processing, RISC assembly, activation of the cleavage-dependent pathway, or multiple rounds of target-RNA cleavage [76–78]. Single-stranded siRNA (containing 5′-phosphates) and pre-miRNA can be loaded on RISC, but not on duplex siRNA [79].

shRNA and mechanism of action

Unlike siRNAs, expression-vector-driven shRNAs need to be transcribed in the nuclei of cells, then processed and transported to the cytoplasm before incorporation into the RISC for activity [80]. The shRNA synthesis and maturation pathway is similar to that of miRNA, and therefore the most efficient shRNAs are designed to follow the rules predicated on the specifics of the cellular machinery for the miRNA. Studies on miRNAs synthetic pathways have provided the groundwork for the designs of shRNA [81], particularly the widely used miR-30 backbone-based shRNAs [82].

The life-cycle of shRNA inside transfected cells is diagrammatically illustrated in Fig. 3. Vector-driven shRNA can be transcribed from either RNA polymerase II or III promoters on the expression cassette. The primary transcript generated from the RNA polymerase II promoter typically contains a hairpin-like stem-loop RNA structure with poly A tails that is processed in the nucleus by a microprocessor complex [83]. The microprocessor complex containing the RNase III enzyme Drosha and the dsRNA-binding-domain protein DGCR8, measures from the loop of the hairpin structure in the primary transcripts and allows processing of the long primary transcripts into individual pre-shRNAs with a 2 nt 3′ overhang [84]. The pre-shRNA molecule is transported to the cytoplasm by Exportin 5, a Ran-GTP-dependent mechanism [85, 86]. In the cytoplasm the pre-shRNA is loaded onto the RNase III enzyme Dicer- and TRBP/PACT-containing complex where the loop of the hairpin is processed off by Dicer to form a double-stranded mature siRNA with a 2 nt 3′

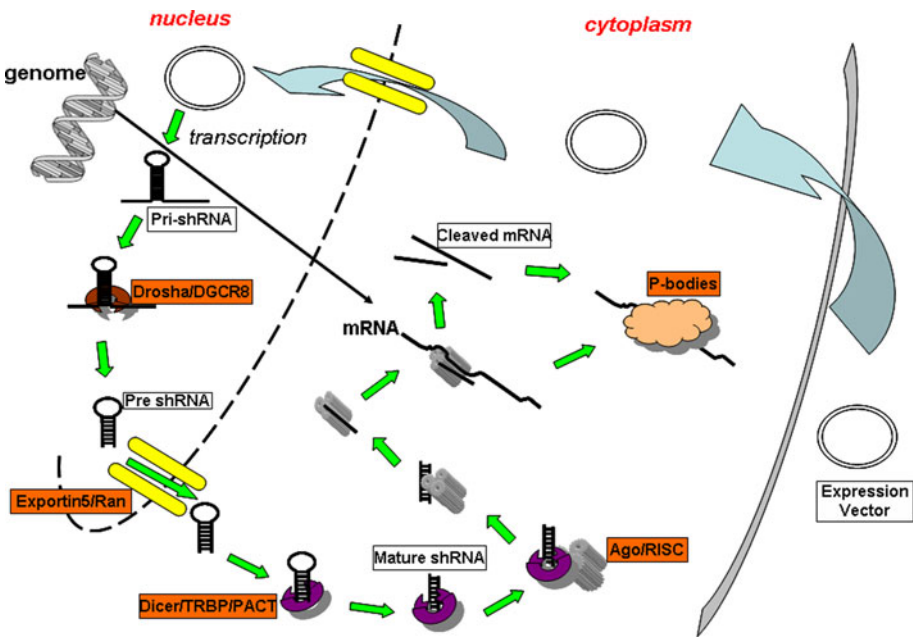

Fig. 3 Schematic of the small hairpin ribonucleic acid (shRNA)-mediated RNA interference pathway. After delivery of the shRNA expression vector into the cytoplasm, the vector needs to be transported into the nucleus for transcription. The primary transcripts (pri-shRNA) follow a similar route, as discovered for the primary transcripts of microRNA. The primary transcripts are processed by the Drosha/DGCR8 complex and form pre shRNAs. Pre-shRNAs are transported to the cytoplasm via Exportin 5, to be loaded onto the Dicer/TRBP/PACT complex, where they are further processed to mature shRNA. Mature shRNAs in the Dicer/TRBP/PACT complex are associated with Argonaute proteins containing RISCs, and they provide the RNA interference function, either through mRNA cleavage and degradation or through translational suppression via p-bodies

overhang at either end of the molecule [87–89]. The Dicer-containing complex then coordinates loading onto the Ago2 protein-containing RISC as described previously for siRNA. Pre-shRNA has been found to be part of the RISC loading complex (RLC); thus, pre-shRNA may potentially directly associate with RLC rather than through a two-step process via a different Dicer/TRBP/PACT complex [90]. After the Dicer processing, mature shRNA is structurally identical to siRNA and should behave in the same way as siRNA in terms of passenger strand departure and subsequent RNAi activity.

The Argonaute family of proteins is the key component of RISC [91, 92]; only Ago2 within the family contains the endonuclease slicer activity necessary to cleave the passenger strand of the double-stranded stem for passenger strand release and to cleave the target mRNA to initiate mRNA degradation [71, 72, 74]. The remaining three members of the Argonaute family, Ago1, Ago3, and Ago4, which do not have identifiable endonuclease slicer activity, also are assembled into RISC and presumably function in a cleavage-independent manner. Thus, RISCs are diverse complexes, and depending on their association with each member of the Ago family of proteins, they can be classified further as cleavage-dependent and cleavage-independent RISCs that may function differently [74].

The Argonaute family of proteins in RISCs are not only involved in the proper loading of siRNA or miRNA but are also implicated in both transcriptional (targeting heterochromatin) and post-transcriptional gene silencing. Ago protein complexes loaded with single-stranded guide-strand siRNA or miRNA seek out complementary recognition sites on mRNAs, where the slicer activity of Ago2 cleaves mRNA to initiate mRNA degradation with the assistance of C3PO [93, 94]. Other Ago-protein-containing complexes without endonucleolytic activity bind predominantly to partially complementary target sites located at the 3′ untranslated region (3′-UTR) for translation repression through mRNA sequestration and degradation in processing bodies (p-bodies) via deadenylation [95–99]. In the cytoplasm, the majority of RISCs are found to be associated with polyribosomes or the small subunit ribosomes [90], strongly suggesting that RISC surveillance is co-compartmentalized with the translational machinery of the cell. Details of the mechanism involving mRNA scanning and target mRNA identification are largely unknown. Whatever the scanning or surveillance mechanism may be, once the target mRNA is identified, it is either cleaved or changed in conformation, and both types of structures then are routed to the p-body for either sequestration or degradation [98, 99]. The active siRNA- or miRNA-loaded complex is then released for additional rounds of gene-silencing activity.

Bifunctional (bi)-shRNA and mechanism of action

A unique RNAi option called “bifunctional-shRNA” (bi-shRNA, for short) was developed by us. The bi-shRNA concept stipulates an shRNA expression design that effectively loads mature shRNAs onto both the cleavage-dependent RISCs and the cleavage-independent RISCs [100]. The synthesis and maturation of bi-shRNA is similar to that described above for shRNA. Each bi-shRNA expression module contains two structurally distinct stem-loops to promote loading of mature shRNAs onto multiple RISCs for action. Simultaneous expression of both types of shRNAs (i.e., siRNA and miRNA) in cells achieved a higher level of efficacy, greater durability of action, and a more rapid onset of gene-expression silencing compared to siRNA and conventional shRNA. As illustrated in Fig. 4, the “bi-functional” shRNA is conceptualized to load onto multiple types of RISCs to enable simultaneously induced degradation of target mRNA and repression of translation through mRNA sequestration. The bi-functional design with two structurally distinct mature shRNAs thermodynamically accommodates passenger strand departure via both cleavage-dependent and cleavage-independent processes, so that RISC loading is functionally guided by the passenger strand rather than being dependent on the Ago protein distribution in the target cell.

This approach is supported by multiple experimental findings. In *Caenorhabditis elegans,* structural features of small RNA precursors determine Argonaute loading [101]. In *Drosophila*, the structure of miRNA leads to two individual pathways, each with dedicated Dicer and Ago proteins, Ago1 preferentially binds to miRNAs that have been excised from imperfectly paired hairpin precursors, whereas those miRNAs that have near-perfectly paired hairpin precursors are bound by Ago2 [102–105]. Recently, Azuma-Mukai and colleagues observed certain miRNAs associated both with human (h)Ago2- and hAgo3-containing RISCs, and they noted that some miRNAs are discriminately loaded onto hAgo2- or hAgo3-containing RISCs [106]. Co-immunprecipitation of different Ago proteins containing RISCs in HEK293 cells transfected with tagged-Ago proteins revealed similar sets of about 600 transcripts bound to Ago1, 2, 3, or 4 [90], suggesting that all four mammalian Ago proteins containing RISCs are involved in the RNAi function with overlapping targets. The bi-functional shRNA approach mimics the natural process by mediating targeted mRNA knockdown through multiple RNAi pathways by multiple RISCs loading to ensure target mRNA knockdown. Conventional siRNA and shRNA, on the other hand, requires slicer activity of Ago2 limiting their activity only to an Ago2-containing RISC.

Using a miR30-scaffold, we tested the “bi-functional” RNAi strategy by first targeting the oncogene, stathmin 1

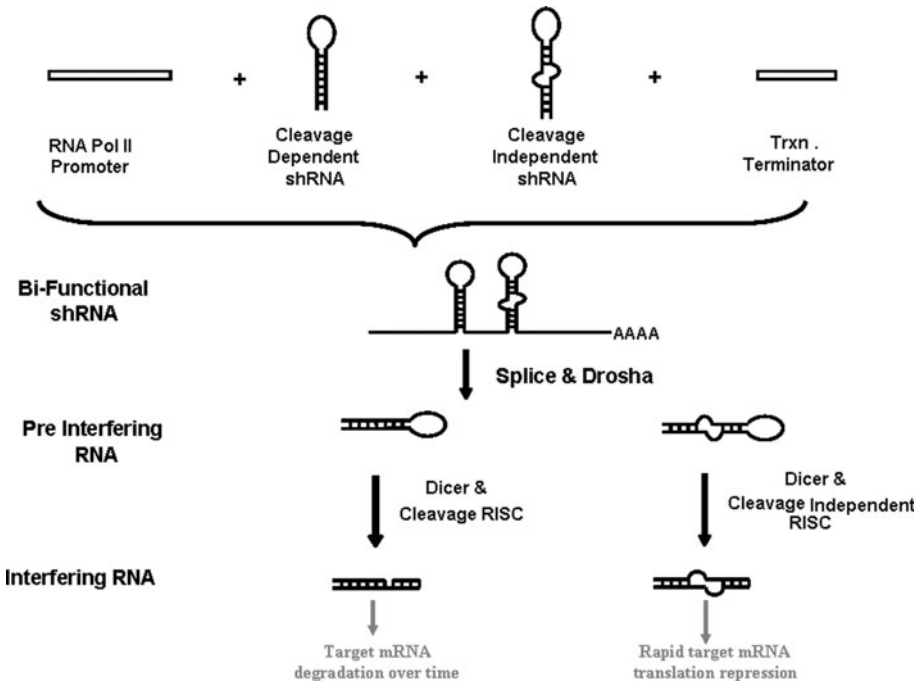

Fig. 4 Schematic of the bi-functional shRNA concept. The bi-functional concept is to design two shRNAs for each targeted messenger (m)RNA; one with perfect match, and one with mismatches at the central location (bases 9–12). The purpose of the bi-functional design is to promote the loading of mature shRNAs onto both cleavage-dependent and cleavage-independent RISCs so that the expression of the target mRNA can be shut down more effectively and efficiently, both through target mRNA degradation and translational repression

(STMN1). Based on transcriptome and proteome couplet signals in tumor/normal tissue specimen analysis, we have shown that STMN1 is differentially overexpressed in cancer patients (83% of 23 patients screened) [8]. Further, the overexpression of STMN1 has been reported in many human cancer cell lines of divergent origin, such as a colon cancer cell line, a breast cancer cell line, and a myelogenous leukemia line. Stathmin 1 is vitally important to mitotic spindle formation and cell division [107, 108]. Stathmin 1 knockdown by siRNA or shRNA resulted in G_2/M cell cycle arrest, inhibition of clonogenicity, and markedly increased apoptosis [108–110]. In an early-stage clinical drug development study [100], we showed effective target knockdown with significant dose response advantage in vitro in tumor cell killing by bi-sh-STMN1 construct versus conventional shRNA and siRNA targeting of the same STMN1 mRNA sequences. Results also confirmed the presence of the predicted mature shRNAs generated both from cleavage-dependent components and cleavage-independent components of the bishRNA design in transfected cells. Involvement of both cleavage-dependent and cleavage-independent mechanisms in mediating the bi-sh-STMN1 function is the most plausible explanation for achieving a high degree of knockdown (93%) of STMN1 in CCL-247 colon cancer cells 48 h after treatment. We also compared bi-sh-STMN1 to conventional siRNA directed against the same STMN1 target site. The bi-sh-STMN1 achieved an IC_{50} in CCL-247 tumor cell growth inhibition at 5 logs lower molar concentration than $siRNA^{STMN1}$ at 48 h post-transfection [100].

Comparison between bi-shRNA and the conventional shRNA targeting the same sequence showed much less pronounced differential in IC_{50} value at 48 h post-transfection, but a strong growth inhibition advantage was observed at a low dose range. The strong advantage of the bi-shRNA over the siRNA could be the result of the difference in effector molecule and mechanisms as described above for siRNA and shRNA. Vector-based DNA achieves stability within the nucleus [111] and steadily produces shRNA via its pol II promoter, which allows for spatial and temporal control, thereby providing a continuous expression of inhibitory effectors chaperoned efficiently through the natural synthetic pathways with a prolonged functional half-life. At the same time, 99% of siRNA was lost within the first 24 h and loading onto the RISC was inefficient. Additionally, the bifunctional strategy acts multifunctionally by cleaving target mRNA, effectively reducing target mRNA concentration, diminishing translation, and sequestering the target mRNA from the translation machinery either with p-body or by cap binding and deadenylation-mediated mechanisms. Thus it provides an efficient and sustained RNA interference mode. The bi-functional shRNA should be much more efficient in the knockdown of the target gene expression, particularly in the lower dose range where conventional shRNA could not achieve similar efficiency in knockdown. Conceptually, the advantage of bi-shRNA is dose response, durability, and efficacy that will provide advantages in the clinical setting.

Other targets that we have designed and are testing with bi-shRNA include PDX-1, SRC1, SRC2, SRC3,

mesothelin, ZIP4, Cyclin E1, and K-RAS mutants, as well as Ewing's sarcoma fusion protein.

Delivery

Use of RNAi as a cancer therapeutic is limited by the effective concentrations of the RNAi mediators (siRNA or vectors expressing shRNA) able to enter into the tumor cells. Clinically, this availability has been primarily dependent on the formulation and delivery method. Ideally, a delivery vehicle should be able to target tumors in a selective and differential manner, infiltrate and homogeneously disperse throughout the entire tumor, and effectively make entry into the tumor cells after systemic administration. Additionally, the formulation needs to be able to avoid detection by the host's acute immune surveillance system for unwanted side effects and be used in multiple administrations. If the endocytosis process is utilized for cell entry, the delivery vehicle has to manage escape from endosomal/lysosomal attack; for shRNA, an additional requirement of nucleus localization of the payload must be met. Because of high transfection efficiency and potential integration of exogenous DNA via viral particles, shRNA may be more effectively delivered by construct into viral vectors; however, support for viral delivery has been waning in recent years because of concerns over safety, immunogenicity, and efficacy involving repeated delivery [112, 113]. Liposomes with biodegradable components have far better safety profiles than do viral delivery systems, but they generally exhibit lower transfection efficiency [114–116]. Non-viral delivery systems for siRNA and shRNA are typically cationic preparations, and the positive charges facilitate complex formation with negatively charged nucleic acids, as well as binding to the negatively charged glycocalyx present on external cell membranes, to initiate endocytosis or membrane fusion. The positive charge on the surface can also be modulated by masking with pegylation. Complexing the delivery vehicle with targeting moieties, such as single-chain monoclonal antibodies, peptides, small-molecule ligands, and aptamers to recognize cell surface markers, can greatly enhance the specificity and efficiency of delivery, particularly for delivery targeting tumors [117, 118]. Once the cell is entered, through endocytosis or fusion, the positive charges on the delivery vehicle facilitate early escape from the endosome [119, 120]. Though the positive charge on these vehicles improves their transfection efficiency, it may also be the culprit for increased toxicity with those delivery vehicles [115, 121].

Several alternative methods are under development to mitigate the various issues related to the delivery of shRNA, siRNA, and bi-shRNA. Non-viral systems are composed of three classes: synthetic polymers, natural/biodegradable polymers, and lipids; with hybridizations of these classes exhibiting potential. One example of this is a cyclodextrin-based cationic polymer shown to be effective in delivery of siRNA targeted to ribonucleotide reductase M2 (RRM2) in various in vivo cancer models [122, 123]. In a Phase I clinical trial now underway, this preparation has been shown to be effective in mediating RNAi in tumors [124]. Lipid-based nanoparticles are exhibiting promise in the delivery of shRNA and siRNA [125]. Two companies, Tekmira (formerly Protiva Biotherapeutics) and Alnylam, have developed liposome-based nanoparticles composed of a lipid-PEG conjugate that is capable of encapsulating nucleic acids for systemic delivery. The lipid-PEG conjugate partially neutralized the positively charged cationic lipids to provide stable formulation. The lipid-PEG conjugate dubbed "SNALP" (stable nucleic acid lipid particles) was used in the first successful administration of siRNA formulation to a non-human primate [126, 127]. The SNALP formulation with two siRNAs targeting kinesin spindle protein (KSP) and vascular endothelial growth factor (VEGF) for liver cancer, ALN-VSP is in use in a dose escalating Phase I clinical trial. The targeted maximum dose of 1.5 mg/kg has been reached with little reported adverse effect. A lipid-based delivery vehicle called AtuPLEX has been introduced by Science Therapeutics for siRNA delivery to endothelial cells. AtuPLEX contains a mix of cationic and fusogenic lipids [128, 129]. In a formulation with siRNAs targeting protein kinase N3, AtuPLEX has been used effectively to knockdown the protein kinase N3 target, inhibiting cancer progression (Atu-027) in murine prostate and pancreatic cancer models [130, 131]. Atu-027, presently in Phase I clinical trial, has exhibited early signs of efficacy. In contrast to SNALP, Atu-027 is a positively charged lipoplex formulation. We have utilized a DOTAP-cholesterol bilamellar invaginated vehicle (BIV) for delivery of the bi-shRNAi [132–134]. This has been used successfully by us and others (BB-IND 10718, BB-IND 13744) in clinical testing [135, 136] and can be conveniently masked and targeted to facilitate cancer-specific entry. A more detailed discussion of delivery vehicles for shRNA [117, 137] and siRNA [116, 138–140], as well as general discussions of organ- and tissue-specific RNAi delivery [113, 141, 142], is beyond the scope of this article. Ongoing clinical trials involving RNAi technology are listed in Table 1.

Off-target effects

The excitement of RNAi technology has built upon the promise of specificity of target gene knockdown without affecting other, non-targeted genes; however, soon after the

Table 1 Clinical trials

RNAi drugs in human clinical trials [170]

Company	Product Name	Disease	Target	Stage
Alnylam	ALN-RSV1	Respiratory syncytial virus infection	Nucleocapsid (N) gene of RSV genome	Expanded phase II
Alnylam	ALN-VSP	Liver cancers and solid tumors	Kinesin spindle protein (KSP), VEGF	Phase I
Benitec/City of Hope	pHIV7-shI-TAR-CCR5RZ	AIDS lymphoma	rHIV7-shI-TAR- CCR5RZ	Phase I
Calando Pharmaceuticals	CALAA-01	Cancer and solid tumors	M2 subunit of ribonucleotide reductase (RRM2)	Phase I
Cequent Pharmaceuticals	CEQ508	Familial adenomatous polyposis	β-Catenin	Phase I
OPKO Health	Bevasiranib	Wet age-related macular degeneration	VEGF	Expanded Phase III
OPKO Health	Bevasiranib	Diabetic macular edema	VEGF	Phase II
Quark Pharmaceuticals	PF4523655/ RTP801i14	Wet age-related macular degeneration	RTP801	Phase II
Quark Pharmaceuticals	PF4523655/ RTP801i14	Diabetic macular edema	RTP801	Phase II
Quark Pharmaceuticals	QPI-1002/Akli5/ I5NP	Acute kidney injury	P53	Phase I/IIa
Quark Pharmaceuticals	QPI-1002/DGFi	Delayed graft function in kidney transplantation	P53	Phase I/II
Sirna Therapeutics (MERCK)/Allergan	Sirna-027/AGN-745	Wet age-related macular degeneration	VEGFRI	Phase II
Silence Therapeutics	Atu027	Lung cancers	Protein kinase N3 (PKN3)	Phase I
Tekmira	ApoB SNALP	High LDL cholesterol	Apo B lipoprotein	Phase I/II
TransDerm, Inc	TD101	Pachyonichia congenita	Keratin 6a (K6a)	Phase I
University of Duisburg-Essen	*BCR-ABL* siRNA	Chronic myeloid leukemia	bcr-abl	Single patient
Gradalis, Inc.	FANG	Advanced cancer	furin	Phase II

discovery numerous studies showed that there are multiple specific and nonspecific mechanisms through which siRNA and shRNA can affect expression of genes other than the intended target genes, so-called off-target effects. Specific off-target effects are brought about by partial sequence recognition by the RNAi-mediating molecules to un-targeted mRNA populations. Nonspecific off-target effects include immune-related and toxicity-related effects that are intrinsic in the RNAi-mediating molecules, the delivery method, and the combinations of RNAi-mediating molecules with the delivery vehicle.

Examination of expression profiles has shown that the siRNA can suppress gene expression of mRNA with partial sequence complement to the passenger or guide strands of siRNA [143]. It has also been noted that the number and identity of the suppressed transcripts is unrelated to the efficiency of the siRNA to silence the target gene [143]. Off-target suppressed genes have been found to contain a region of sequence complementary to the guide strand of siRNA; this region could be as small as seven nucleotides [144]. Both siRNA and shRNA, with the same sequence in the "seed region" (nucleotides in positions 2–8 from the 5′ end), were later demonstrated to produce the same off-target expression profiles, even across cell lines and independent of delivery method [145].

The location of the region of complementarity within the RNAi molecules and the mRNA transcript are important predictors of potential for suppression. Complementarity within nucleotides positions 2–8 at the siRNA's 5′ end of passenger of guide strands has been shown to be a key region in producing off-target effects [57]. This particular region of the siRNA is similar to the "seed region" of the microRNA. The seed region of miRNA is a heptameric sequence beginning at the second position from the 5′ end of the miRNA that recognizes sites in the 3′-untranslated region (3′-UTR) of mRNA through sequence complement to guide target gene silencing [98, 146, 147].

In vitro studies demonstrated that shRNA has fewer off-target effects than siRNA. Both shRNA and siRNA of the same targeting sequence for *TP53* knockdown were applied to HCT-116 colon carcinoma cells in concentrations necessary to achieve equivalent levels of target gene

expression knockdown. Expression profile analysis with microarray demonstrated a significantly higher number of genes that are upregulated and downregulated in siRNA-transfected cells than in shRNA-transfected cells [148]. It was suggested that the differences may be due to the fact that shRNA is transcribed in the nucleus with subsequent processing and transport via native pathways utilized by the endogenous miRNA, and siRNA is ectopically applied under more artificial conditions. Furthermore, the majority of the siRNA is susceptible to degradation in the cytoplasm (as illustrated above in the section titled siRNA Mechanism of Action), for which the breakdown siRNA may lead to more off-target silencing [149].

Off-target effects not resulting from the direct interaction of an RNAi mediating molecules with an mRNA transcript are often referred to as "nonspecific off-target effects." In this grouping are interferon (IFN) and other immune system responses to RNAi molecules, cellular toxicities elicited because the nature of nucleic acids molecules, and the delivery vehicle itself or combinations thereof.

Introduction of dsRNA longer than 29–30 bp into mammalian cells is well known to induce the innate immune system via PKR, similar to the mammalian cell and body defense mechanism in response to viral infection [150]. Activation of the innate immune response by pattern recognition receptors sensitive to exogenous nucleic acids can lead to global degradation of mRNA and broad inhibition of translation, as well as global upregulation of IFN-stimulated gene expression. Although shorter siRNA less than 29–30 bp have been shown to avoid receptor-mediated immune activation and were originally thought to be non-immunogenic [10], subsequent in vitro data have shown that the introduction of synthetic siRNA oligomers and shRNA expression vectors can elicit a partial interferon response [151–153]. Such partial responses may be dependent on specific sequence pattern (e.g., GU-dependent, 5′-UGUGU-3′ and GU-independent, 5′-GUCCUU-CAA-3′). Those identified sequence patterns should be avoided when designing siRNA or shRNA vectors. Activation of the innate immune system in the case of exogenous applied RNAi mediating molecules is likely activated through several cytoplasmic and endosomal recognition systems that are attuned to recognize foreign nucleic acids from infectious agents. Toll-like receptors (TLRs) 7 and 8 (typically activated by ssRNA), TLR 9 (via unmethylated CpG activation) and TLR 3 (via dsRNA), are well identified as the key pattern-recognition effectors associated with endosomes [154–158]. Immune activation by nucleic acids at the cytoplasmic level, on the other hand, is more likely to be mediated through RNA-sensing receptors such as RIG-I and MDA-5 [59, 159]. Mechanisms surrounding immune activation by RNAi are reviewed more thoroughly elsewhere [160, 161]. Immune activation appears to be related to the method of delivery and cell entry. A delivery method that bypasses endosomes should be able to avoid TLR activation and the necessity for endosome escape. Unmethylated CpG motifs in the shRNA expression vectors, which are also typically found in bacterial DNA, are readily recognized by TLR 9 located in enodosomes [162, 163]. Careful shRNA-encoding plasmid design to avoid unmethylated CpG motifs can effectively eliminate TLR 9-mediated endosomal immunoactivation [164].

Endogenous miRNA maturation and processing systems utilized by shRNA provide an advantage over the exogenously delivered siRNA in terms of the propensity of the latter for induction of IFN; however, the oversaturation of the endogenous systems has been shown to have other consequences that are not affected by siRNA. In a key in vivo study of the toxic effects of long-term expression of vector-based shRNA in the livers of adult mice, type 8 adeno-associated virus (AAV), was used to deliver RNA polymerase III promoter driven expression of 49 different shRNAs of different lengths and sequences directed against six targets. Thirty-six (36) of the expression constructs tested resulted the in a dose-related liver injury that was further determined to be associated with the pan-reduction of the expression of critical endogenous miRNAs. The degree of reduced expression of miRNA was related neither to the shRNA sequence nor to the degree of down-regulation of the target mRNA [165]. This observation alarmed investigators in the RNAi field and significantly curtailed the development of RNAi-related clinical development, particularly the vector-based approach. Subsequent transfection studies have suggested the generalized reduction in miRNA expression was resulting from a competitive bottleneck in shared miRNA processing and maturation pathways, most likely at the level of exportin-5 (the nuclear membrane export protein that transports pre-miRNAs into the cytoplasm) and Ago2 [166]. A similar in vivo study for which systematically introduced siRNAs targeting two hepatocyte-specific genes (apolipoprotein B and factor VII) and a scramble control to mice and one siRNA targeting the hepatocyte-expressed gene Scap to hamsters suppressed target gene expression in all cases; the level of the hepatocyte miR-122 was not changed in the mice or the hamsters, nor were the broadly expressed miRNAs, such as miR-16 and let-7a, found in the mice [167]. This result further suggests that siRNA, without passage through miRNA maturation pathways, does not result in observed toxicity.

When using shRNA as an effector of RNAi, care should be taken to minimize the potential for nonspecific off-target effects mediated by over-saturation of exportin-5 and the miRNA maturation processing machinery. In one in vitro study, overexpression of the exportin-5 protein was shown

to eliminate the nuclear export bottleneck and allow cells to tolerate higher expression of shRNA without toxicity [168]. Another study showed that an adeno-associated virus construct using an RNA Pol-II promoter was able to achieve stable target gene suppression at high multiplicity of infection (MOI) for over one year after the initial dosing [169]. Data to date suggest that both promoter selection (e.g., pol II versus pol III) and limiting the dosage of shRNA expression vectors constitute important factors that can be used to avoid the toxicity resulting from competitive inhibition of the endogenous miRNA biogenesis machinery.

Summary

In summary, both the mechanism of delivery and preclinical safety have been extensively demonstrated for several distinct variations of RNAi technology (i.e., si, sh, bi-sh). Remarkable target specificity and anti-cancer activity have been demonstrated in animal models, and platforms for safe systemic delivery are being explored. RNAi technology is now "evolving" into the stage of clinical assessment. Phase I experience is only now becoming available, but preliminary results support acceptable safety. This technology will likely have a significant impact on the development of personalized therapeutics as the work unfolds.

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