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Chapter 8

Combinatorial RNAi approaches

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Combinatorial RNAi approaches

Although potent gene knockdown can be obtained with a single RNAi-inducer, some clinical applications will require a combinatorial RNAi approach. Sometimes multiple genes need to be targeted simultaneously or intensified silencing of a single gene is required to obtain a biological effect. In mammalian cells, the natural RNAi pathway can be triggered by expression of transcripts that resemble the pre-miRNA structure: short hairpin RNAs (shRNAs) of 19-21 bp (Fig. 1A) (1-3). Recently, these shRNAs have been optimized by inclusion of RNA structural motifs that mimic pri-miRNAs (Fig. 1A) (4). These artificial miRNAs seem to be more efficient than the simple shRNAs in inducing RNAi-mediated silencing (5,6). In order to express multiple RNAi effectors simultaneously, several combinatorial RNAi strategies have been developed. This chapter summarizes the latest experimental advances in combinatorial RNAi-based gene therapy approaches and the potential for clinical application will be discussed.

Multiple short hairpin RNAs for basic research purposes

To induce combinatorial RNAi, the most straightforward strategy is to simultaneously express multiple shRNAs from several gene cassettes (Fig. 1B). If an identical shRNA is multiplexed, one obtains intensified RNAi of a single gene. If different shRNAs are multiplexed, one achieves combinatorial RNAi.

Using a U6-driven 2-shRNA expression vector, Yu et al. (7) simultaneously inhibited the α- and β-isoforms of glycogen synthase kinase, 2 related kinases involved in the regulation of a variety of cellular processes. Increased GSK-3 α/β levels are associated with type 2 diabetes and Alzheimer's disease (8), whereas decreased GSK-3 activity results in accumulation of β-catenin, which has been associated with colon cancer and melanomas (9). GSK-3 phosphorylates β-catenin and causes its destabilization. Knocking down GSK-3 α or GSK-3β resulted in increased β-catenin levels and simultaneous inhibition of both kinases led to a further increase in β-catenin expression. These results demonstrate the usefulness of combinatorial RNAi in dissecting the functional roles of 2 kinases with partially redundant functions.

Jazag et al. used combinatorial RNAi to dissect the functions of the transcription factors Smad 2, 3 and 4 using multiple shRNA cassettes with a U6 promoter (10). These transcription factors mediate the growth inhibitory effect of TGF-β in many cell types. Loss of the TGF-β cytostatic response by various genetic and epigenetic alterations of the TGF-β-Smad pathway has been described for several human cancers (11-13). Simultaneous knockdown of Smad 2, 3 and 4 resulted in distinct phenotypic changes in TGF-β-dependent cellular functions, including invasion, wound healing and apoptosis.

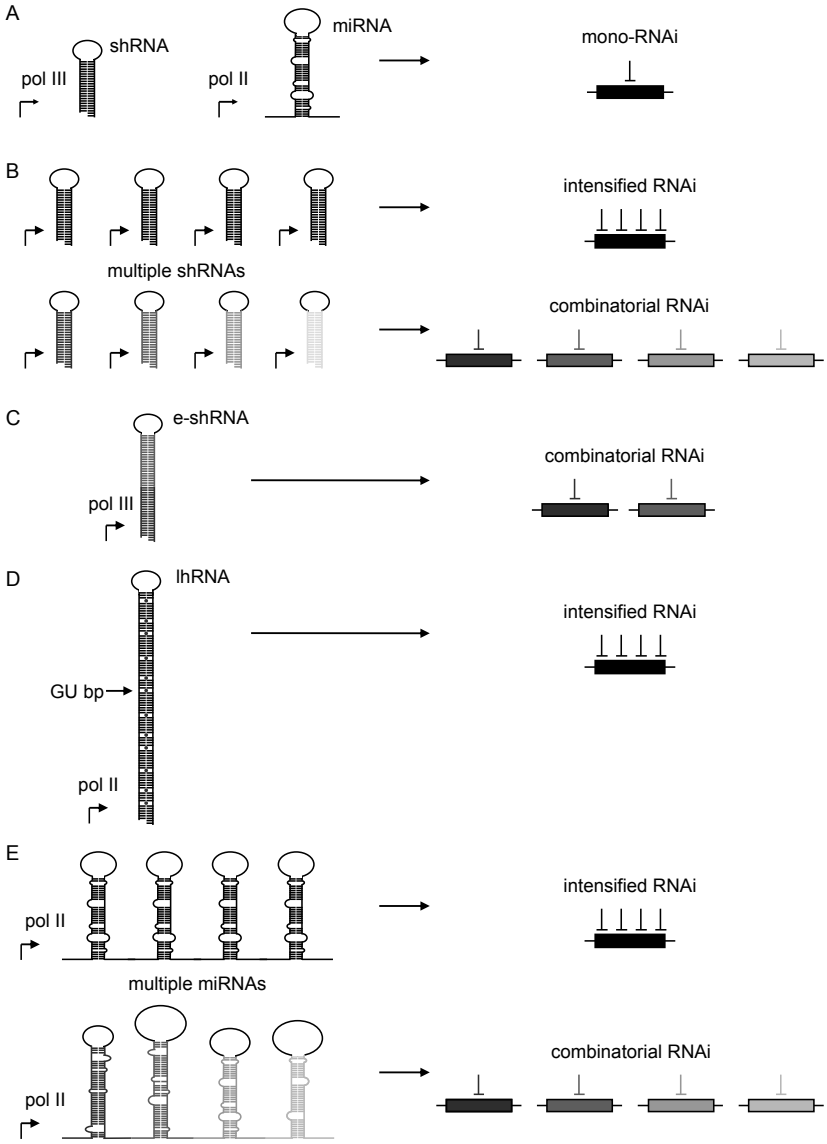

Figure 1. Expression strategies for RNAi inducers. (**A**) Mono-RNAi therapy with an shRNA vector driven by an RNA polymerase III promoter or an artificial miRNA driven by an RNA polymerase II. (**B**) Combinatorial RNAi with multiple identical or different shRNA expression cassettes in a single vector to intensify the RNAi effect or to simultaneously target different genes. (**C**) Combinatorial RNAi with an e-shRNA expressed from an RNA polymerase III promoter to generate multiple siRNAs that inhibit different genes. (**D**) Intensified RNAi with a lhRNA to generate multiple siRNAs to inhibit multiple adjacent targets. (**E**) Combinatorial RNAi with a miRNA-like polycistron expressed from an RNA polymerase II promoter to intensify the RNAi effect or to target different genes at once.

Multiple short hairpin RNAs in antitumor RNAi therapeutics

The potential of combinatorial RNAi to treat chronic myeloid leukemia (CML) was analyzed by targeting the constitutively active Bcr-Abl tyrosine kinase and the downstream signalling molecules Shp2, Stat5 and Gab2 (14,15). Bcr-Abl modulates intracellular signalling cascades, thereby enhancing survival and proliferation of leukemic cells (16). Treatment of CML with a drug that inhibits the Bcr-Abl oncoprotein leads to the selection of drug-resistant cancer cells, especially in advanced disease (17). Therefore, the downstream signalling molecules were targeted as an alternative therapeutic option. Increased inhibitory efficacy was obtained with lentiviral vectors expressing 2 versus a single shRNA, without loss of specificity. Interestingly, simultaneous knockdown of Shp2 and Stat5 triggered the specific depletion of Bcr-Abl-expressing cells, indicating that it is not necessary to knock down the Bcr-Abl oncoprotein itself.

For laryngeal squamous carcinoma, a combinatorial RNAi approach with shRNAs against the VEGF, TERT and Bcl-xl functions was reported. These factors are upregulated in cancer cells and stimulate tumor growth. Simultaneous targeting of these 3 genes produced a more prominent inhibitory effect on the laryngeal squamous carcinoma cells *in vitro* and *in vivo* than an RNAi mono-therapy (18,19).

Multiple short hairpin RNAs to intensify the RNAi activity

Another rationale to use multiple RNAi inducers is to intensify the RNAi-mediated suppression of an individual gene. An extremely low expression level of the target gene is sometimes required to establish a biological effect. If a single shRNA is multiplexed, one obtains intensified RNAi of a single gene (Fig. 1B).

To achieve maximal target knockdown, Gonzalez et al. (20) designed DNA expression plasmids encoding up to 6 copies of U6-shRNA cassettes targeting the HLA class I genes in cultured and primary human T cells. Downregulation of HLA genes using combinatorial RNAi could be applied to avoid T-cell mediated rejection of immunogenic and/or HLA-divergent allografts. Consistent with these results, Gou et al. (21) observed an intensified RNAi effect against eGFP using a DNA vector encoding 4 shRNAs. Inhibition of HIV-1 replication by silencing of the LEDGF/p75 cellular cofactor provides another example where an intensified RNAi attack was needed to score an antiviral effect (22). Recently, Nagao et al. (23) constructed 2 types of combination vectors encoding either 3 different or 3 identical shRNA expression cassettes targeting the DNA repair factor XPA. This target was chosen because it is not essential for cell viability and functional knockdown can easily be measured since XPA deficient cells are sensitive to UV-irradiation. XPA suppression was significantly increased in cells expressing the multiple shRNA vectors compared to cells expressing a single shRNA vector. Increased XPA knockdown indeed resulted in decreased UV-induced DNA damage repair and an increased sensitivity to UV-irradiation.

Multiple short hairpin RNAs against viruses

Other interesting targets for combinatorial RNAi therapy include escape-prone viruses such as poliovirus, human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV) and hepatitis C virus (HCV). RNAi mono-therapy against these viruses faces the same obstacle as mono-therapy with antiviral drugs, which is the selection of viral escape variants (24-28). The sequence-specificity of RNAi now turns into a disadvantage because a single point mutation in the target is sufficient to overcome RNAi-mediated inhibition. Viral escape was described by Gitlin et al. (24,25) in poliovirus-infected cells treated with siRNAs. Similarly, HCV escape mutants with multiple point mutations in the target sequence emerged upon successive mono-siRNA treatment in the HCV replicon system (26). Several groups reported the emergence of HIV-1 variants with a point mutation in the target sequence upon elicitation of RNAi pressure by a single inhibitor (27-29). Interestingly, HIV-1 can also escape by mutation of an upstream nucleotide that triggers a change in local RNA secondary structure of the target (30), although this may represent a rather unique escape route (31). This induced RNA structure occludes the target sequence for binding to the complementary siRNA, thus explaining the resistance phenotype (32).

A mono-RNAi therapy is not likely to yield long-term inhibition of viruses that establish a chronic infection in the host. To obtain a permanent block of virus replication, a combinatorial attack is needed that simultaneously targets several viral sequences. Ideally, such targets should encode essential functions and its sequence should be well conserved among different virus strains, thus avoiding that genetic variation will impair the therapy (33). Another therapeutic possibility against escape-prone viruses is to inhibit cellular cofactors that are essential for viral replication, e.g. mediating the process of viral entry by targeting the receptor molecules. Peripheral blood mononuclear cells (PBMCs) that stably co-express shRNAs against the HIV-1 co-receptors CXCR4 and CCR5 were protected against viral infection (34). The therapeutic potential of such inhibitors has to be validated to exclude the possibility that novel HIV-1 variants are selected that use new receptors for cell entry.

Chang et al. (35) used lentiviral vectors encoding anti-HIV shRNAs against highly conserved sequences in the pol, int and vpu genes and demonstrated efficient inhibition of virus replication in acutely and chronically infected cells and PBMCs. The combination of 3 vectors was much more efficient than treatment with an individual shRNA vector. However, the possible prevention of viral escape was not evaluated in that study.

Ter Brake et al. (33) performed a study using 86 shRNA gene constructs against highly conserved HIV-1 sequences. Multiple effective shRNAs against 8 targets within the HIV-1 RNA genome were identified. Virus production in cells that stably express 3 shRNAs from a single lentiviral vector was strongly reduced compared to the cells expressing a single shRNA. Furthermore, cells that stably express 2 shRNAs showed a more durable inhibition compared to

cells expressing a single shRNA construct. In a follow-up study (36), different promoters were used to express 4 shRNAs against HIV-1. This approach was used to avoid recombination-mediated deletion of shRNA cassettes on repeated H1 promoter sequences (37). In contrast to cells expressing a single shRNA, no HIV-1 escape was observed in 4-shRNA transduced cells. Notably, these results were obtained with a considerable dose of challenge virus in stably transduced cells with only a single copy of the therapeutic vector. A low shRNA expression level is important to avoid the risk of toxicity due to saturation of the RNAi pathway. These results indicate that a combinatorial RNAi approach against HIV-1 could be safe and effective, but further studies are needed to validate the therapeutic potential *in vivo*.

Combinatorial RNAi against HCV and a cellular cofactor was induced with a 3-shRNA lentiviral vector (38). These vectors specifically inhibited HCV replication for approximately 17 days. Interestingly, a 2-shRNA vector targeting a host factor (CD81) and HCV (NS5B) showed reduced efficacy compared to vectors that target 2 viral sequences (NS5B and IRES). Whether this reduced efficacy is perhaps caused by a relatively poor efficacy of the individual shRNAs remains to be tested.

Taken together, these results demonstrate the effectiveness of combinatorial RNAi by multiple shRNA expression from the same or different promoters. The use of identical promoters in a single vector should be avoided as it can cause recombination or deletion due to sequence similarity. To circumvent this problem, different promoters can be used for the expression of multiple hairpin RNAs. However, a careful selection of promoters is necessary to ensure an approximately equal expression level of each hairpin. Unequal RNAi pressure on different target sites may not be ideal to obtain the desired RNAi effect. However, important safety issues including targeting of other genes (off-target effect) and saturation of the RNAi pathway need to be analyzed in detail to determine the clinical usefulness of this approach.

Extended short hairpin RNAs

A novel combinatorial RNAi approach combines multiple shRNA units in a single transcript by stacking them on top of each other (Fig. 1C). We combined 2 highly effective shRNAs targeting 2 conserved HIV-1 regions in a single extended e-shRNA construct expressed from an H1 promoter (39) (chapter 2). This approach of shRNA-stacking requires a very careful design, because the guide strands of the 2 shRNAs do not target a contiguous sequence of the target mRNA. Thus, imprecise Dicer cleavage will generate a second siRNA that is not necessary perfectly complementary to the target, and thus loosing effectivity. We made a set of e-shRNAs with increasing stem length and observed that the first siRNA is always produced and fully active. However, the second siRNA was only produced when the hairpin stem reached a certain length (chapter 2). In a follow up study, we further extended these e-shRNAs and showed that this design can maximally produce 3 active siRNAs (chapter

3). Furthermore, we showed that HIV-1 replication is durably inhibited in T cells expressing an e-shRNA encoding multiple active siRNAs, whereas early break-through of viral replication was observed in cells that express a single shRNA. Importantly, e-shRNAs do not elicit an IFN response in 293T cells at the efficacious dose. Other groups generated similar hairpin RNA constructs that encode 2 or 3 active siRNAs against HIV-1 and showed that efficient virus inhibition can be obtained using these constructs (40,41). A major disadvantage of this strategy is that a gradient of siRNAs is produced from the base towards the top of the hairpin, possibly due to reduced Dicer processing. Furthermore, each shRNA combination may require optimization of the precise e-shRNA design.

Long hairpin RNAs against viruses

Another method to induce combinatorial RNAi is to express long hairpin RNAs (lhRNAs) driven by an RNA polymerase II promoter (Fig. 1D). These lengthy RNA duplexes are designed to be processed into numerous siRNAs. Compared to the e-shRNA design, the regular lhRNA has an extended stem that encodes additional siRNAs that are complementary to mRNA sequences that are immediately adjacent to the target of the siRNA from the base of the lhRNA. This design has the main advantage that any siRNA produced from the lhRNA, independent of the precise position of Dicer cleavage, is fully complementary to the target mRNA, and thus potentially an inhibitor. An obvious disadvantage is that not all processed siRNA will turn out to be effective inhibitors.

In contrast to transfection of dsRNA molecules larger than 30 bp, intracellular expression of lhRNA at an effective dose does not readily elicit an IFN response (42). Furthermore, inclusion of point-mutations in the sense strand of the hairpin that result in G-U base pairs can avoid this immunostimulatory effect (43,44). Thus far, lhRNA expression vectors have been used to inhibit several escape-prone viruses including HIV-1 and HCV (29,42-45). Efficient knockdown of HIV-1 was reported using lhRNAs of 50, 53, 60, 80 and 300 bp without IFN induction (29,41,42,45). Konstantinova et al. (42) measured reduced antiviral activity for a 300-bp lhRNA compared to a regular shRNA-inhibitor, although most of the inhibitory power of this particular lhRNA transcript may be due to a non-RNAi mechanism induced by sense HIV-1 leader sequences (46). Efficient inhibition of HCV was also demonstrated using 50, 100 and 197-bp hairpins with mutations in the sense strand (43,44). Importantly, these lhRNAs can silence both wild-type and shRNA escape viruses, indicating that multiple effective siRNAs were generated from the single lhRNA precursor (29,42,44).

The first direct evidence that a 50-bp hairpin produces 2 siRNAs was reported by Akashi et al. (43). A detailed analysis of a 62-bp lhRNA against HBV by Weinberg et al. (47) revealed that siRNAs are mainly produced from the base of the hairpin. It seems likely that for other lhRNAs the siRNA production is also diminished from the base towards the top of the lhRNA due to less

efficient processing by Dicer, but no further studies thus far looked into the number of active siRNAs that are produced by this design. The results presented in chapter 3 strongly argue that there is an upper limit to the size of an active lhRNA molecule. As such, mammalian lhRNAs loose most of the power of truly lengthy antiviral lhRNA molecules as originally designed in plants.

Multiple artificial miRNAs to intensify the RNAi activity

Another, elegant strategy to induce combinatorial RNAi is to express transcripts that encode multiple artificial miRNAs under control of an RNA polymerase II promoter (Fig. 1E). These transcripts resemble a natural miRNA cluster that is transcribed as a single transcription unit (polycistron). In contrast to the multiple shRNA approach, this approach allows expression of several miRNA precursors from a single promoter. This approach has been used in several studies for different purposes: to intensify RNAi, to knock down disease-associated genes or to inhibit multiple viral genes.

Zhou et al. (48) designed a modified miR-30 transcript to target human superoxide dismutase 1 (SOD1), a gene that is mutated in the neurological disorder amyotrophic lateral sclerosis (49). To intensify the RNAi effect, 2 miRNA-like hairpins were concatenated in a single transcript (separated by 100 nt) and expressed from the cytomegalovirus (CMV) immediate early promoter. Surprisingly, inhibition by the 2-miRNA vector was decreased compared to the single miRNA vector. This could be due to processing problems of the tandem-miRNA transcript, which highlights the importance of a careful design when multiplexing diferent miRNAs.

In contrast, Sun et al. (50) observed an increase in siRNA levels and a more profound inhibition when 2 miR-30 based hairpins were concatenated compared to a single miRNA. However, addition of a third hairpin only modestly increased the RNAi activity. Interestingly, the efficacy of a single hairpin is even enhanced upon multimerization with an irrelevant second hairpin, arguing for a general effect on transcript stability or the efficiency of miRNA processing. Consistently, Chung et al. (51) demonstrated a gradual increase in RNAi activity using up to 8 miR-155 modified miRNAs against the luciferase mRNA reporter.

To study the isoform redundancy of cAMP-dependent kinases, Zhu et al. (52) used multiple miR-30 based hairpins and demonstrated functional consequences of depleting multiple endogenous target genes. These results highlight the possibility to intensify the RNAi effect using multi-miRNA expression vectors for effective knockdown of a single gene or multiple genes.

Besides the ability to co-express multiple miRNAs, another major advantage of using an RNA polymerase II promoter is the possibility for conditional RNAi (5). For example, Shin et al. (53) expressed artificial miRNAs from a lentiviral vector using a tetracycline-regulated promoter. Using this vector encoding 2 artificial miRNAs, conditional knockdown of the heterotrimeric G proteins Gα12 and Gα13 was obtained.

A recent report by McLaughlin et al. (54) showed the efficacy of combinations of miRNA mimics targeting several sites within the Abl-coding sequences for the treatment of leukemia. Previously, this group studied the potential of shRNAs to suppress leukemic cell growth *in vivo* by targeting the Bcr-Abl transcript, but significant levels of Bcr-Abl kinase activity remained. A lentiviral vector encoding 3 anti-Abl miRNAs could effectively suppress oncogene expression and activation of the Bcr-Abl pathway to such an extend that regrowth of leukemic cells was prevented *in vitro* and *in vivo*.

Multiple artificial miRNAs against viruses

Recently, Snyder et al. (55) expressed anti-HBV miRNAs under control of a liver-specific promoter. A polycistronic construct encoding 4 different anti-HBV miRNAs was generated and demonstrated to be efficient in inhibiting HBV production. The functionality of each antiviral miRNA was tested on a specific luciferase reporter. Modest inhibition was obtained per miRNA, suggesting that the potent inhibition of HBV production was an additive effect of all antiviral miRNAs.

We used a multiplex miRNA expression strategy based on the mir-17-92 cluster to target HIV-1 at different highly conserved regions (chapter 4). First, we generated a large set of anti-HIV-miRNAs and selected the best inhibitors for construction of the polycistronic plasmid. Second, we generated constructs encoding 2, 3 and 4 hairpins. Interestingly, consistent with the results from Sun et al. (50), we observed a greater inhibitory activity of a miRNA when it is concatenated to other, even irrelevant hairpins in a single transcript. The increased inhibitory effect correlated well with increased production of the mature miRNAs. Our results indicated that the intrinsic activity of a miRNA is in fact higher than that of an shRNA inhibitor. Furthermore, we showed that HIV-1 replication is inhibited in T cell lines that stably express 4 antiviral miRNAs.

In summary, these results indicate that the multi-miRNA approach can be used in combinatorial RNAi therapeutics. However, careful design for concatemerization of the hairpins is necessary to ensure proper processing and optimal inhibitory activity. Further studies are needed to test the impact of many variables (hairpin positioning, hairpin number, spacer length, type of miRNA scaffold etc.) to optimize this approach.

RNAi side effects and off-target effects

To apply combinatorial RNAi in the clinic, critical issues including safety and efficacy have to be carefully addressed. Safety is the major concern, since a mono-RNAi treatment can already produce an unwanted side-effect, including IFN induction (56), off-target effects on other mRNAs (57) and toxicity due to saturation of the endogenous RNAi pathway (58). These risks may be increased in a combinatorial RNAi setting, in particular the off-target effect

and saturation of the RNAi pathway. Off-targeting can be due to pairing of the seed area of the siRNA/miRNA guide strand with small complementary stretches in the 3′ UTR of non-target mRNAs. To minimize the risk of toxicity, the concentration of RNAi inducer should ideally be fine-tuned. The use of a cell-type specific polymerase II promoter has several advantages in this respect. First, expression of the RNAi inducer will only occur in the cells of interest. Second, it will allow regulated shRNA/miRNA expression. Recently, miRNA-like hairpins were shown to be more active than simple shRNAs, thus allowing a lower dose (5,6). Interestingly, the miRNA-scaffold also resulted in significantly reduced neurotoxicity in mice compared to the respective shRNA (59). Thus, artificial miRNAs may provide a suitable approach to enhance RNAi activity and at the same time reduce toxicity. However, further studies are needed to elaborate on these initial findings.

The concerns regarding combinatorial RNAi need to be carefully addressed using the lowest efficacious dose in appropriate *in vivo* animal models. Interestingly, some mammalian viruses can use cellular or viral miRNAs for their own benefit (60). These viruses were reported to encode RNAi suppressors to inhibit the antiviral RNAi response (61). Whether these RNAi suppressors will diminish the RNAi effect induced by shRNAs/artificial miRNAs remains to be tested. However, given the power of induced antiviral RNAi therapies using shRNAs or artificial miRNAs, it is not likely a serious problem for RNAi therapeutics.

Taken together, a growing body of evidence indicates that combinatorial RNAi has great potential to attack challenging escape-prone targets, such as HCV, HIV and many other human pathogenic viruses. Preliminary studies also show the potency for treatment of metabolic, genetic or blood disorders. Furthermore, combinatorial RNAi may provide an effective tool to unravel roles of different redundant factors in complex cell signalling and transformation processes. Although combinatorial RNAi is very attractive, further studies must be carried out to better understand the unwanted side effects and efficacy of combinatorial RNAi therapies *in vivo*.

References

1. Brummelkamp,T.R., Bernards,R. and Agami,R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, **296**, 550-553.
2. Siolas,D., Lerner,C., Burchard,J., Ge,W., Linsley,P.S., Paddison,P.J., Hannon,G.J. and Cleary,M.A. (2005) Synthetic shRNAs as potent RNAi triggers. *Nat.Biotechnol.*, **23**, 227-231.
3. Paddison,P.J., Caudy,A.A., Bernstein,E., Hannon,G.J. and Conklin,D.S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.*, **16**, 948-958.
4. Zeng,Y., Wagner,E.J. and Cullen,B.R. (2002) Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol.Cell*, **9**, 1327-1333.
5. Silva,J.M., Li,M.Z., Chang,K., Ge,W., Golding,M.C., Rickles,R.J., Siolas,D., Hu,G., Paddison,P.J., Schlabach,M.R. *et al.* (2005) Second-generation shRNA libraries covering the mouse and human genomes. *Nat.Genet.*, **37**, 1281-1288.

6. Liu,Y.P., Haasnoot,J., Ter Brake,O., Berkhout,B. and Konstantinova,P. (2008) Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucleic Acids Res*, **36**, 2811-2824.
7. Yu,J.Y., Taylor,J., DeRuiter,S.L., Vojtek,A.B. and Turner,D.L. (2003) Simultaneous inhibition of GSK3alpha and GSK3beta using hairpin siRNA expression vectors. *Mol Ther.*, **7**, 228-236.
8. Eldar-Finkelman,H. (2002) Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol Med*, **8**, 126-132.
9. Polakis,P. (2000) Wnt signaling and cancer. *Genes Dev.*, **14**, 1837-1851.
10. Jazag,A., Kanai,F., Ijichi,H., Tateishi,K., Ikenoue,T., Tanaka,Y., Ohta,M., Imamura,J., Guleng,B., Asaoka,Y. *et al.* (2005) Single small-interfering RNA expression vector for silencing multiple transforming growth factor-beta pathway components. *Nucleic Acids Res*, **33**, e131.
11. Siegel,P.M. and Massague,J. (2003) Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat.Rev.Cancer*, **3**, 807-821.
12. Miyazono,K., ten Dijke,P. and Heldin,C.H. (2000) TGF-beta signaling by Smad proteins. *Adv.Immunol*, **75**, 115-157.
13. Hahn,S.A., Schutte,M., Hoque,A.T., Moskaluk,C.A., da Costa,L.T., Rozenblum,E., Weinstein,C.L., Fischer,A., Yeo,C.J., Hruban,R.H. *et al.* (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science*, **271**, 350-353.
14. Scherr,M., Chaturvedi,A., Battmer,K., Dallmann,I., Schultheis,B., Ganser,A. and Eder,M. (2006) Enhanced sensitivity to inhibition of SHP2, STAT5, and Gab2 expression in chronic myeloid leukemia (CML). *Blood*, **107**, 3279-3287.
15. Chaturvedi,A., Battmer,K., Schaefer,D., Ganser,A., Eder,M. and Scherr,M. (2007) Comparison between molecularly defined and conventional therapeutics in a conditional BCR-ABL cell culture model. *Oligonucleotides.*, **17**, 22-34.
16. Ren,R. (2005) Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat.Rev.Cancer*, **5**, 172-183.
17. Chu,S., Xu,H., Shah,N.P., Snyder,D.S., Forman,S.J., Sawyers,C.L. and Bhatia,R. (2005) Detection of BCR-ABL kinase mutations in CD34+ cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment. *Blood*, **105**, 2093-2098.
18. Wang,Y., Tao,Z.Z., Chen,S.M., Xiao,B.K., Zhou,X.H. and Liu,J.P. (2008) Application of combination of short hairpin RNA segments for silencing VEGF, TERT, and Bcl-xl expression in laryngeal squamous carcinoma. *Cancer Biol Ther.*, **7**, 896-901.
19. Chen,S.M., Wang,Y., Xiao,B.K. and Tao,Z.Z. (2007) Effect of blocking VEGF, hTERT and Bcl-xl by multiple shRNA expression vectors on the human laryngeal squamous carcinoma xenograft in nude mice. *Cancer Biol Ther.*, **7**, 734-739.
20. Gou,D., Weng,T., Wang,Y., Wang,Z., Zhang,H., Gao,L., Chen,Z., Wang,P. and Liu,L. (2007) A novel approach for the construction of multiple shRNA expression vectors. *J Gene Med*, **9**, 751-763.
21. Gonzalez,S., Castanotto,D., Li,H., Olivares,S., Jensen,M.C., Forman,S.J., Rossi,J.J. and Cooper,L.J. (2005) Amplification of RNAi--targeting HLA mRNAs. *Mol Ther.*, **11**, 811-818.
22. Marshall,H.M., Ronen,K., Berry,C., Llano,M., Sutherland,H., Saenz,D., Bickmore,W., Poeschla,E. and Bushman,F.D. (2007) Role of PSIP1/LEDGF/p75 in lentiviral infectivity and integration targeting. *PLoS.ONE.*, **2**, e1340.
23. Nagao,A., Zhao,X., Takegami,T., Nakagawa,H., Matsui,S., Matsunaga,T. and Ishigaki,Y. (2008) Multiple shRNA expressions in a single plasmid vector improve RNAi against the XPA gene. *Biochem Biophys.Res Commun.*, **370**, 301-305.
24. Gitlin,L., Karelsky,S. and Andino,R. (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature*, **418**, 430-434.
25. Gitlin,L., Stone,J.K. and Andino,R. (2005) Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. *J.Virol.*, **79**, 1027-1035.

26. Wilson,J.A. and Richardson,C.D. (2005) Hepatitis C virus replicons escape RNA interference induced by a short interfering RNA directed against the NS5b coding region. *J.Virol.*, **79**, 7050-7058.
27. Boden,D., Pusch,O., Lee,F., Tucker,L. and Ramratnam,B. (2003) Human immunodeficiency virus type 1 escape from RNA interference. *J.Virol.*, **77**, 11531-11535.
28. Das,A.T., Brummelkamp,T.R., Westerhout,E.M., Vink,M., Madiredjo,M., Bernards,R. and Berkhout,B. (2004) Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J.Virol.*, **78**, 2601-2605.
29. Nishitsuji,H., Kohara,M., Kannagi,M. and Masuda,T. (2006) Effective suppression of human immunodeficiency virus type 1 through a combination of short- or long-hairpin RNAs targeting essential sequences for retroviral integration. *J.Virol.*, **80**, 7658-7666.
30. Westerhout,E.M., Ooms,M., Vink,M., Das,A.T. and Berkhout,B. (2005) HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res.*, **33**, 796-804.
31. von Eije,K.J., Ter Brake,O. and Berkhout,B. (2008) Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. *J Virol*, **82**, 2895-2903.
32. Westerhout,E.M. and Berkhout,B. (2007) A systematic analysis of the effect of target RNA structure on RNA interference. *Nucleic Acids Res.*, **35**, 4322-4330.
33. Ter Brake,O., Konstantinova,P., Ceylan,M. and Berkhout,B. (2006) Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol.Ther.*, **14**, 883-892.
34. Anderson,J. and Akkina,R. (2005) CXCR4 and CCR5 shRNA transgenic CD34+ cell derived macrophages are functionally normal and resist HIV-1 infection. *Retrovirology.*, **2**, 53.
35. Chang,L.J., Liu,X. and He,J. (2005) Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1. *Gene Ther.*, **12**, 1133-1144.
36. Ter Brake,O., 't Hooft,K., Liu,Y.P., Centlivre,M., von Eije,K.J. and Berkhout,B. (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 Inhibition. *Mol Ther.*, **16**, 557-564.
37. Ter Brake,O. and Berkhout,B. (2007) Lentiviral vectors that carry anti-HIV shRNAs: problems and solutions. *J Gene Med*, **9**, 743-750.
38. Henry,S.D., van der Wegen,P., Metselaar,H.J., Tilanus,H.W., Scholte,B.J. and van der Laan,LJ.W. (2006) Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA Interference expression cassettes. *Mol.Ther.*, **14**, 485-493.
39. Liu,Y.P., Haasnoot,J. and Berkhout,B. (2007) Design of extended short hairpin RNAs for HIV-1 inhibition. *Nucleic Acids Res*, **35**, 5683-5693.
40. Saayman,S., Barichievy,S., Capovilla,A., Morris,K.V., Arbuthnot,P. and Weinberg,M.S. (2008) The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA. *PLoS.ONE.*, **3**, e2602.
41. Sano,M., Li,H., Nakanishi,M. and Rossi,J.J. (2008) Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. *Mol Ther.*, **16**, 170-177.
42. Konstantinova,P., de Vries,W., Haasnoot,J., Ter Brake,O., de Haan,P. and Berkhout,B. (2006) Inhibition of human immunodeficiency virus type 1 by RNA interference using long-hairpin RNA. *Gene Ther.*, **13**, 1403-1413.
43. Akashi,H., Miyagishi,M., Yokota,T., Watanabe,H., Hino,T., Nishina,K., Kohara,M. and Taira,K. (2005) Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. *Molecular BioSystems*, **1**, 382-390.
44. Watanabe,T., Sudoh,M., Miyagishi,M., Akashi,H., Arai,M., Inoue,K., Taira,K., Yoshiba,M. and Kohara,M. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. *Gene Ther.*, **13**, 883-892.
45. Barichievy,S., Saayman,S., von Eije,K.J., Morris,K.V., Arbuthnot,P. and Weinberg,M.S. (2007) The inhibitory efficacy of RNA POL III-expressed long hairpin RNAs targeted to untranslated regions of the HIV-1 5' long terminal repeat. *Oligonucleotides*, **17**, 419-431.

46. Berkhout,B. and van Wamel,J.L. (1995) Inhibition of human immunodeficiency virus expression by sense transcripts encoding the retroviral leader RNA. *Antiviral Res.*, **26**, 101-115.

47. Weinberg,M.S., Ely,A., Barichievy,S., Crowther,C., Mufamadi,S., Carmona,S. and Arbuthnot,P. (2007) Specific inhibition of HBV replication *in vitro* and in vivo with expressed long hairpin RNA. *Mol Ther.*, **15**, 534-541.

48. Zhou,H., Xia,X.G. and Xu,Z. (2005) An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acids Res*, **33**, e62.

49. Andersen,P.M. (2006) Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr.Neurol.Neurosci.Rep.*, **6**, 37-46.

50. Sun,D., Melegari,M., Sridhar,S., Rogler,C.E. and Zhu,L. (2006) Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotechn.*, **41**, 59-63.

51. Chung,K.H., Hart,C.C., Al Bassam,S., Avery,A., Taylor,J., Patel,P.D., Vojtek,A.B. and Turner,D.L. (2006) Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res*, **34**, e53.

52. Zhu,X., Santat,L.A., Chang,M.S., Liu,J., Zavzavadjian,J.R., Wall,E.A., Kivork,C., Simon,M.I. and Fraser,I.D. (2007) A versatile approach to multiple gene RNA interference using microRNA-based short hairpin RNAs. *BMC Mol Biol*, **8**, 98.

53. Shin,K.J., Wall,E.A., Zavzavadjian,J.R., Santat,L.A., Liu,J., Hwang,J.I., Rebres,R., Roach,T., Seaman,W., Simon,M.I. *et al.* (2006) A single lentiviral vector platform for microRNA-based conditional RNA interference and coordinated transgene expression. *Proc Natl Acad Sci USA*, **103**, 13759-13764.

54. McLaughlin,J., Cheng,D., Singer,O., Lukacs,R.U., Radu,C.G., Verma,I.M. and Witte,O.N. (2007) Sustained suppression of Bcr-Abl-driven lymphoid leukemia by microRNA mimics. *Proc Natl Acad Sci U S A*, **104**, 20501-20506.

55. Snyder,L.L., Esser,J.M., Pachuk,C.J. and Steel,L.F. (2008) Vector design for liver-specific expression of multiple interfering RNAs that target hepatitis B virus transcripts. *Antiviral Res*.

56. Sledz,C.A., Holko,M., de Veer,M.J., Silverman,R.H. and Williams,B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat.Cell Biol*, **5**, 834-839.

57. Jackson,A.L. and Linsley,P.S. (2004) Noise amidst the silence: off-target effects of siRNAs? *Trends Genet.*, **20**, 521-524.

58. Grimm,D., Streetz,K.L., Jopling,C.L., Storm,T.A., Pandey,K., Davis,C.R., Marion,P., Salazar,F. and Kay,M.A. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*, **441**, 537-541.

59. McBride,J.L., Boudreau,R.L., Harper,S.Q., Staber,P.D., Monteys,A.M., Martins,I., Gilmore,B.L., Burstein,H., Peluso,R.W., Polisky,B. *et al.* (2008) Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci U S A*, **105**, 5868-5873.

60. Berkhout,B. and Jeang,K.T. (2007) RISCy business: microRNAs, pathogenesis, and viruses. *J Biol Chem*, **282**, 26641-26645.

61. Haasnoot,J., Westerhout,E.M. and Berkhout,B. (2007) RNA interference against viruses: strike and counterstrike. *Nat.Biotechnol.*, **25**, 1435-1443.