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Exploring cell type-specific internalizing antibodies for targeted delivery of siRNA

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Abstract

A major challenge to the development of therapeutic small interfering RNAs (siRNAs) is specific and efficient *in vivo* delivery to target cells. Recent studies suggest that cell type-specific gene silencing *in vivo* can be achieved by combining siRNAs with cell type-specific affinity ligands such as monoclonal antibodies. The antibody-directed siRNA complex enters target cells through receptor endocytosis and is subsequently released to the cytosol to specifically silence target gene expression through biologically conserved RNA interference (RNAi) pathways. Antibody fragments fused with a small basic nucleic-acid-binding protein and antibody fragment-directed nano-immunoliposomes are two examples of effective delivery vehicles *in vivo*. The demonstrated specificity of *in vivo* gene silencing and the lack of nonspecific immune activation and systemic toxicity encourage further development of therapies based on cell type-specific delivery of siRNA.

Keywords: *Therapeutic siRNA; internalizing antibody; systemic delivery; cell type-specific gene silencing*

INTRODUCTION

There is a continuous search for new approaches that allow efficient and specific modulation of gene expression in disease cells. The use of small interfering RNAs (siRNAs) to specifically silence target gene expression through biologically conserved RNA interference (RNAi) pathways is one such approach that is receiving increasing attention [1, 2]. The RNAi pathway was discovered when Fire *et al*. [3] found that double stranded (dsRNA) introduced into *Caenorhabditis elegans* silenced expression of a homologous target gene, and did so much more efficiently than the corresponding antisense RNA. SiRNAs, typically 19–23 nucleotides (nt) long, are intermediates in the RNAi pathway [4–6]. SiRNAs, generated by Dicer or introduced exogenously, are bound by a multiprotein complex, the RNA-induced silencing complex (RISC), and the complex is directed to the homologous site on the target mRNA [4, 7–10]. Only one of the two strands of the siRNA can direct RISC-mediated cleavage [11, 12]. Details of the RNAi pathway have been reviewed elsewhere [4, 8, 13].

To harness the RNAi machinery to inhibit gene expression, siRNAs can be directly introduced into cells [14–18]. Alternatively, plasmids or viral vectors can be used to express short hairpin (sh)RNAs (resembling endogenous microRNA precursors) that are processed by the endogenous microRNA machinery into siRNAs [9, 19–26]. This gene therapy approach has advantages and drawbacks that are reviewed extensively elsewhere [20, 27]. This review focuses on the opportunities and challenges for developing siRNA-based therapeutics.

A major challenge to the development of therapeutic siRNA is specific and efficient *in vivo* delivery to target cells [13, 20]. Unlike *C. elegans*, which expresses a membrane receptor for dsRNAs, most mammalian cells do not take up siRNA [13]. As such, intravenous injection of naked siRNA often fails to achieve significant silencing effects [13, 20]. Thus, an active uptake mechanism is required for effective siRNA therapeutics. Targeted systemic delivery of siRNAs provides such a mechanism, as well as tissue specificity in gene silencing, but faces many of the same issues of targeted delivery of other

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Table 1: Summary of studies on antibody-mediated targeted siRNA delivery covered in this review. Pro, protamine; ils: immunoliposomes; HA-LFA-1, high affinity LFA-1 epitope; TfR, transferrin receptor; PBMC, peripheral blood mononuclear cells

Targeting antibody	Target antigens	Target cells	Targeting vehicles	Target genes	*In vitro*/ *in vivo*	References
FI05-Fab	HIV *env* (gpI60)	HIV infected HeLa-GFP & CD4 T cells	Fab-Pro	EGFP & HIV *gag*	*In vitro*	[28]
FI05-Fab	HIV *env*	BI6 melanoma cells expressing gpI60 (gpI60-BI6)	Fab-Pro	c-myc, MDM2 & VEGF	*In vitro*	[28]
FI05-Fab	HIV *env*	Xenograft of gpI60-BI6 cells	Fab-Pro	Mix of c-myc, MDM2 & VEGF	*In vivo*	[28]
ML39-scFv	ErbB2	SKBR3 Xenograft	ScFv-Pro	Ku70	*In vivo*	[28]
AL-57-scFv	HA-LFA-1	Stimulated PBMC, primary lymphocytes & memory T cells	ScFv-Pro	Ku70, CD4, CCR5, Ku70 & Cyclin DI	*In vitro*	[29]
AL-57-scFv	HA-LFA-1	Stimulated K562 expressing LFA-1	ScFv-Pro	Ku70	*In vitro*	[29]
AL-57-scFv	HA-LFA-1	Xenograft of K562 cells expressing HA-LFA-1	ScFv-Pro	*Cy3*- siRNA	*In vivo*	[29]
TfRscFv	TfR	PANC-1 & MDA-MB-435	ScFv-iLs	HER2	*In vitro*	[30, 31, 57]
TfRscFv	TfR	MDA-MB-435/LCC6 xenograft	ScFv-iLs	6-FAM-siRNA	*In vivo*	[30, 31]
TfRscFv	TfR	MDA-MB-435 & PANC-1 xenografts	ScFv-iLs	HER2	*In vivo*	[30, 57]

therapeutics such as small molecule drugs, including serum stability, target tissue accumulation and penetration, pharmacokinetics and biodistributions. In addition, unlike many small molecule drugs that can passively diffuse into surrounding cells, siRNAs need to be internalized and then delivered to the proper subcellular localization, i.e. the cytosol, to achieve effective gene silencing.

Despite these challenges, a series of recent studies suggest that targeted *in vivo* delivery of siRNA is feasible (Table 1 and Figure 1). Song *et al*. [28] and Peer *et al*. [29] combined the nucleic-acid-binding properties of the small basic protein, protamine, with the specific ligand-recognition properties of antibodies to achieve cell-type-specific siRNA delivery *in vitro* and *in vivo*. Using a different delivery vehicle, Pirollo *et al*. [30, 31] constructed a nanosized, anti-transferrin receptor single-chain antibody fragment (scFv)-directed nanoimmunoliposome to deliver siRNA *in vivo*, and showed that an anti-HER-2-directed siRNA complex can silence the target gene and sensitize tumor cell lines to chemotherapeutics *in vitro* and *in vivo*. Both studies exploited antibodies targeting cell surface molecules and the receptor endocytosis pathway to achieve intracellular targeted delivery of siRNA *in vivo*. We will first outline a phage antibody-based approach that can be utilized to identify internalizing cell type-specific antibodies. We will then review these recent studies that utilized internalizing antibodies for *in vivo* siRNA delivery, and discuss challenges that remain to be resolved in order to translate antibody-mediated delivery of siRNA into therapeutics (Table 2).

CELL TYPE-SPECIFIC SURFACE EPITOPES AND INTERNALIZING ANTIBODIES

Due to relatively easy accessibility to extracellularly administered agents, cell type-specific surface molecules are often exploited for targeted delivery of therapeutic agents. The identification of these molecules and particularly tumour-specific cell surface antigens, however, is hampered by the complexity of the epitope space at the cell surface. In addition to proteins, relevant antigens include carbohydrates and other post-translational modifications that may not be readily predicted from studies of genomic copy numbers or mRNA expression levels. Where extensive modifications are not involved, tumour cells may process normal gene products abnormally and generate neo-epitopes on the cell surface. For example, nucleolin, a nuclear and cytoplasmic protein, has been found to locate at the surface of certain tumour cells and tumour-associated endothelial cells [32, 33]. Intracellular nucleolin regulates the maturation of rRNA and the ribosome assembly. However, a phosphorylated form of nucleolin on the surface of cells acts as a receptor for a variety of ligands, including growth factors and chemokines [34–36].

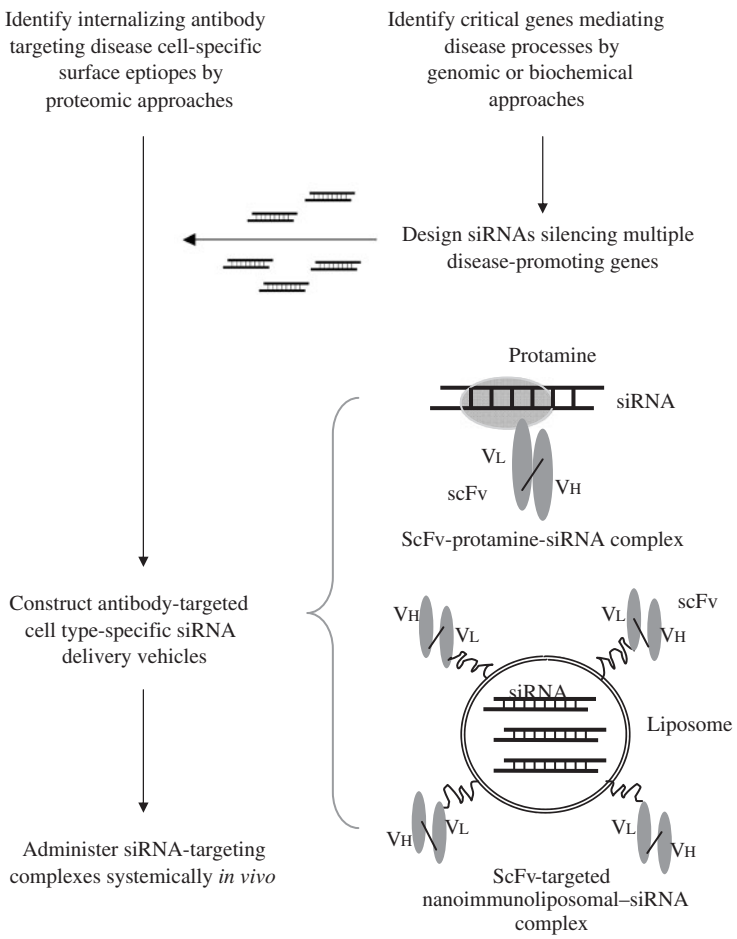

Figure 1: Development of targeted systemic delivery of siRNA *in vivo*. The diagram shows the two delivery strategies covered in this review: a fusion between the small nucleic-acid-binding protein protamine and an internalizing antibody fragment, and an scFv-directed nanoimmunoliposome – siRNA complex.

Table 2: Design considerations for developing targeted *in vivo* siRNA delivery vehicles

Variables	Issues
Targeting antibodies and target antigens	Antibody fragments (e.g. scFvs and Fabs) with well-defined cell type specificity and internalizing function are desired. A high level of target antigen/epitope expression on the target cell surface is desired. Antibody fragments with favourable pharmacokinetics and biodistributions are candidates for further development.
Targeting vehicles	The targeting vehicles should retain (or increase) targeting specificity and internalizing function of that of the targeting antibody. They should have favourable pharmacokinetics and biodistributions. Components should be simple to produce and the assembly method needs to be reproducible.
Target genes	The choice of target genes provides an additional level of selectivity for the siRNA targeting strategy. Multiple genes in a disease pathway may be targeted simultaneously.
Post-internalization	The siRNAs need to be released from encapsulating carriers (e.g. liposomes) and entrapping cellular structures such as the endosome to reach the proper subcellular location, i.e. the cytosol, to achieve gene silencing.
Stability	Modifications are often introduced to improve stability of siRNA in serum. Those modifications may affect the activity of siRNAs and need to be studied on a case-by-case basis.
Off-target effects and systemic toxicity	The siRNA sequences need to be carefully designed to minimize overlaps with homologous genes to reduce off-target effects, and to avoid immunostimulatory sequences that are known to induce nonspecific activation of the immune system.

Nucleolin does not have a signal sequence and is localized to the cell surface through an unknown mechanism [34].

In principle, regardless of its exact chemical makeup, the tumour-specific epitope space can be mapped directly by complementary monoclonal antibodies. Antibodies, major histocompatibility complex (MHC) molecules, and T cell antigen receptors are the three classes of molecules used by the immune system to recognize antigens. Of these three, antibodies recognize the widest range of antigenic structures, show the greatest ability to discriminate between antigens with subtle structural and conformational differences (specificity), and bind to antigen with the greatest strength (affinity). Specific, high affinity antibodies have been made against a broad spectrum of antigens, including polypeptides, nucleic acids, carbohydrates, heptans, and small organic and inorganic molecules. Cell type-specific surface variations can thus be readily probed using this class of affinity agents. In addition, monoclonal antibodies (mAbs) are an important class of drugs for treatment of diseases caused by infectious agents, autoimmunity and cancer [37–41]. Tumour-specific mAbs provide exquisite targeting specificity and a common framework from which a variety of therapeutics are derived [42–45].

Antibody phage display has evolved into an effective approach for the identification and development of human mAb against both known and novel tumour antigens with complex composition including post-translational modifications [46, 47]. Given the high transformation efficiency of bacteria, it is possible to make libraries of millions to billions of different antibodies, allowing full coverage of the entire immune response to an antigen. Moreover, a large phage display library can be made from naïve B cells, resulting in much smaller bias against self-antigens conserved across species [48–50]. Naïve phage display libraries therefore are more efficient in isolating antibodies specific to complex surface antigens on specific cell types. Selection of a phage antibody library can therefore lead to the identification of lead compounds for therapeutics and diagnostics, as well as affinity reagents for basic research [51].

Phage antibody libraries have been selected directly on live tumour cells to generate tumour-specific single-chain variable regions fragments (scFvs). Because the reaction takes place *in vitro*, selection conditions can be manipulated to isolate scFvs with desired function such as the ability to trigger receptor-mediated endocytosis [52]. For example, internalizing scFvs targeting known cell surface receptors such as erbB2 and EGFR have been identified and used to deliver liposomal drugs to tumour cells [53–55]. In another example, a panel of internalizing scFvs against novel prostate cancer cell surface antigens has also been identified [56]. These internalizing antibodies define a spectrum of tumour cell surface receptors that are 'drugable' since they are potential gateways for intracellular delivery of anti-neoplastic agents. These studies demonstrated that phage antibody libraries are an effective tool for identifying cell type-specific antibodies as well as antibodies with desired functionality such as internalization and payload delivery.

TARGETED *IN VIVO* DELIVERY OF siRNA USING ANTIBODY-PROTAMINE FUSION

Song *et al.* [28] performed the first antibody-mediated targeted delivery of siRNA *in vivo*. The authors fused Fab and scFv fragments with a small nucleic-acid-binding protein protamine for targeted delivery of siRNAs, and demonstrated specific delivery *in vitro* and *in vivo*. Using an anti-HIV envelope protein Fab-protamine fusion, the authors were able to introduce siRNAs into hard-to-transfect primary CD4+ T cells and suppressed HIV production in infected cells. The authors further tested this delivery strategy using an engineered melanoma model expressing the anti-HIV envelope protein. When a mixture of siRNAs targeting tumour-related genes (*c-myc*, *MDM2* and vascular endothelial growth factor) was administered in this way *in vivo*, tumour growth was inhibited, demonstrating the therapeutic potential of this approach. The authors also constructed an anti-ErbB2 scFv-protamine fusion and showed that it delivered siRNA to 32% of ErbB2-expressing tumour cells in a breast cancer xenograft model *in vivo*. To evaluate nonspecific inflammatory responses, the authors measured gene expression of interferon β and two other key interferon response genes and found that siRNA delivery did not trigger interferon responses.

In a recent study that also utilizes the protamine fusion strategy, Lieberman, Shimaoka and

colleagues [29] showed that targeting the human integrin lymphocyte function-associated antigen-1 (LFA-1) allows efficient delivery of siRNAs and cell type-specific gene silencing in primary lymphocytes, monocytes and dendritic cells. To achieve specific gene silencing only in activated leukocytes, the authors constructed a protamine fusion protein from a scFv that preferentially recognizes activation-dependent conformational changes in LFA-1. In a series of elegant experiments, the authors demonstrated that this siRNA-fusion protein selectively targets activated leukocytes, suppressing gene expression and cell proliferation only in activated lymphocytes. To evaluate the potential *in vivo* applicability of LFA-1-directed siRNA delivery, the authors administered intravenous injections of fusion protein–siRNA complexes to SCID mice carrying cells expressing latent or constitutively activated LFA-1, and showed that fusion complexes constructed from scFv targeting activated LFA-1 delivered siRNA preferentially to grafted cells expressing activated LFA-1. Consistent with the previous study, the siRNA–fusion protein complexes did not cause lymphocyte activation or induce interferon responses. This study showed that it may be feasible to silence gene expression specifically in activated cells to suppress pathogenic immune stimulation without global immunosuppressive effects on bystander immune cells.

Taken together, these studies demonstrate the potential for *in vivo* systemic, cell type-specific, antibody-mediated siRNA delivery. The protein fusion strategy is simple and broadly applicable to antibodies and antibody fragments. The size of siRNA–protein fusion complexes is above the threshold for renal clearance, and thus they are expected to have longer circulating half-life than naked siRNA. Further studies are needed to determine pharmacokinetics and biodistribution (including penetrance and relative accumulation in target cells/tissues) of the fusion complex. Given that each protamine protein binds to a limited number (~6) of siRNA molecules [28], and only a fraction of the surface-bound scFvs are likely to be internalized and released into the cytoplasm, the actual number of siRNA entering the RNAi pathway may be heavily dependent on receptor density, antibody affinity, and the rate of endocytosis for a given antibody–receptor pair. Small nucleic-acid-binding proteins other than protamine may also be studied to increase the production level of the fusion protein and to optimize binding and release of siRNA.

TARGETED *IN VIVO* DELIVERY OF siRNA USING NANOIMMUNOLIPOSOMES

Pirollo *et al.* [30] took advantage of elevated transferrin receptor levels on tumour cells and used a nanosized, anti-transferrin receptor scFv-directed nanoimmunoliposome to deliver siRNA *in vivo*. Several modifications were made to increase the delivery efficiency of the nanoimmunoliposomal siRNA. To enhance post-internalization endosomal escape, the authors included a pH-sensitive histidine-lysine peptide in the complex. To increase stability and potency, the siRNAs were modified by replacing the sense RNA with DNA (hybrid), and by further modifying the DNA by 2′-OMe and 2′-deoxyinosine substitutions (modified hybrid) [57]. The authors showed that anti-transferrin receptor-targeted nanoimmunoliposomal anti-HER-2 siRNA complex can sensitize a pancreatic cancer line to gemcitabine and a breast cancer line to docetaxel *in vitro*, and silence the target HER-2 gene *in vivo*. The authors further showed that when combined with gemcitabine, systemic delivery of the nanoimmunoliposome anti-HER-2 siRNA complex can significantly inhibit tumour growth in a pancreatic cancer model.

This study showed that delivery vehicles previously developed for gene therapy can be adapted to deliver siRNA *in vivo*, and demonstrated several important features with regards to nanoimmunoliposome-based systemic siRNA delivery. First, the study shows that endosome escape is an important factor influencing the potency of siRNA delivered. Inclusion of the pH-sensitive peptides increased the effectiveness of gene silencing by 3-fold. Secondly, the study demonstrates that the potency of siRNA can be significantly improved by chemical modifications. The authors first showed that a blunt ended DNA–RNA hybrid was slightly more effective than the standard duplex siRNA. They then modified the sense DNA strand in the DNA–RNA hybrid with centrally located 2′-OMe moieties and flanking DNA that contains 2′-deoxyinosine residues to block possible RNase H-mediated sense strand off-target cleavage, and to abrogate immunogenic side effects of siRNAs [57]. These modifications significantly

increased the potency of the siRNA both *in vitro* and *in vivo*. For example, the authors showed that the systemically delivered nanoimmunoliposomal DNA–RNA hybrid (unmodified) resulted in a 50% decrease in target HER-2 gene expression *in vivo* compared with untreated control, whereas treatment with the modified hybrid virtually eliminated HER-2 expression. The authors noticed no systemic toxicity or weight loss, although there was no data on interferon production. Finally, this study demonstrates a multi-layered approach to achieving specificity in siRNA-based therapeutics: in addition to the targeting antibody which determines the specificity of delivery, the choice of target genes can also influence the specificity and effectiveness of siRNA-based therapeutics. HER-2 gene function is required for growth of tumour but not normal cells such as the fibroblasts. While the authors did not measure the uptake of the nanoimmunoliposome–siRNA complex by normal fibroblasts, they showed that the transferrin receptor-directed, anti-Her-2 siRNA complex did not affect the growth of normal human fibroblasts. This added specificity may be an important consideration for siRNA-based therapeutics as antibodies with absolute tumour specificity may be difficult to identify.

This study demonstrates a feasible form of therapeutic based on the combination of cell-specific gene silencing via targeted siRNA delivery and conventional chemotherapeutics. This scheme may one day prove to be useful in the clinic. While the authors showed that the viability of control cells such as normal human fibroblasts was not affected by the single agent treatment (anti-Her-2 siRNA complex), it remains to be determined if these cells are nonetheless sensitized to chemotherapeutics.

CONCLUSION AND FUTURE PERSPECTIVES

These studies demonstrated that internalizing cell type-specific antibodies can be used to systemically deliver siRNA *in vivo*. The demonstrated specificity and efficiency of gene silencing and the lack of nonspecific immune activation or systemic toxicity are encouraging. Further studies are needed to determine how silencing is affected by the internalizing activity of the targeting antibody, and by the release of the complex to the cytosol. Biodistribution studies are also needed to further determine targeting specificity and penetrance to the target tissue. Ultimately, the efficiency and specificity of *in vivo* siRNA delivery depends on the targeting antibodies or other cell type-specific affinity ligands. Several novel phage antibody selection methods have been developed that allow the identification of internalizing antibodies targeting tumour cells *in situ* [58], as opposed to cell lines, increasing clinical relevance. These antibodies are expected to be able to target payloads more accurately to disease causing cells in actual cases, and to be useful in constructing additional siRNA delivery vehicles. To summarize, the studies discussed above demonstrate that the barrier for *in vivo* siRNA delivery can be overcome, significantly raising the likelihood of siRNA-based therapeutics against a broad spectrum of human diseases.

Key Points

- A major challenge to the development of therapeutic siRNAs is specific and efficient *in vivo* delivery.
- Cell type-specific internalizing antibodies can be used to deliver payload intracellularly to target cells.
- Recent studies suggest that cell type-specific gene silencing *in vivo* can be achieved by combining siRNAs with cell type-specific internalizing antibody fragments.
- The demonstrated specificity and efficiency of *in vivo* gene silencing and the lack of systemic toxicity suggest that the barrier to *in vivo* siRNA delivery can be overcome, encouraging further development of therapeutics based on targeted delivery of siRNAs.

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