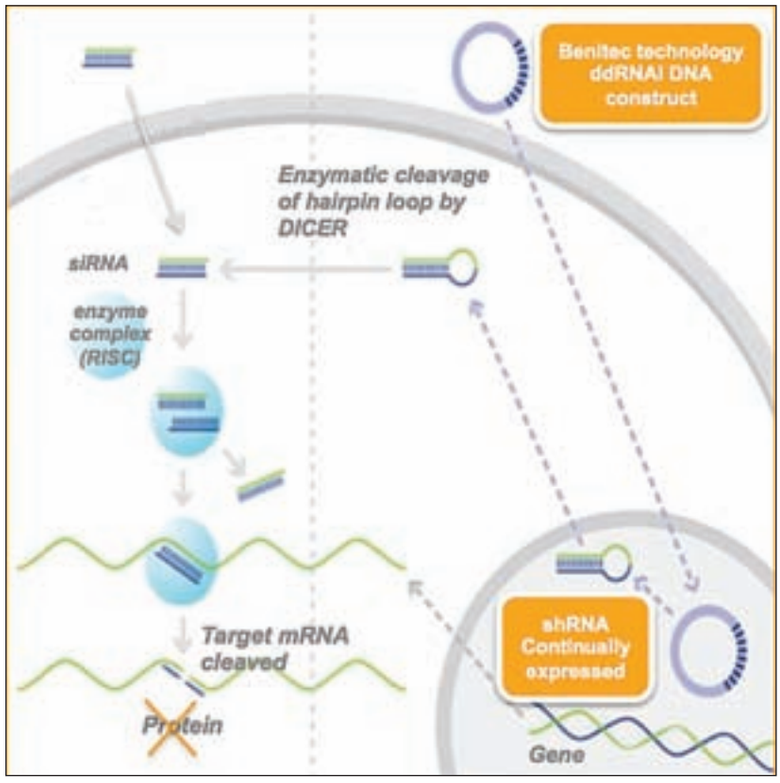
In it for the long haul: Vector mediated therapeutics based upon DNA-directed RNA Interference

A decade and a half after being first described to occur in mammalian cells, numerous trials are indicating that RNA interference can be harnessed to treat human disease. This article argues that positive results are now starting to emerge for the application of vector-based ddRNAi.

By Dr David Suhy

RNA interference (RNAi) is an evolutionarily conserved mechanism of sequence-specific gene silencing. It is mediated by small interfering RNAs (siRNAs), which are comprised of double-stranded RNAs of approximately 21bp, with 2bp 3' OH overhanging ends. To employ this mechanism for therapeutic purposes, one can utilise a 'delivered approach' of introducing synthetic siRNAs into cells.Alternatively, an 'expressed approach' relies on the use of introducing DNA templates (ddRNAi) to utilise the cells' endogenous transcriptional machinery to produce short hairpin RNAs (shRNAs) that are then processed by the endogenous RNAi machinery into siRNAs (**Figure 1**).

As is the case for any nucleic acid-based drug, the ability to deliver therapeutically relevant concentrations into the appropriate target tissue continues to be the largest technical hurdle to the field of RNAi therapeutics. In the burgeoning siRNA therapeutics community, one of the few organs that can be readily transfected is the liver. Systemic administration of siRNA using small, lipid-based nanoparticles, polyconjugates or GalNAc-siRNA conjugates to deliver the siRNA duplexes results in small size of the spheroids, typically less than 100nm in diameter and permits the extravasation of systemically administered formulations into liver tissues by entry through the fenestrations in the liver. Because use of these delivery agents results in near complete transfection of hepatocytes, it is not surprising that the pipeline programmes of siRNA-based therapeutics companies are disproportionally skewed towards treating liver-based diseases such as Hepatitis B and genetic-based metabolic disorders of the liver such as TTR mediated amyloidosis, primary hyperoxaluria and hepatic porphyrias among a wide range of others.



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Because ddRNAi relies upon the transcription of shRNA from DNA expression constructs instead of delivering small synthesised RNA duplexes, a wide variety of delivery tools typical of gene therapy approaches, including the use of non-replicating viral vectors, can be employed to target a wide variety of tissue types. Although delivery of ddRNAi agents is not limited to viral vectors, the current discussion will focus on that delivery mechanism. Characterisation of naturally occurring serotypes of viruses isolated have shown a broad swath of biodistribution when introduced *in vivo* including liver, muscle, brain, CNS, retina, cardiac and cancer cells[1]. Viruses may also have their tissue targeting properties changed by introducing novel proteins, also known as pseudotyping, to change the specificity of the delivery vehicles for specific tissue or cell types opening up a broad range of potential tissues that can be transduced[2,3].

This aspect, in combination with its broad cell and tissue tropism, and limited viral host response has made it an attractive vector system for gene therapy. The viral protein capsid, the primary interface with the host, is the main determinant for these phenotypes, is highly variable,and is most subject to pressures during replication.

Yet, the use of viral vectors for ddRNAi delivery is not without its own set of challenges, particularly from an immunologic standpoint. For instance, the patient cannot have been previously exposed to the specific serotype of vector that is being used as the therapeutic delivery vehicle, as pre-existing immunity would almost undoubtedly neutralise the agent before target tissues could be appropriately transduced. In addition, administering high levels of viral vectors has the potential to elicit cell-mediated immune responses in the tissues that it transduces that must be monitored and treated accordingly[4]. Even the eye, thought to be largely immune privileged and able to tolerate introduction of foreign antigens without triggering an inflammatory cascade, has shown some evidence of an immune response to capsid protein of the viral vector when administered at high doses[5]. Yet controlling these immune responses is not a new issue for the field of gene therapy. Once identified, capsid-mediated inflammation can be readily treated with a short course of corticosteroids. A number of different approaches have been considered to treat subjects with pre-existing immunity and extend the range of patients that can be treated, including apheris, a transient course of immunosuppression agents, as well as pre-dosing with empty capsids to absorb any pre-existing neutralising antibodies[6]. Finally, one technique that is sure to generate a whole new toolkit of viral vectors to choose from is the ability to use engineered virus capsids, in which directed evolution or rationale design is imposed upon naturally occurring serotypes of the viral vectors in order to create vectors with new tissue tropisms, improved tissue targeting and reduced immunogenicity[7].

Once inside of the appropriate cell, the benefit of a ddRNAi approach is extended by the fact that the regulatory elements within genetic construct can be easily manipulated to fine tune the expression of shRNA from the DNA template. This is particularly important since early studies that coupled shRNA expression following delivery with viral vectors demonstrated that inappropriately high levels of expression of shRNA could lead to animal mortality in a mouse model that appeared to be related to saturation of components within the endogenous miRNA pathway[8]. While this finding was confirmed in a non-human primate model, it was also demonstrated that using regulatory elements that resulted in significantly reduced levels of transcription could abrogate toxicity and lead to a potentially broad therapeutic window[9]. The ability to express shRNA from a DNA template also permits additional safety features to be built into

the therapeutic construct. Because viral vectors do not always selectively only transduce the target tissues of interest, additional safety features can be built into the recombinant expression cassettes to restrict expression of the shRNA including the use of tissue-specific Pol II promoters or modelling the expression of the therapeutic shRNAs within the context of an endogenous miRNA cluster[10].

In most 'delivered'-based siRNA approaches, pre-synthesised duplexes designed against a single targeted region are delivered to the cells. Yet because the 'expressed' ddRNAi approach relies upon transcription from a DNA template, expression constructs can be designed that result in the production of multiple therapeutic shRNAs. The ability to express multiple shRNA hairpins from a single genetic construct to target multiple genes in multiple cellular pathways lends itself to treating genetic diseases that are not simple monogenic disorders such as cancer, diabetes and heart disease. If using a viral delivery vehicle, the number of hairpins that can be included in a therapeutic construct is simply limited by the packaging capacity of contained within the recombinant viral vector; AAV can package approximately 4.5Kb, Lentivirus approximately 9Kb and adenovirus vectors can package up to 30Kb[11]. Given that individual pol III promoter typically encompass 300-400 nucleotides and the shRNA that is expressed comprises as little as 75 nucleotides, each of those viral delivery systems could accommodate a large number of independent shRNA expression cassettes.

Having additional space in the DNA expression construct also permits shRNA to be paired up with other therapeutic modalities. For instance, Gradalis, a Texas-based company, has developed the FANG vaccine as a therapeutic treatment for advanced solid cancers and has just recently completed Phase I studies and is in the midst of several Phase II studies[12]. Initially designed for treatment of subjects with hepatocellular carcinoma, FANG contains an expression cassette for an shRNA targeting the Furin enzyme responsible for reducing the levels of TGF isoforms, as well as containing an expression cassette for the human granulocyte-macrophage colony stimulating factor (GM-CSF).

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Taken together, FANG has been designed to enhance the ability of the body's own immune system to attack tumour cells.

Alternatively, having the additional space within the genetic construct has permitted the development of suppress and replace strategies for rare genetic disorders. Difficulties in having RNAi-mediated therapeutics against gain of function autosomal dominant genes have been well documented[13]. Specifically, it has been difficult to inhibit a gene on the basis of a single nucleotide change that may lead to the diseased phenotype while leaving the expression levels of the wildtype gene relatively unperturbed. In 'suppress and replace' strategies, the shRNA is typically designed to inhibit both the disease causing allele as well as its wildtype counterpart. Additional space within the recombinant DNA construct is reserved for an expression cassette containing a codon optimised wildtype protein that takes advantage of codon degeneracy to encode for wild type protein with a sequence that is not susceptible to the therapeutic shRNA. Genable, an Irish-based therapeutics company, is developing an AAV-based vector that encompasses a suppression and replacement strategy for the treatment of Rhodopsin-linked autosomal dominant retinitis pigmentosa (Rho-AdRP)[14].

For the application of ddRNAi to treat viral disease, the ability to express multiple shRNA hairpins at different locations on the viral genome[15] is analogous to the emergence of highly active antiretroviral therapy (HAART) to treat HIV. Due to the inability to control viral escape mutants, clinicians did not gain ground on treating the diseases such as HIV until a therapeutic regimen was developed involving combinations of multiple drugs that were administered simultaneously. In a similar approach, researchers at the City of Hope demonstrated the utility of an approach in which CD34(+) hematopoietic progenitor cells were transduced *ex vivo* with a lentivirus vector that expressed multiple therapeutic RNA molecules, including an shRNA against tat/rev as well as a TAR decoy and a CCR5 ribozyme, in patients with AIDS lymphoma[16]. Currently, the San Diego-based company Calimmune is in the midst of a Phase I/II clinical trial in which CD4+ T lymphocytes and autologous hematopoietic progenitor cells from HIV-1 infected patients are transduced with a recombinant lentivirus vector that expresses a shRNA to inhibit expression of the CCR5 receptor as produced a small HIV-1 derived peptide to inhibit HIV-1 fusion and entry into uninfected cells[17].

One of the principle benefits of using viral-based gene therapy approaches for delivery of ddRNAi constructs is the duration of expression that can be achieved. Though the choice of vector and tissue type has a significant impact, it is possible to produce robust expression that lasts months or years following a single administration of the viral vector. In one such recent example, hemophiliac patients treated with a single intravenous administration of a recombinant Adeno Associated Virus (AAV) that expresses the human Factor IX protein, responsible for blood clotting, results in years of persistent expression of corrective levels of the protein and thus has the ability to moderate the severity of the disease[4]. In light of the previous discussion on immunogenicity, long-term expression from a single injection is important as the foreign proteins in the viral vectors undoubtedly serve as antigens to trigger an immune response and thus prevent redosing the treated patient with the same viral vector.

Conversely, much like small molecule drugs, siRNA have a limited durability of persistence within the cell. The dose administered is calibrated to achieve therapeutic levels within the cell, over time the concentration wanes as the siRNA is broken down within the cell[18,19]. Thus if the therapeutic regimen requires knockdown beyond a few days or weeks, the drug must be continuously administered over time to continue to boost siRNA levels into therapeutic range and maintain efficacy of the compound. Careful maintenance of intracellular drug levels is an especially challenging issue for the treatment of viral diseases. Improper dosing levels and/or 'drugs holidays', in which the patient either forgets or purposefully declines to take the recommended drug, can compromise the clinical outcome. Prolonged periods of drug at subtherapeutic levels can drive the generation of viral escape mutants that can circumvent further treatment with the same agents[20]. One of the clear advantages of ddRNAi strategies is that once the appropriate target tissues have been transduced, the cell's transcriptional machinery can produce steady state levels of shRNA. Furthermore, because treatment of patients with a gene therapy vector represent a 'one and done' administration, patient compliance and drug holidays are taken out of the equation altogether. This is particularly critical since patient compliance in taking medications for extended periods of time has been estimated by WHO to be as little as 50% in developed countries, and substantially less than that in non-developed countries[21]. The ability to produce durable therapeutic responses from a

one-time treatment may also represent a significant advantage in isolated communities in which access to traditional healthcare and continuous follow-up treatment is restricted.

Despite the spate of novel direct acting antiviral compounds that have been recently introduced into the market to treat individuals chronically infected with the Hepatitis C virus (HCV), an ideal treatment would involve much shorter timeframes, ideally a single dose that cures the disease. Benitec Biopharma has recently entered into the clinic and is currently dosing subjects in a Phase I/IIa study with a compound that embodies many of the concepts of ddRNAi and represents the first in human study in which an AAV vector delivering shRNA has been administered systemically. TT-034 utilises a single administration of an AAV serotype with preferential biodistribution into hepatocytes to deliver a recombinant DNA vector that expresses persistent, steady state levels of three different shRNAs against target sequences within the RNA genome of the virus[9]. With no known extra-hepatic site of HCV replication, elimination of the virus in infected hepatocytes should completely resolve the HCV infection in subjects with chronic hepatitis C. The ability to cleave the HCV RNA genome at three different positions, which provides redundancy, added efficacy and perhaps most importantly makes it much harder for the virus to escape through mutations[22].

Yet, long term persistence of expression of therapeutic agents from viral vectors might be considered a double-edged sword, particularly if there is a serious adverse event coupled with administration of the compound. In most cases, once a viral vector is administered, it cannot be withdrawn and thus necessitates a more cautious approach to clinical development. Thus pairing up RNA interference with delivery mechanisms more typically employed in a gene therapy context, creates additional stringent regulatory hurdles that must be cleared before testing in humans can be initiated and places restrictions on how quickly trials can proceed. As such, development of ddRNAi programmes is regulated by the Center for Biological Evaluation and Research within the US Food & Drug Administration and often requires additional oversight from the Recombinant DNA Advisory Committee (more commonly known as the RAC) within the National Institute of Health Office of Biotechnology Activities.

Now over a decade and a half after RNAi was first described to occur in mammalian cells, a large number of clinical trials involving a wide variety of disease indications are yielding positive data and demonstrating that RNA interference can be harnessed to treat human disease. Although siRNA-based therapies have dominated the clinical space, the number of shRNA-based therapies involved in human testing have been slowly catching up. Given the optimal product profile of long term therapeutic benefit from a single administration, there is a tremendous amount of promise for application of vector-based ddRNAi. The results from the ongoing trials that have been described will be instrumental in guiding this field moving forward. DDW

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