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Vector-based delivery of siRNAs: *In vitro* and *in vivo* challenges

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TABLE OF CONTENT

1. ABSTRACT

RNA interference (RNAi) induced by small interfering RNAs (siRNAs) has recently become a powerful tool to knock-down gene expression in a sequence-specific manner. In addition to chemically made siRNAs, stable expression of siRNA in the form of short hairpin RNAs (shRNAs) expressed from an RNA polymerase III (pol III) promoter is now widely used approach for the application of RNAi in mammalian cells. However, long-term suppression using constitutive promoters can be problematic and emerging evidence indicates that siRNAs can cause several side effects in human cells. Here we review the recent advances in developing controllable expression vectors in order to accelerate the therapeutic applications of RNAi.

2. INTRODUCTION

In 1998, Fire *et al.* reported the discovery of a double-stranded (ds) RNA-based mechanism that silence gene expression in a sequence-specific manner. Only a few dsRNA molecules per cell were needed to silence the targeted gene. However, one of the drawbacks of the use long ds RNA in somatic mammalian cells, is the activation of the interferon pathway. By using short double-stranded RNAs (21 nucleotides in length) known as small interfering RNAs (siRNAs) Thomas Tüschl and colleagues presented an efficient system to knock down gene expression and bypassing the interferon pathway (1). As a result of their potency, siRNAs have rapidly gained ground as an indispensable tool to study gene function both *in vitro* and

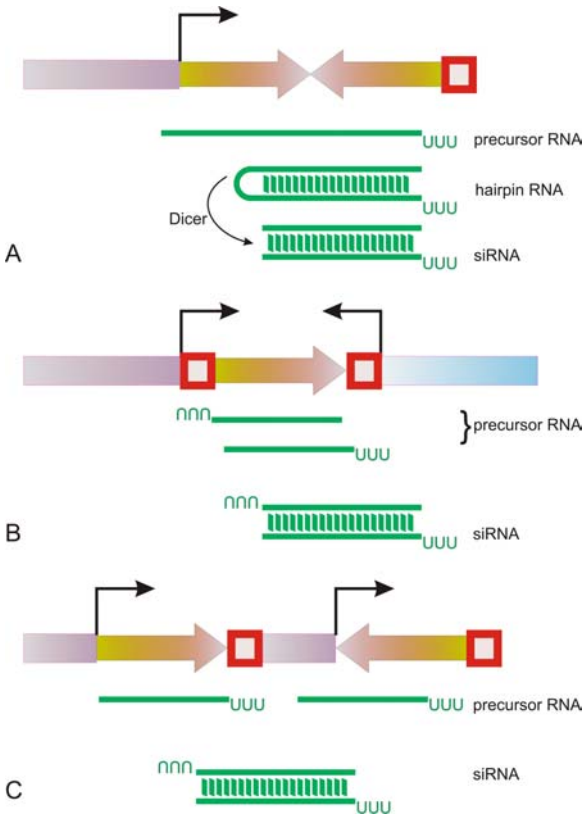

Figure 1. Common strategies to express shRNAs. See the text for detailed description.

in vivo. Of note, most of the technologies available in molecular biology result from application of basic research discoveries. In the case of siRNAs, even if the principle was described early in plants, the study of regulatory RNAs in biology and diseases has taken off since the discovery of siRNA-mediated gene silencing in human cells.

In plants and animals, such as *Caenorhabditis elegans* and *Drosophila,* RNAi can be activated by long ds RNAs, which are processed by Dicer to active siRNAs (3). However, in mammalian long ds RNAs (> than 30 nucleotides) induce general translational inhibition and unspecific cellular RNA degradation (4). The activation of host-defense mechanisms by RNAs can interfere with RNAi pathway (5-7). An ideal RNA-mediated mRNA degradation method would therefore have to escape the "immune radar" (8). Although siRNAs were though to avoid immune recognition, recent data indicate that they can be sensed by TLR7/8 in human immune cells (8). Thus, data obtained with certain siRNAs should be interpreted with caution (9).

Despite these side effects, siRNA technology has been widely adopted in basic research and somatic gene therapy applications (10). Technically, two strategies are mainly used: The first strategy method relies on chemically synthesized siRNAs that can be delivered to target cells as a drug. In the second strategy system, precursors of siRNAs are expressed endogenously following transfection of expression cassettes of short hairpin RNA (shRNA) molecules that are processed by Dicer to yield functional siRNAs. Now it has become common practice to induce RNAi in mammalian cells using vectors encoding shRNAs.

3. DESIGN OF VECTOR-BASED siRNA

For RNAi-based gene therapy applications, an ideal method would be the endogenous expression of siRNAs. To express siRNAs, several strategies were used (11-14). Active siRNAs can be produced in cells after transfection of expression vectors encoding siRNA sense and antisense strands or vector encoding shRNAs that are processed to produce functional siRNAs. In both cases, the RNA was expressed from an RNA polymerase pol III promoter such as U6, H1 promoter or tRNA promoter (14, 15). Figure 1 A and B illustrate the expression strategy. Alternatively, active siRNA molecules can be generated by using two promoters oriented in the opposite direction as illustrated in Figure 1C (16, 17)). In all cases, the aim was to generate ds RNAs that would be transported to the cytosol and processed into siRNAs capable of entering the RNAi pathway. Of note, pol III promoters have relatively small size and the transcription is conveniently terminated within a stretch of four or more uridines. The resulting shRNAs are processed by Dicer to generate small RNA duplexes that resemble the active siRNA duplexes with 2-3 U 3' overhangs. This favors loading of the siRNA molecules into the effector complex RISC, which contains the endonuclease Argonaute 2 (AGO2), which cleaves bound mRNA provided that it is perfectly complementary to the guide strand (18). In general, the presence of 2 bases 3' overhang protects siRNAs or miroRNAs (miRNAs), genome encoded small RNAs, for being recognized by cytoplamic sensors of RNAs (8). In addition to H1 and U6 promoters, functional siRNAs have been expressed from a pol II promoter (12, 19, 20). Also, pol II and pol III promoters were used in tandem and shown to be more efficient than H1 alone in certain cell types (21). Pol I promoter was also used to create a species-specific construct (22).

4. CONTROL OF SIRNA EXPRESSION

The development of siRNA as human therapeutics requires the imperative regulation of siRNA function in cells. As mentioned above, constitutive expression of siRNAs can produce side effects such as the induction of the interferon pathway and silencing of unwanted genes (23). Furthermore, endogenously expressed siRNAs could saturate the cell's RNAi pathway, thereby inhibiting the function of endogenous miRNAs (24). To control siRNA expression, several methods have been described (Fig. 2).

4.1. Promoter modifications

As mentioned above, the human U6 small nuclear RNA promoter and human H1 promoter are among the common pol III promoters used for expressing siRNAs. One of the advantages of using these promoter is their constitutive expression in all cell types (21)). Therefore,

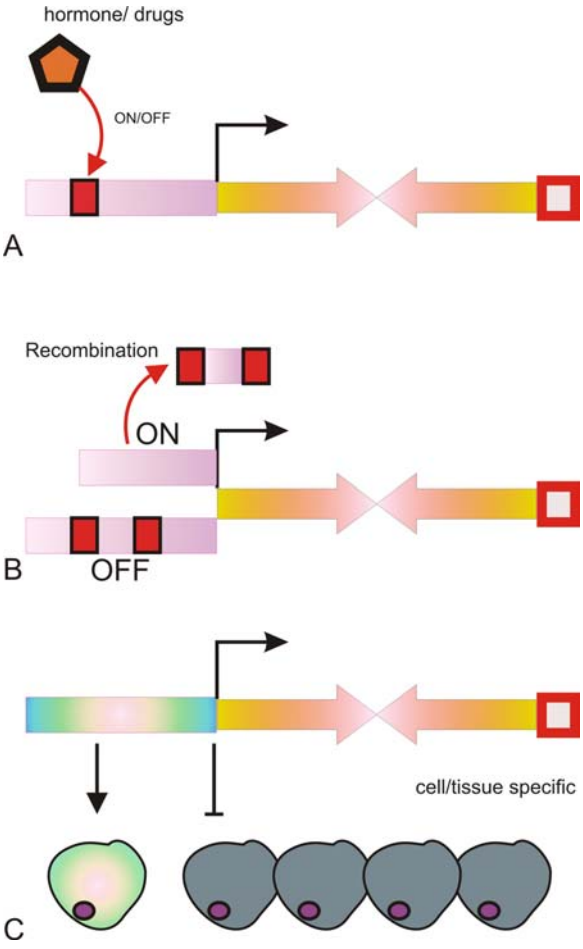

Figure 2. Exogenous control of shRNA transcription. (A) The promoter containing a binding cassette can be turned on or off upon addition of the ligand. (B). Subsequent to recombination, the siRNA-promoter is unmasked and the transcription of the shRNA is activated. (C) A shRNA under the control of a tissue specific promoter can be transfected in all cells, but it will be activated only in the cell where the promoter is transcriptionally active.

they are ideal to produce important amount of siRNA molecules. However, in some case one might want to control the expression of siRNAs temporally. The first example of siRNA-based transcriptional modifications was the addition of tetracycline operator cassettes on a H-1 promoter (25, 26). When this vector-based siRNA was cotransfected with a vector encoding for the tet-operator (a protein that will block or release the promoter depending on the presence of tetracycline), the cleavage a given mRNA could be controlled both temporally and quantitatively. Subsequently, the same strategy was used in an ecdysone-controlled promoter (27). Taken together these innovations gave rise to a series of constructs that can be controlled by the simple addition of an external product as illustrated in Figure 2A. A second strategy was described by Coumoul and colleagues where the promoters was blocked by the addition of a neomycin gene flanked by LoxP (28). However, the construct has to be inserted in a target cell to be turned on, otherwise it will be constitutively inhibited. In the presence of Cre-reconbinase, this cassette is deleted, thus leading to the activation of the promoter and synthesis of siRNA molecules (Figure 2B). Importantly, the system was found to work *in vivo* (29).

4.2. Promoter specificities

Another way to regulate a promoter is to use its inherent specificity. The main benefit provided by this strategy is that the target gene knockdown cannot only be restricted temporally but also spatially because the activation of the promoter will depend on its environment. By using a modified Pol II promoter, Rossi and coworkers demonstrated that the target gene (HIV) triggers the expression of the specific siRNAs in infected cells (30). This elegant construct is then turned on only in infected T cells, reducing the risk of off-target and other side effects.

Notably, severe and often therapy-limiting side effects are a major obstacle in cancer therapy. As the most used delivery agents can enter all cell types, specificity must be built into the delivery agents or the expressed shRNAs. Recently**,** we have developed a cancer cell-specific vector that expresses siRNAs from the human survivin promoter (31). The levels of inhibition were comparable to that obtained with the constitutively active U6 promoter. Transcriptional targeting employs regulatory sequences isolated from cancer-specific genes to control shRNAs and/or miRNAs expressed by the vectors. Song and colleagues also reported on the expression of siRNAs under the control of the PSA promoter (32). Interestingly, reduced gene expression was achieved in a tissue-specific and hormone-dependent manner.

4.3. Product modifications

The above described modifications are directed at the promoter's level. However, some studies reported on the direct control on the siRNA sequences. By placing two loxP sites at the level of the loop of the hairpin, the formation of the active siRNA was blocked. However, the activation of LoxP recombination by the addition of a fusion protein containing the Cre domain siRNA leads to the transcription of siRNAs (33). A recent approach based on RNA genetic switches has been presented. Here the authors have replaced the hairpin loop with a RNA aptamer that is recognized by theophylline (34). In the presence of theophylline the RNAi activity was blocked. Recent innovations in modifying the gene product itself have been described. Wang and co-workers have designed a vector containing multiple siRNA expression cassettes (35). This construct can be used for the study of a single gene or multiple genes targeted by siRNAs. The construct is composed of up to six U6 promoter-siRNA cassettes. A summary of the different strategies is shown in Figure 3.

5. APPLICATIONS

5.1. Standard assays

As discussed by Dykxhoorn and colleagues (36), a weak point of the vector-based siRNA approach is their poor transfection efficiency when compared to synthetic siRNAs. In model cells such as human cervix carcinoma Hela cells or human embryonic kidney HEK-293 cells,

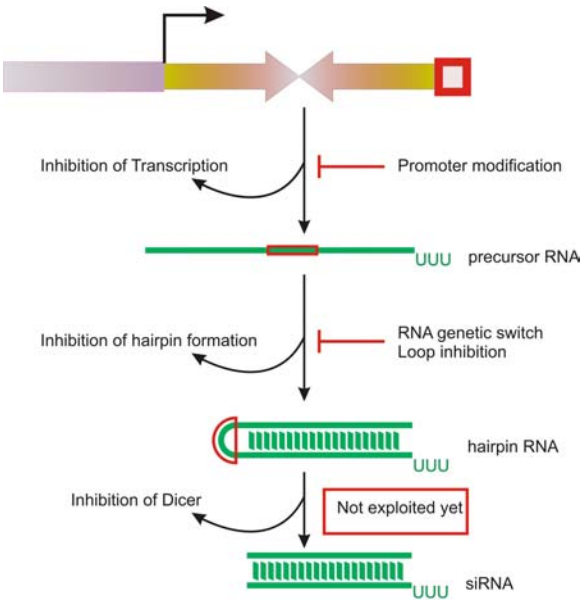

Figure 3. Summary of the available methods to control shRNA expression during processing and maturation.

DNA transfection can reach 70% to 90% efficiency depending on the type of transfection reagent used. On the other hand, in primary cells as well as in the broadly used mouse fibroblasts NIH3T3 cells, transfection becomes a delicate issue in terms of efficiency. Thus, one can argue that for the development of siRNA-based therapeutics, the plasmid-based method might be unproductive. However, the development of viral delivery vectors should overcome this potential problem. In this respect, three main types of viral vectors have been effectively employed to deliver siRNA into mammalian cells: the retrovirus (37, 38), adeno- and adeno associated virus (12, 39), and lentivirus (40). Retroviruses are restricted in their potency because they infect only dividing cells. Nevertheless they can be useful in cancer therapy. In a target-discovery assays and functional genomics, retrovirus have been found to be an ideal support for a genome-based siRNA library (41).

5.2. Medical applications

Adenoviruses and lentiviruses have the advantage of infecting both dividing and quiescent cells, which render them attractive when non-dividing cells are targeted. Recent reports have demonstrated the efficiency of these delivery systems. Adenoviruses were used *in vitro* to deliver siRNAs in specific cells of the pancreas (42). Also, adeno associated viruses (AAV) have been used to deliver siRNAs in specific brains area of mice (43). Using AAV vector and model-mice for polyglutamine-induced neurodegeneration, it was demonstrated that siRNA can specifically knockdown the expression of a mutated allele, thus improving the clinical score in an ataxia mice model (44). Also, efficient delivery of siRNA was achieved with lentiviral vectors. In this respect, primary effusion lymphoma could be specifically destroyed by lentivirus-delivered siRNAs (45). Interestingly, virus infection was restricted to the peritoneal region, promoting the use of this delivery system to treat peritoneum infiltrated cancer cells. It should be noted that viral particles have a great potential since they are easy to produce and to test. However, the necessity of targeting the virus to the selected tissues still represents a major challenge (46). Although several targeting strategies have been described, the modification of the viral capsid represents a promising strategy. For example, viral particles were modified in order to display so-called carrier peptides that do not impede the biology of the virus (47, 48). These peptides serve as ligands for receptors expressed on cell of interest. So far, viruses with modified capsids have not been used for delivering siRNA, but one can speculate that the combination of these two technologies will generate new opportunities in gene-based therapy.

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Abbreviations : RNAi: RNA interference, siRNA: small interfering RNAs, RISC: RNA-induced gene silencing complex. miRNA: microRNA.

Key Words: RNAi; siRNAs; Tissue-specific promoter, Review

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