Small Interfering RNA for Effective Cancer Therapies

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Abstract: Small interfering RNA (siRNA) has become a specific and powerful tool to turn off the expression of target genes, and has turned into a promising tool in molecular medicine. It can be targeted against cancer by several strategies. These include the suppression of overexpressed oncogenes, retarding cell division by interfering with cyclins and related genes or enhancing apoptosis by inhibiting anti-apoptotic genes. RNA interference (RNAi) against multidrug resistance (MDR) genes or chemo/radio-resistance and angiogenesis targets may also provide beneficial cancer treatments. Successful cancer therapy by siRNA *in vitro* and *in vivo* provides the enthusiasm for potential therapeutic applications of this technique. Here, we review RNAi in cancer therapy, highlighting recent progress and examining the hurdles that must be overcome before this promising technology is ready for clinical use.

Keywords: Small interfering RNA, pharmacodynamics, cancer, oncogene, apoptosis, cell cycle, angiogenesis, multidrug resistance.

1. INTRODUCTION

Small interfering RNA (siRNA), sometimes known as short interfering RNA, is a class of 21-23 nucleotide-long double-stranded RNA (dsRNA) with 2-nt 3' overhangs on either end (Fig. **1**) [1-3]. These siRNAs intermediates are involved in the RNA interference (RNAi) pathway where siRNAs bind to the RNA induced silencing complex (RISC) and then selectively degrade the complementary single-stranded target RNA in a sequence-specific manner. Since the discovery of RNAi activity in mammalian cells, not only is siRNA being adopted for the discovery and validation gene function through cell culture and animal model studies [3], the growing success as a research tool and the favorable outcomes of several early phase clinical trials have also stirred up tremendous interest in using siRNA as a potential therapeutic agents [4-10]. With the explosion in knowledge generated by a growing understanding of the human genome and the development of high-throughput gene expression profiling of cancer cells, more and more genes that contribute to the tumor transformation and metastasis are discovered [11-15]. RNAi has been applied in this setting to suppress the expression of dominant mutant oncogenes, gene amplifications, translocations and viral oncogenes as a novel cancer therapy strategy and improve the efficacy of existing chemotherapeutic agents by specifically targeting and silencing resistance-associated genes.

While traditional therapeutic approaches have been widely used for cancer therapy, there are limitations to these approaches. Most small molecule inhibitors are not specific with regard to their targets. For example, protein tyrosine kinase inhibitors, many of which are ATP competitive inhibitors and have alternative targets that compromise their specificity and contribute to toxicity [16-17]. Additionally, the structures of some target proteins are unavailable, which excludes the possibility of rational design of corresponding small molecule inhibitors. The advantages of siRNA over other therapeutic modalities include its high specificity, versatility, and efficiency, which enable the use of RNAi to selectively knock-down expression of alleles carrying point mutations, insertions, or deletions, and thereby to produce cells with reduced expression of essential genes. The duration of knockdown by siRNA can typically last for 7-10 days, and has been shown to transfer to daughter cells [18]. Of further note, siRNA is effective at quantities much lower than alternative gene silencing methodologies, including antisense and ribozyme based strategies and can be works in the nanomolar range. Furthermore, siRNA is less likely to interfere with gene regulation by endogenous microRNAs [19-20]. Therefore, siRNA provides a novel therapeutic approach to silence cancer-causing genes otherwise not amenable to conventional therapeutics, such as small molecules, proteins, or monoclonal antibodies.

2. RNAi MECHANISM

The mechanism of RNAi has been well characterized and reviewed [1-3, 21-22]. Here we only provide a brief account of this process.

The RNAi pathway is initiated by an RNase III–type enzyme, Dicer, which cleaves long dsRNA molecules into short RNA of between 19 and 21bp which have symmetric 2–3 nucleotide overhangs and 5’ phosphate and 3’ hydroxyl groups, although examples of shorter and longer duplexes

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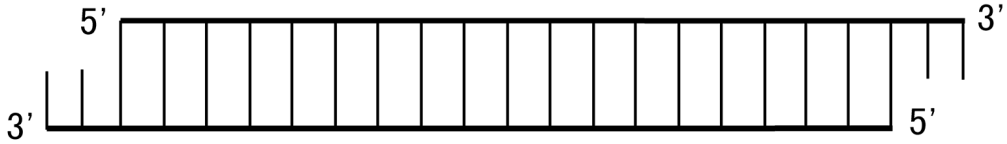

Fig. (1). Schematic representation of a siRNA molecule : a ~ 19-21 base pair RNA core duplex that is followed by a 2 nucleotide 3' overhang on each strand.

have been reported [23]. This process is ATP dependent and thought to be restricted to the cytoplasm. The generated siRNA associates with the RNA-induced silencing complexes (RISC) composed of four different subunits, a helicase, exonuclease, endonuclease, and homology searching domains. The siRNA duplex is then unwound by the helicase in RISC and then one strand of duplex (the "passage strand") is cleaved and discarded, while the other strand, the one with the lowest duplex stability at its 5' end which is usually the antisense one, guides RISC to its complementary or near-complementary mRNA, with target cleavage occurring at a single site in the centre of the duplex region between the guide siRNA and the target mRNA, 10 nucleotides from the 5' end of the siRNA. An RNA endonuclease within the complex, Argonaute 2 (Ago2), cleaves the target mRNA that loses its protection against RNases and gets degraded *via* its PIWI domain, which adopts an RNase H– like structure [24]. Importantly, the guide strand loaded by RISC is protected from degradation and can direct the cleavage of many mRNA molecules [25-26]. This cleavage does not require ATP, but does require magnesium ions. Exogenous synthetic siRNA can also be directly incorporated into RISC, thereby bypassing the requirement for dsRNA processing by Dicer, and modulate transcript levels by rapid, Ago-2 mediated transcript cleavage (Fig. **2**). SiRNA can also induce moderate level of gene knockdown through translational repression due to partial complementarity to 3' un-translated region (3' UTR) of the mRNA [27-29]. This silencing mechanism may be responsible for the ''off-target'' effects observable during RNAi experiments which can be minimized by including design parameters that select siRNA with low 3'UTR seed complement frequencies.

3. PHARMACODYNAMICS

Synthetic siRNA is usually used as effective therapeutics for oncology applications without inducing an interferon response. Unmodified naked siRNA has a half-life of less than 1 hour in human plasma and is cleared rapidly through the liver and kidneys due to their small size. Stabilizing chemical modifications (e.g., phosphorothioate, 4-thio, cholesterol) without affecting RNA-interference effect increase binding to serum proteins and the circulating half-life *in vivo* [30]. This allows more opportunity for the siRNA to enter target tissues. Morrissey *et al*. reported that the plasma half-life of an unmodified siRNA duplex in mice was 0.03 h, the half-life of a modified duplex was 0.8 h, and the half-life of a modified duplex packaged in a stabilized nucleic acid lipid particles (SNALP) was 6.5 h [31]. Similarly, Soutschek *et al*. reported studies using modified siRNAs in rats in which an unconjugated duplex had an elimination half-life of 0.1 h, while a cholesterol-conjugated duplex had a half-life of 1.5 h [32].

Even when chemical modifications are employed to stabilize a duplex from degradation, plasma clearance may still be rapid. Incorporation siRNA with particulate carriers appears to be a promising approach to improve pharmacokinetics because they are prevented from being excreted from the kidney due to their size. Furthermore, surface modifications of particulate vectors with aptamer/ targeted ligand /antibody leads to enhanced potency relative to non-targeted formulations and enables the cell-selective targeting of siRNAs in animals [33-36]. Bartlett *et al*. reported that transferrin-targeted siRNA cyclodextrin nanoparticles reduced tumor luciferase activity by approximately 50% relative to non-targeted siRNA nanoparticles and demonstrated that primary advantage of targeted nanoparticles is associated with processes involved in cellular uptake in tumor cells rather than overall tumor localization [37]. Landen *et al.* investigated the uptake of siRNA against EphA2 using tail vein injection of siRNA complexed with DOTAP liposomes [38]. They showed successful application of siRNA delivered by DOPC liposomes to reach and treat intra peritoneally growing ovarian cancer in mice and found that 30% of tumor cells can be reached within 1 hour of injection. Even ten days after the administration, fluorescent siRNA molecules were still found in tumor cells.

Recently, Svensson *et al*. have described a facile assay that facilitates the evaluation of the pharmacodynamics of a systemically delivered siRNA formulation using a mouse strain containing robust ubiquitous expression of firefly luciferase [39]. The primary advantage of this approach is that siRNA efficacy against a nonessential target can be easily evaluated in any tissue. This method can enhance the ability to rapidly screen, compare and optimize various siRNA formulations for tissue-targeted systemic delivery in a preclinical development setting.

4. RNAi AND CANCER

Most cancers are characterized by abnormal gene expression, which is thought to contribute to the tumor tansformation and metastasis. Silencing such genes by siRNA would appear to be a rational approach to the therapy of cancer. Indeed, *in vitro* and *in vivo* findings have demonstrated the efficacy of siRNA knocking down of genes that are pivotal for carcinogenesis, tumor growth, metastasis, angiogenesis, and chemo-resistance, leading to tumor growth inhibition. Molecules that indirectly contribute to tumor growth are also potential candidates for RNAi intervention. In most cases, suppression of a single gene is not enough to cure the disease and it could be necessary to inhibit multiple targets simultaneously or to combine gene silencing with other therapies such as chemotherapy and radiotherapy.

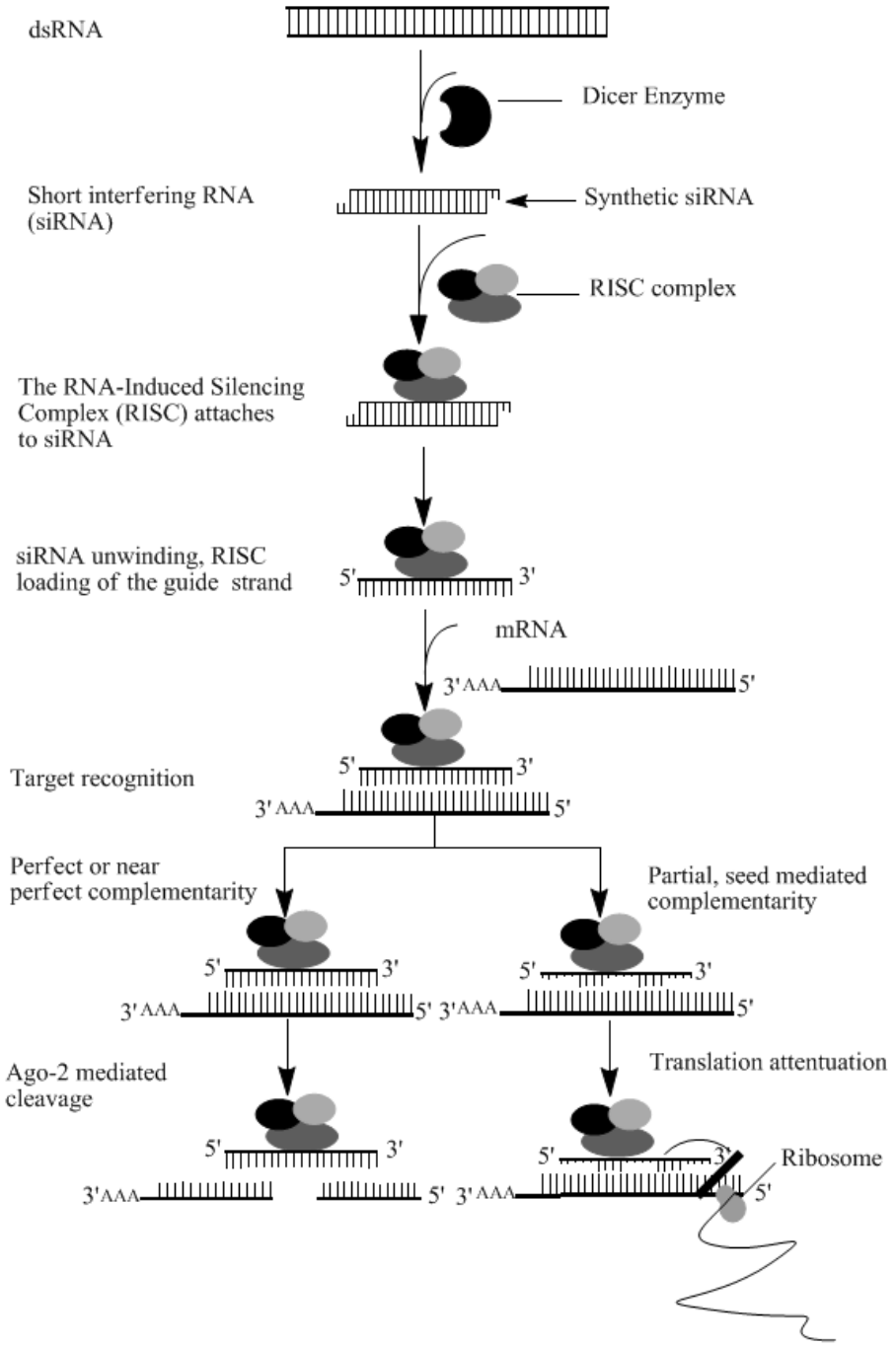

Fig. (2). Proposed mechanism of RNAi. The initial dsRNA is cleaved into siRNA by a nuclease called Dicer in an ATP-dependent reaction. SiRNA is then incorporated into RISC, which contains a helicase that unwinds the duplex. The antisense strand of the duplex guides the active RISC to the complementary mRNA. In the case of perfect complementarity, the target is cleaved by an Ago2-mediated mechanism. The cleaved mRNA is degraded rapidly and the protein that it encodes is not produced. SiRNA can also induce moderate levels of gene knockdown by seed-mediated translation attenuation. This process of off-targeting can be minimized by optimization of the siRNA design parameters. (This figure is adapted from the reference [28] with the permission of Nature Publishing Group).

4.1. Carcinogenesis Pathways

Viral oncogenes, Ras, p53, the receptor protein tyrosine kinases (PTKs) and p53 are the main components contributing to the cancer development and progression. Herein we first try to review the recent progress in RNAi based cancer therapy targeting against these pathways.

4.1.1. Viral Oncogenes

Infection with viruses has been detected in several human cancers, and the functions of virally-encoded genes have been implicated in the development and maintenance of those malignancies. For example, More than 90% of cervical carcinomas contain genome sequences derived from human

papillomavirus (HPV), and the viral E6 and E7 genes are supposed to co-operate in mediating deregulated cell proliferation and survival [40]. Targeting of these genes with siRNAs resulted in attenuation of cell proliferation, increased apoptosis, and reduced tumor size in a mouse model and these phenotypic outcomes were not manifested in HPV-negative cells [40-44]. The development of several other types of cancer are also associated with viral infection, for example Epstein-Barr Virus (EBV) in nasopharyngeal carcinoma, Kaposi's sarcoma-associated herpesvirus (KSHV) in primary effusion lymphoma, and human T-cell leukemia virus type 1 (HTLV-1) in adult T-cell leukaemia, and in models of each of these diseases RNAi targeting of virally-encoded genes resulted in changes in invasive behavior, tumor cell apoptosis or reduced tumorigenicity in a mouse model [45-49].

4.1.2. Ras Pathways

Ras is one of the most commonly mutated oncogenes in human malignancies (about 30% of all human tumors) [50], making it difficult to target. One major obstacle regarding designing a sequence-specific anti-Ras therapy simply lies in the nature of its activation: a single point mutation suffices to turn the protein on and convert it to a dominant oncoprotein [51]. Depletion of K-rasV12 using RNAi resulted in almost completely abrogated anchorage-independent growth and failure to produce tumors not only in pancreatic cancer cell culture but, encouragingly, in an animal *in vivo* model as well. Importantly, expression of the wild-type K-Ras gene was unaffected [52]. Zhou *et al*. reported that silencing of N-Ras gene expression using RNAi decreased transformation efficiency and tumor growth in transformed cells [53]. A major advantage of RNAi-mediated anti-Ras cancer therapy, in contrast to other Ras-based therapies, is the specific targeting of oncogenic Ras. This might be crucial, since inhibition of Ras function in cells could interfere with normal cell viability.

4.1.3. PTKs Pathways

PTKs are key molecules involved in cellular signal transduction and are frequently mutated in human cancers. When mutated or altered structurally, PTKs become potent oncoproteins, leading to cellular transformation from constitutive/enhenced kinase activity [54]. Such transformation can occur due to genomic rearrangements such as chromosomal translocations, which produce oncogenic fusion proteins that include a PTK catalytic domain and an unrelated protein that provides constitutive activity to the tyrosine kinase [55]. One example is the Bcr-Abl fusion protein, which is commonly observed in chronic myeloid leukemia. Transfection of leukemia cells with siRNA targeting a Bcr-Abl fusion transcript induced apoptosis [56-58]. Noticeably, leukemic cells without Bcr-Abl rearrangement were not killed by anti-Bcr-Abl siRNA. Moreover, the introduction of Bcr-Abl siRNA increases the sensitivity of Bcr-Abl overexpressing cells to the chemotherapeutic agent imatinib [56-57, 59]. Wohlbold *et al.* found that treatment of cells with imatinib mesylate and a suboptimal dose of BCR/ABL siRNA resulted in regained sensitivity to imatinib in formerly resistant leukemic cells as well as increased sensitivity to gamma irradiation [60].

Non-small-cell lung cancers (NSCLC) often have mutations within the epidermal growth factor receptor's (EGFR) catalytic protein tyrosine kinase domain, resulting in constitutive activation. The oncogenic activity of EGFR promotes cell proliferation and survival. RNAi mediated knocking-down the mutant EGFR *in vitro* results in apoptosis [61-63]. Sequence-specific siRNAs target specifically the mRNA of the mutant EGFR allele and have no effect on the expression of the wild-type EGFR. These studies indicate that the expression of the mutant EGFR is essential for the oncogenicity in lung cancers harboring these mutations, and that the RNAi-mediated down-regulation of the mutant EGFR results in specific and extensive apoptosis of NSCLC cells.

4.1.4. p53 Pathways

The tumor suppressor protein p53 is a frequent target for point mutations associated with oncogenesis. Since mutated p53 forms have dominant negative effects on wild type p53 in addition to intrinsic properties such as an altered binding partner repertoire, a specific inhibition of the mutant forms might restore wild type p53 functions [53]. Indeed, it has been demonstrated that transfected siRNAs suppress a mutant p53 carrying a point mutation without affecting the expression of its wild type counterpart [64]. As a consequence, p53-dependent apoptosis can be induced**.**

p53 pathway interacts with several other signal transduction pathways, and a number of positive and negative autoregulatory feedback loops act upon the p53 response. Several molecules participated in the p53 pathway have been investigated using RNAi technology and some of these may serve as potential targets for RNAi based cancer therapy. Hdm2, which is a key p53 negative regulator, has been found to be overexpressed in many human cancers [65-67]. RNAi-mediated silencing of Hdm2 significantly inhibits the growth potential of human breast cancer cells in a p53- dependent manner [65, 68]. Similarly, knocking-down the expression of Notch-1[69], Delta-like-1 or Jagged-1 within the p53 pathway by RNAi also induces apoptosis and suppresses cell proliferation in multiple glioma cells transfected with siRNAs [70].

PPM1D, a negative regulator of the p38 MAP kinase-p53 pathway, is an oncogene that is overexpressed in many cancers and is also proposed to participate in other critical cell survival pathways [71]. Down-regulation of PPM1D by siRNA induced reduced cell proliferation and apoptosis in MCF7 and ZR-75-1 cells carrying wild-type p53 but not in BT-474 carrying mutant p53 [71]. Thus, targeting PPM1D by siRNA may be another useful strategy for tumors with normal p53.

4.1.5. Other Oncogenesis Pathways

Other cellular pathways altered in cancer include glioma-associated oncogene (GLI) pathway, adenomatous polyposis coli (APC) pathway, hypoxia-inducible transcription factor (HIF) pathway, and so on. For example, more than 60 putative direct HIF-1 target genes have been identified to play important roles in tumor progression [72]. Knocking-down the expression of HIF-1a using RNAi enhanced hypoxiamediated tumor cell apoptosis *in vitro*, prevented tumor growth and resulted in increase of chemosensitivity [73-75]. There-

fore, targeting the HIF-1alpha may be useful treatment modality for cancers.

β-Catenin is a member of the APC signaling pathway which is crucially involved in the regulation of cellular proliferation. Mutation of this gene results in increased β-catenin protein level which enhances cellular proliferation and support colon cancer development. Knocking-down of the overexpressed β-catenin by siRNAs decreased the expression of β-catenin-dependent genes and reduced the proliferation rates of colon cancer cells [76-77]. Another potential target of cancer therapy includes inhibiting gene amplification exemplified by N-MYC (neuroblastoma-myelocytomatosis viral oncogene) expressed at late stages [78] or in aggressive forms of neuroblastoma and erbB-2 (verbB-2 avian erythroblastic leukemia viral oncogene homolog 2) [79-81]. In conclusion, although a thorough discussion of each gene involved in carcinogenesis pathways is almost impossible, many reports have showed silencing of various genes results in the suppression of proliferation and/or enhanced apoptosis of cancer cells, which emphasizes the potential of RNAi as a therapeutic method to treat malignant tumors.

4.2. Cell Cycle

RNAi can be directed against cell-cycle control genes to block cell division and promote apoptosis. Polo-like kinase-1 (PLK1) is a key cell-cycle regulator that is overexpressed in various human tumors [82-83]. Spänkuch-Schmitt *et al.* used RNA interference to silence the PLK1 in breast cancer, cervical cancer, colon cancer and lung cancer cell lines [82]. Several cells manifest abrogation of spindle formation and mitosis was arrested at various time points dependent on the cell line Treatment with siRNA also led to a statistically significant anti-proliferative activity. Additionally, Nogawa *et al.* showed that intravesical administration of PLK1-targeted siRNA carried by cationic liposomes prevented tumor growth in an animal model of bladder carcinoma [84]. More importantly, RNA interference-mediated silencing of the Polo-like Kinase 1 enhanced cancer cell chemo- and radiation sensitivity [85-87].

An increase of Skp-2, which is involved in the degradation of cell-cycle regulators, is thought to be one of the potential mechanisms underlying cell-cycle derangement in various types of cancer. Anti-Skp-2 RNAi was delivered to a human small-cell lung carcinoma cells with increased Skp-2 and knocking-down the expression of Skp2 resulted in efficient inhibition of the *in vitro* cell growth [88]. However, no significant effect on the growth of cells with low Skp-2 levels was observed upon administration with Skp-2 siRNA [88]. Overexpression of p28GANK increases the phosphorylation of pRB and inhibits p16INK4a activity, thus accelerating cell-cycle progression. RNAi was used to inhibit p28GANK expression in HCC [89]. The results showed that the depletion of p28GANK led to the decrease of pRB phosphorylation, the enhancement of caspase 8- and 9-mediated apoptosis, and the suppression of HCC tumor growth in nude mice.

Other molecules targeted by siRNA are the cyclins that are important for phase specific progression. Li *et al.* showed that depletion of cyclin E by RNAi promoted apoptosis of hepatocellular carcinoma cells, blocked cell proliferation and inhibited hepatocellular carcinoma tumor growth in nude mice [90]. Cyclin A_2 is critically involved in two steps in the cell cycle, both in the entry of G1 cells into S phase and in the G2 - M transition [91-92]. Increased expression of cyclin A_2 has been detected in many types of cancers [93]. Wang *et al.* indicated that transient transfection of chronic myelogenous leukemia cell lines with siRNA suppressed cyclin A_2 overexpression, thereby inhibiting cell growth and promoting apoptosis [91]. The role of cyclin B_1 in progression through the G2/M phase of the cell cycle is well characterized, and its expression is also known to be aberrant in a number of malignant tumors [93]. The reduction of cyclin B_1 levels in HeLa cells by siRNA caused inhibition of proliferation and apoptosis [94]. Cells with reduced cyclin B_1 were more susceptible to the administration of taxol and showed inhibited tumor growth in nude mice [94].

4.3. Cell Senescence

In contrast to normal somatic cells, cancer cells overexpress telomerase which maintains the length of telomeres, preserving genome stability [95]. Silencing the human telomerase reverse transcriptase (hTER) by siRNA can results in cancer cell growth impairment [96-97]. Inhibition of hTERT by RNAi in a model of bladder cancer resulted in decreased cell number of S phase, increased cell population of G0/G1 phase both *in vitro* and *in vivo*, and attenuated tumor growth of xenograft mice model compared with controls [98]. Moreover, Nakamura *et al.* showed that knocking down the expression of hTER by siRNA sensitized cancer cells to ionizing radiation and chemotherapy [99].

Other molecules associated with cellular senescence may also serve as targets for RNAi cancer therapy. The mammalian heterogeneous nuclear ribonucleoparticule A1 and A2 proteins bind to the G-tails of telomeres with high affinity. siRNA mediated down-regulation of A1/A2 protein expression in various human cancer cells, such as those derived from cervical, colon, breast, ovarian and brain cancers, induces specific and rapid apoptosis but not in normal cells [100-101].

4.4. Apoptosis

Over-expression of anti-apoptotic factors characterizes cancer cells. These anti-apoptotic proteins include Bcl-2, Bcl-xL, surviving, X chromosome-linked IAP (XIAP) and Fas-associated death domain-like interleukin-1b-converting enzyme-like inhibitory protein (FLIP). This property enables them to survive under abnormal growth stimuli and confers resistance to different apoptotic triggers, like oxidative stress, chemo- and radio-therapeutic agents. Therefore, the restoration of apoptosis by using RNAi to target key anti-apoptotic proteins expressed by cancer cells would have important therapeutic implications. SiRNA against Bcl-2 had a strong ant-tumor activity in a mouse model of liver metastasis and in a xenograft model of human prostate cancer [102]. Specific suppression of Bcl-2 and c-Raf by RNAi induced apoptosis and enhanced the efficacy of the chemotherapeutic agent Etoposide on myeloid leukemia cells [103]. Remarkably, synergistic anti-tumor effects have also been

obtained *in vivo* by co-administering cisplatin and Rad51-targeted siRNA [104] . Down-regulation of survivin by RNAi inhibited tumor growth *in vitro* and *in vivo* through induction of apoptosis [105-106]. RNA interference targeting the XIAP, leading to an elevation of caspase-3 activity, promotes radiation-induced apoptosis [107-108]. Other molecules with anti-apoptotic function, though not directly related to the apoptosis pathway, may also serve as targets for RNAi technology.

4.5. Angiogenesis

For tumors to become prolific and metastasize, they must be well vascularized. The process of tumor angiogenesis has been investigated as an important target for siRNA against cancer. VEGF plays a critical role in the pathological angiogenesis that occurs in a number of cancers. Takei *et al*. have shown that siRNA against VEGF mixed with atelocollagen suppressed the *in vivo* growth of PC-3 prostate cancer cells [109]. Suppression of VEGF by RNAi in colorectal carcinoma, osteosarcoma, hepatocellular carcinoma and retinoblastoma can also lead to the inhibition of tumor growth and metastasis [110-113].

CD31/PECAM-1 (Platelet endothelial cell adhesion molecule-1) is also a major constituent of the endothelial cell intercellular junction. Santel *et al*. systematically administered lipoplexed siRNA anti-CD31 to mice with establishes tumors grown subcutaneously and found a decrease in the total amount of CD31 positive vessels and in the vessels length [114]. Notably, tumor growth inhibition as well as reduction in the volume of lymph node metastases was observed even when tumors were grown orthotopically.

4.6. Cellular Adhesion and Invasion

RNAi technology can be used to target the molecules involved in tumor invasion and metastasis. Urokinase plasminogen activator receptor (uPAR) and cathepsin B are proteases implicated in the extracellular matrix degradation, a characteristic feature of tumor progression. Some investigators used the RNAi approach to silence uPAR and cathepsin B. They found that RNAi of uPAR and cathepsin B reduces glioma cell invasion and angiogenesis both *in vitro* and *in vivo* [115-119].

The metalloproteinase family of proteins is involved in cellular migration and invasion [120-121]. Jia *et al.* reported that down-regulation of metalloproteinase 11 by siRNA inhibited the metastatic capability of murine hepatocarcinoma cell Hca-F to lymph nodes [122]. Blackburn *et al.* showed that RNA interference inhibition of matrix metalloproteinase-1 prevents melanoma metastasis by reducing tumor collagenase activity and angiogenesis [123]. Sanceau *et al.* employed siRNA to inhibit matrix metalloproteinase- 9 and demonstrated that suppression of this protein led to a change in phenotype for Ewing's sarcoma COH cells and the treated cells became less migration, aggregated in clusters, and expressed increased levels of E-cadherin and other cytoskeletal proteins associated with cell-cell adhesion [124].

The elevated glycolysis and proton secretion in tumors is also supposed to contribute to the transformation, proliferation and metastasis of cancer cells. Proton pump V-ATPase is the enzyme that can pump protons into extracellular environment and is important in solid tumors for maintaining neutral cytosolic pH and acidic extracellular pH of cancer cells [125]. Inhibition of V-ATPase activity by down-regulation of ATP6L by RNAi has been shown to effectively retard cancer growth and suppressed cancer metastasis [125].

CXC chemokine receptor-4 (CXCR4) plays an active role in the adhesion/metastatic process. SiRNA against CXCR4 effectively suppressed CXCR4 expression in salivary gland mucoepidermoid carcinoma cells [126] and human breast carcinoma cells[127], leading to significant decrease in malignant cell invasiveness *in vitro*. More importantly, direct injection of a pool of naked siRNA against CXCR4 has been found to prevent tumorigenesis of a breast cancer cells in SCID mice [127]. Thus, siRNA targeting CXCR4 may represent a potentially novel preventive and therapeutic strategy for cancer treatment.

Over-expression of RhoA and RhoC is associated with poor prognosis of breast cancer, due to increased tumor cell proliferation and invasion. *In vitro*, RhoA and RhoC siRNAs inhibited human breast cancer cells proliferation and invasion more effectively than conventional blockers of Rho cell signaling [128]. Moreover, in a nude mouse model, intratumoral injections of anti-RhoA siRNA almost totally inhibited the growth and angiogenesis of xenografted human breast tumors [128].

Overexpression of receptor tyrosine-kinase RON mediates the transformed phenotypes in immortalized colon epithelial cells. Knocking down the expression of RON by siRNA significantly affected cancer cell proliferation and motility and led to increased apoptotic cell death [129]. Focus-forming activities and anchorage-independent growth of colon cancer cells were also dramatically reduced [129].

Heparanase is an endoglycosidase which cleaves heparan sulfate and participates in degradation and remodeling of the extracellular matrix (ECM) [130]. It is preferentially expressed in human cancers. Administration of siRNA against heparanase into breast carcinoma and hepatocellular carcinoma cells led to a profoundly reduced angiogenesis, invasion and adhesion ability both *in vitro* and *in vivo* [131-133].

The a6b4 integrin has been implicated to play an important role in the invasive phenotype of many carcinomas. SiRNA targeting the a6b4 integrin reduced the expression of this integrin and resulted in decreased invasiveness of breast carcinoma cells [134]. Osteopontin, a ligand for integrin-αVβ3 and CD44 receptors, is also involved with adhesion/metastasis in several cancers. Inhibition the expression of osteopontin by siRNA suppressed the metastasis of colon cancer, gastric cancer and pancreatic cancer [135-137].

4.7. Radiotherapy/Chemotherapy Resistance

Improving the efficacy of radiotherapy and chemotherapy in cancer has been another potential application of RNAi. Inhibition the expression of HIF-1 by RNAi led to the increased sensitivity of tumor cell to ionizing radiation [138-139]. It is known that defects in repair of DNA double-strand breaks makes cells hypersensitive to ionizing radiation. Peng *et al*. used RNAi to target Prkdc that encodes for the cata-

lytic subunit of DNA repair protein kinase DNA-Pkcs and found that radio-sensitivity was increased particularly in low-dose region of 0–1 Gray in human fibroblasts [140]. Furthermore, a combined treatment with radiation and siRNA targeting the PI3 kinase P110α and p85β isoforms, or Akt-1, in cancer cells has also been shown to enhance cell killing [141].

Multi-drugs resistance (MDR) is a well-known phenomenon limiting the efficacy of several therapeutic regimens. A hallmark of multidrug resistant cancers is the overexpression of the MDR-1 gene product, the P-glycoprotein [142-144]. Knockdown the expression of the MDR1 by siRNA was shown to enhance the intracellular accumulation of various chemotherapeutic drugs and selectively restore chemo-sensitivity of malignant cells [142-146]. RNA interference targeting the M2 subunit of ribonucleotide reductase enhanced chemo-sensitivity to gemcitabine and resulted in tumor growth retardation and significant tumor mass reduction in the nude mouse xenograft model [147]. Treatment of hepatocellular carcinoma cells [148-149], pancreatic carcinoma cells [150] and melanoma cells [151] with siRNA directed against Mcl-1 sensitized cells to apoptosis and significantly enhanced chemo-sensitivity. In addition, RNAi to phosphatidylinositol 3-kinase (PI3K) components was found to enhance apoptosis of resistant colorectal cancers upon treatment with TRAIL and suppressed metastatic growth *in vivo* [152]. Niu *et al*. showed that siRNA-mediated type 1 insulin-like growth factor receptor (IGF1R) silencing increases liver cancer cells sensitivity to adriamycin and promotes apoptosis [153]. Tao *et al.* indicated that suppression of Aurora-A kinase using RNAi enhances radiation response in p53-deficient cancer cells [154]. These results suggest that multidrug-resistant cancers may be efficiently combated by a combination of chemotherapy and RNA interference.

5. CHALLENGES AND PROSPECTS

Since the discovery that siRNA can silence genes in mammalian cells, RNAi has been considered as a potential cancer treatment. Although many important oncogenic targets have been identified and RNAi-mediated gene silencing of these genes has produced favorable outcomes *in vitro* and *in vivo*, several issues have not been fully resolved. These include off-target effects, competition with cellular RNAi components and tissue specific targeting delivery *in vivo*.

Off-targeting is one of the challenges facing siRNA as a cancer therapy tool. Although the process of RNAi causes degradation of target mRNA, it can also prevent the translation of other somewhat mismatched mRNA wherein the siRNA is functioning as a miRNA, leading to potential toxicity. This issue can be partly addressed by refinement of the algorithms used for designing siRNA, as well as through chemical modification of the siRNA or even encapsulation in nano carriers [155-156].

Another issue is what effect an excess of siRNA from outside the cell may have on the function of the RISC, the components at the central of the RNAi pathways [157-158]. The number of RISCs in the cell is unknown, thus it is difficult to determine the exact titre of siRNA required to mediate efficient knock down of target gene, since over administration could create problems in exhausting the pools of Dicer, a known limiting factor for normal gene regulation. As more is gleaned about the detailed mechanism by which RNAi regulates gene expression, this issue can be partially or fully solved in the future.

The biggest challenge to the potential use of siRNA-based cancer therapies is the requirement for safe and efficient delivery of siRNA to cancer cell tissues *in vivo* [156, 159-160]. Cell-specific targeting of RNAi can be achieved by particulate carriers coated with receptor-recognized ligand and receptor-targeting aptamers or antibody. For specific targeting, angiogenesis and metastasis can also be exploited for the differences between cancerous cells and normal cells, which include uncontrolled proliferation, insensitivity to negative growth regulation and antigrowth signals.

ACKNOWLEDGEMENTS

The authors thank NSFC (20831003, 90813001, 20833006, 90913007, 20903086), 973 Project (2011CB93 6004), and Funds from the Chinese Academy of Sciences for the financial support.

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Received: August 08, 2010 Revised: November 10, 2010 Accepted: January 06, 2011