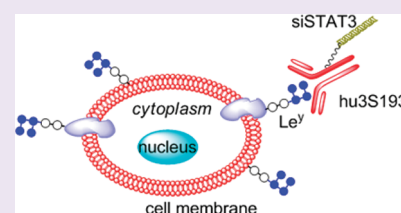


Humanized Lewis-Y Specific Antibody Based Delivery of *STAT3* siRNAYuelong Ma,[†] Claudia M. Kowolik,[†] Piotr M. Swiderski,[†] Marcin Kortylewski,[‡] Hua Yu,[‡] David A. Horne,^{*,†} Richard Jove,[†] Otavia L. Caballero,[§] Andrew J. G. Simpson,[§] Fook-Thean Lee,^{||} Vinochani Pillay,^{||} and Andrew M. Scott^{*,||}[†]Department of Molecular Medicine and [‡]Department of Cancer Immunotherapeutics and Tumor Immunology, Beckman Research Institute at City of Hope, Duarte, California 91010, United States[§]Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, New York 10021, United States^{||}Ludwig Institute for Cancer Research, Melbourne-Austin Branch, Austin Hospital, Heidelberg, VIC, 3084, Australia

S Supporting Information

ABSTRACT: The clinical application of siRNA is limited largely by the lack of efficient, cell-specific delivery systems. Antibodies are attractive delivery vehicles for targeted therapy due to their high specificity. In this study we describe the use of a humanized monoclonal antibody (mAb), hu3S193, against Lewis-Y (Le^Y), as a delivery vehicle for *STAT3* siRNA. This mAb is rapidly internalized into Le^Y-expressing cancer cells *via* antigen recognition, and when coupled to *STAT3* siRNA, a potentially powerful molecularly targeted delivery agent is created. Selective silencing of *STAT3* is associated with tumor suppression. Two hu3S193 based siRNA delivery systems using *STAT3* siRNA as a prototype were developed and tested in Le^Y-positive cancer cells: (a) a covalent construct based on a reductive disulfide linker that is expected to undergo cleavage within cells and (b) a noncovalent construct based on (D-arginine)₉ (9r) modified hu3S193. Le^Y-specific binding and internalization of both the covalent and noncovalent constructs were confirmed by flow cytometry and confocal microscopy. Both the covalent and the noncovalent system led to efficient *STAT3* silencing in Le^Y-positive cancer cells (A431) but not in Le^Y-negative cancer cells (MDA-MB-435). The covalent construct, however, required co-treatment with reagents such as chloroquine or 9r that facilitate the escape of the siRNA from endosomes to achieve significant gene silencing. The 9r modified noncovalent construct induced ~70% *STAT3* knockdown at submicromolar siRNA concentrations when used at an optimal vehicle-to-siRNA ratio of 5:1. The *STAT3* knockdown also led to ~50% inhibition of cell proliferation of Le^Y-positive cells. Noncovalent linked *STAT3* siRNA-hu3S193 has great promise for targeted knockdown of *STAT3* in tumor cells.



Small interfering RNAs (siRNAs) are a class of short double-stranded RNAs that can induce RNA interference (RNAi). After processing and incorporation into the RNA induced silencing complex (RISC), the antisense strand of the siRNA selectively binds to its complementary mRNA and induces its degradation in the cytoplasm.^{1–4} Synthetic siRNAs of 19–23 bp in length can also induce RNAi;^{5,6} however, it has been reported that 25- to 27-nt long siRNAs have greater potency than the corresponding 21-nt siRNAs. These longer, so-called Dicer substrate siRNAs (D-siRNAs) must be processed by Dicer before they can be incorporated into RISC.⁷ The increased potency of D-siRNAs has been attributed to a more efficient RISC incorporation by Dicer processing.^{3,7}

siRNAs have been considered by many scientists as potential “superdrugs”;⁸ however, the lack of efficient and specific delivery methodologies has impeded the development of this promising class of therapeutics.² Initial clinical trials focused on locally administered “naked” siRNA.² The first targeted siRNA delivery in humans was recently reported and employed human transferrin (Tf) modified cyclodextrin polymer nanoparticles for the specific recognition and treatment of Tf receptor expressing cancers.⁹ Successful siRNA delivery systems require efficient

transport to the target organs, uptake by the target cells, and escape of the siRNA from endosome vesicles into the cytoplasm.^{1,10} The size and the negative charge of siRNA make the accomplishment of this goal difficult. Viral and nonviral delivery vectors along with physical interventions (e.g., electroporation) have been used to facilitate siRNA delivery.^{1,2,11,12} Most of the current research is focused on the development of nonviral vehicles such as liposomes, nanoparticles, and peptides because of their lower costs, easier assembly, and greater safety compared to viral vectors.² From a synthetic point of view, there are two kinds of strategies for building nonviral-based delivery vehicles: covalent and noncovalent constructs with siRNA.¹³ Covalent constructs typically contain a cleavable (disulfide or other sessile bond) or a noncleavable linker between the siRNA and its vehicle. Most of the noncovalent constructs are based on electrostatic interactions between the positively charged vehicles and negatively charged siRNA. Conjugation of those covalent or noncovalent constructs with specific ligands would generate targeted delivery systems.¹²

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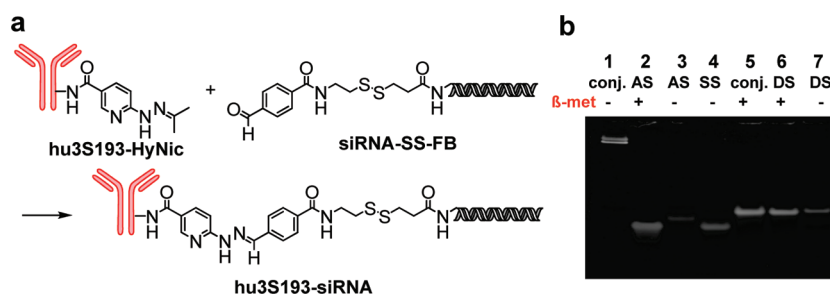


Figure 1. Synthesis and characterization of the covalent construct hu3S193-siRNA. (a) Synthetic scheme for the preparation of the covalent construct. (b) PAGE analysis of mAb-siRNA conjugate. Lanes 1 and 5: hu3S193-siRNA conjugates; lane 2 and 3: SS-FB-antisense strand (AS); lane 4: sense strand (SS); lanes 6 and 7: double strand siRNA-SS-FB (DS). Lanes 2, 5, and 6: treatment with β -mercaptoethanol (5%).

In principle, ligands that could facilitate targeted siRNA delivery include antibodies, aptamers, small molecules, and other proteins or short peptides.¹² Although costly, monoclonal antibodies or antibody fragments are still considered excellent delivery platforms for siRNAs because of their high specificity.^{2,14} For example, Lieberman's group used a protamine modified antibody fragment to deliver noncovalently bound siRNA into target cells.¹⁵ Kumar *et al.* reported a siRNA delivery strategy based on a CD7 single-chain antibody fragment (scFv) conjugated to (D-arginine)₉ (9r).¹⁶ Chang's group used a cationic liposome mixture modified with an antitransferrin receptor scFv for the delivery of anti-HER-2 siRNA,¹⁷ and Pardridge's group conjugated a streptavidin modified antibody with biotin modified siRNA to deliver siRNA.¹⁸

Hu3S193, an anti-Lewis-Y (Le^y) monoclonal antibody (mAb),¹⁹ was used in our study for the development of a siRNA delivery vehicle for several reasons: First, Le^y antigen is a blood group-related antigen expressed in over 70% of epithelial cancers including breast, colon, ovary, prostate and lung cancers. However, its expression on normal cells and normal tissues is limited.^{19–23} Second, this humanized Le^y-specific mAb is well tolerated and selectively targets Le^y-expressing tumors, with minimal normal tissue uptake, as demonstrated in a recent first-in-human trial.²⁴ Third, the Le^y-hu3S193 complex is a feasible choice for drug delivery since it is internalized after antigen recognition.²⁵ Selective delivery and promising bioactivity have been previously reported when a hu3S193-calicheamicin conjugate was used as specific drug delivery vehicle in a xenograft mouse model.²⁶

It has been reported that STAT3 is overexpressed and/or persistently activated in a wide diversity of solid tumors and blood malignancies.^{27–31} Activated STAT3 enhances tumor cell proliferation and survival, stimulates tumor angiogenesis and suppresses antitumor immune responses in the tumor microenvironment.^{27–31} Thus, the STAT3 pathway contributes to both tumor-cell intrinsic and extrinsic mechanisms of malignancy, making it an important target for cancer therapy. There are several strategies to inhibit STAT3 (or its signaling pathway) including reducing STAT3 levels (knockdown of gene expression), decreasing upstream phosphorylation (tyrosine kinase inhibitors) and blocking protein–protein interactions (inhibition of STAT3 recruitment to activated receptor complex, dimerization or STAT3-DNA promoter binding).^{27–31} Initial studies using siRNA/shRNA for the knockdown of STAT3 expression in cancer cells or in the tumor microenvironment have already showed promising results in cancer regression *in vitro* or *in vivo*.^{32–40} For example, an intratumoral injection of *Stat3* siRNA with a transfection reagent effectively inhibited tumor growth in a

mouse study.³⁹ CpG oligonucleotide-conjugated D-siRNA selectively silenced *Stat3* expression in toll-like receptor 9 positive cells, which led to the activation of tumor-associated immune cells and potent antitumor immune responses *in vivo*.⁴⁰

Although both covalent and noncovalent constructs have previously been used for siRNA delivery, these two strategies have not been compared within the same system. Especially for mAb based siRNA delivery, studies on covalent systems are very limited compared with noncovalent systems.^{13,18} Herein, we report the construction and efficacy of two hu3S193 based *STAT3* D-siRNA delivery vehicles: (a) a covalent construct consisting of mAb and siRNA conjugated by a reductive disulfide linker, which could be cleaved within the cells and (b) a noncovalent construct, which could bind siRNA *via* electrostatic interactions after the mAb was modified with a (D-arginine)₉ peptide. The tumor cell binding, internalization, and *STAT3* knockdown efficiencies were evaluated for both constructs.

RESULTS AND DISCUSSION

Synthesis and Characterization of hu3S193 siRNA Constructs. *Covalent Construct (hu3S193-siRNA).* The linker in the covalent construct contains a disulfide bond, which is expected to be reduced within the cytoplasm of the cell, and a stable bis-arylhydrazone bond with a specific UV wavelength at 350 nm, which facilitated detection and purification (Figure 1a). To synthesize this construct, hu3S193 and siRNA were first modified with commercially available linkers hydrazinonicotinate acetone hydrazone (HyNic) and disulfide formyl benzoate (SS-FB), respectively. This afforded the corresponding products hu3S193-HyNic and siRNA-SS-FB. Hu3S193-HyNic batches with different linker numbers ($N = 1–5$) per mAb were prepared by modulating the concentration of the reactants. Individual hu3S193-HyNic batches with different linker ratios were allowed to react with siRNA-SS-FB, which produced the covalent hu3S193-siRNA constructs (see Table 1 for description of antibody siRNA constructs). Regardless of the number of HyNic per mAb, the primary products after reaction with excess siRNA were mono hu3S193-siRNA conjugates. Lowering the HyNic-to-mAb ratio resulted in significantly reduced coupling yields with the siRNA (Supplementary Figure S1). Considering the significant reduction in chemical coupling yields with the siRNA when a low number of HyNic ($N < 5$) was attached to hu3S193, hu3S193-HyNic ($N = 5$) was used for all further syntheses. PAGE analysis of hu3S193-siRNA after disulfide reduction showed a signal corresponding to double stranded siRNA, which suggests the feasibility of a similar release mechanism of siRNA within cells

Table 1. Description of Antibody siRNA Constructs

construct	description
hu3S193	parent antibody
hu3S193-siRNA	covalent antibody siRNA construct
hu3S193-HyNic	HyNic linker modified antibody
hu3S193-siRNA(FL)	fluorescein-labeled hu3S193-siRNA
9r	(D-arginine) ₉ peptide
15r	(D-arginine) ₁₅ peptide
hu3S193-9r(1)	9r modified antibody with antibody:9r ratio = 1:1
hu3S193-9r(4)	9r modified antibody with antibody:9r ratio = 1:4
siRNA(FL)	fluorescein-labeled siRNA
hu3S193-9r(1):siRNA	noncovalent construct based on hu3S193-9r(1)
hu3S193-15r(1)	15r modified antibody with antibody: 15r ratio = 1:1
siSTAT3	STAT3 siRNA
siLuci	luciferase siRNA

(Figure 1b). For flow cytometry and confocal microscopy studies we also synthesized a fluorescein-labeled hu3S193-siRNA (hu3S193-siRNA(FL)) covalent conjugate using a fluorescein-labeled amino modified RNA strand.

Noncovalent Construct (hu3S193-9r:siRNA). In addition to the covalent system, a (D-arginine)₉ based noncovalent siRNA delivery strategy was developed. Arginine-rich peptides have been used for siRNA delivery as free peptide⁴¹ or conjugated with target ligands.^{16,42} The delivery mechanisms are not fully understood. It has been suggested that arginine-rich peptides improve siRNA delivery by facilitating the escape of siRNAs from endosomes^{43,44} or by protecting siRNAs from degradation.⁴² *d*-Peptides were favored due to their increased stability toward proteolysis.⁴⁵ To synthesize the noncovalent construct, hu3S193 was first thiolated using Traut's reagent and then allowed to react with activated (D-arginine)₉ (9r) to produce hu3S193-9r (Figure 2a). Two batches of 9r modified hu3S193 with 9r-to-mAb ratios of 1:1 (hu3S193-9r(1)) and 4:1 (hu3S193-9r(4)) were synthesized. Upon mixing with siRNA, hu3S193-9r associates with siRNA *via* electrostatic interactions. Electrophoretic gel mobility-shift assays (EMSA) confirmed that siRNA efficiently binds to hu3S193-9r(1) but not to the unmodified hu3S193 (Figure 2b). Due to the increased number of 9r per mAb, hu3S193-9r(4) displayed stronger binding to siRNA (Figure 2c). A 15r peptide modified hu3S193, hu3S193-15r(1), was also synthesized using the same methodology. EMSA results indicated that hu3S193-15r(1) binds siRNA similarly to hu3S193-9r(1) (data not shown). For flow cytometry or confocal imaging studies, fluorescein-labeled siRNA (siRNA(FL)) was used instead of regular siRNA in the preparation of the noncovalent construct.

Specific Delivery of siRNA into Le^y-Expressing Cells by Covalent and Noncovalent Constructs. Using fluorescein-labeled hu3S193 (hu3S193(FL)), we first confirmed by flow cytometry analysis previous reports^{46,47} that A431 cells (human squamous cell carcinoma) expressed high levels of Le^y, while MDA-MB-435 cells (breast cancer) were Le^y-negative (Figure 3a). To determine whether the constructs could efficiently and specifically bind to Le^y-positive cells, MDA-MB-435 and A431 cells were incubated with hu3S193-siRNA(FL) or hu3S193-9r:siRNA(FL) and analyzed by flow cytometry. As shown in Figure 3b, the covalent conjugate could specifically bind to Le^y-expressing

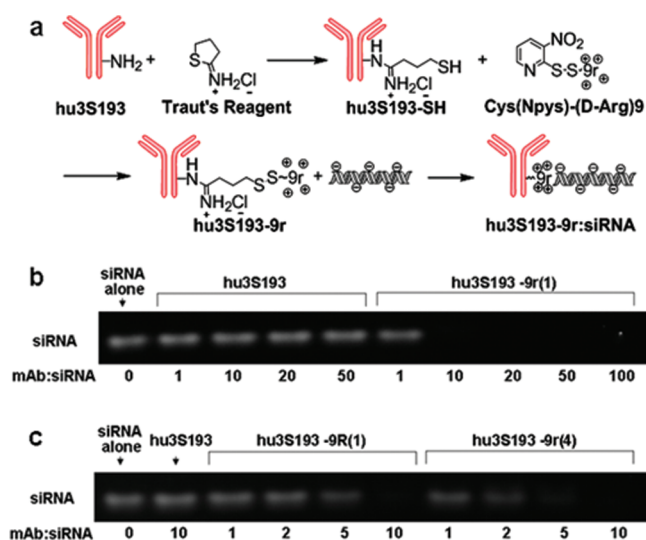


Figure 2. Synthesis and characterization of the noncovalent construct hu3S193-9r:siRNA. (a) Synthetic scheme for the preparation of the noncovalent construct. (b) siRNA binding to hu3S193-9r(1). Hu3S193 or hu3S193-9r(1) was mixed with siRNA at the indicated molar ratios and analyzed by electrophoresis. (c) Comparison of siRNA binding to hu3S193-9r(1) or hu3S193-9r(4). The constructs hu3S193-9r(1) and hu3S193-9r(4) (modified with one or four 9r peptides per antibody, respectively) were incubated with siRNA at the indicated molar ratios and analyzed by electrophoresis.

A431 cells but not to Le^y-negative MDA-MB-435 cells. Flow cytometry analysis of the two noncovalent constructs, hu3S193-9r(1) and hu3S193-9r(4), modified with either one or four 9r per mAb, respectively, showed dramatically different results. When A431 and MDA-MB-435 cells were treated with various ratios of hu3S193-9r(1)-to-siRNA(FL) ranging from 1:1 to 1:10, only the percentage of fluorescein-positive A431 cells increased with higher hu3S193-9r(1)-to-siRNA(FL) ratios (Figure 3c). No significant siRNA binding was observed after treatment of A431 cells with siRNA(FL) alone or siRNA(FL) mixed with hu3S193 and/or 9r (Supplementary Figure S2a). In addition, pretreatment of A431 cells with the unmodified hu3S193 antibody partially blocked cell binding of hu3S193-9r(1):siSTAT3(FL)(5:1). The percentage of positive cells was reduced by ~70% by the pretreatment (Supplementary Figure S2b).

On the other hand, when the cells were treated with hu3S193-9r(4):siRNA(FL) the percentage of both fluorescein-positive A431 and MDA-MB-435 cells increased with higher hu3S193-9r(4)-to-siRNA(FL) ratios (Figure 3d). These results suggest that increasing the amount of 9r per mAb led to nonspecific binding to Le^y-negative cells. Thus, hu3S193-9r(1) modified with one 9r peptide per mAb was used for all further experiments with the noncovalent construct.

Cell internalization of the covalent and noncovalent constructs was confirmed by confocal imaging. Fluorescence signals were observed on the surface and within Le^y-expressing A431 cells but not within Le^y-negative MDA-MB-435 cells for both hu3S193-siRNA(FL) (Figure 4a) and hu3S193-9r(1):siRNA(FL) (Figure 4b). No internalization was observed in Le^y-expressing cells with siRNA(FL) alone or siRNA(FL) mixed with hu3S193 and/or 9r (data not shown).

Silencing Efficiency of the Covalent and Noncovalent Constructs. To test *STAT3* silencing efficiency Le^y-expressing

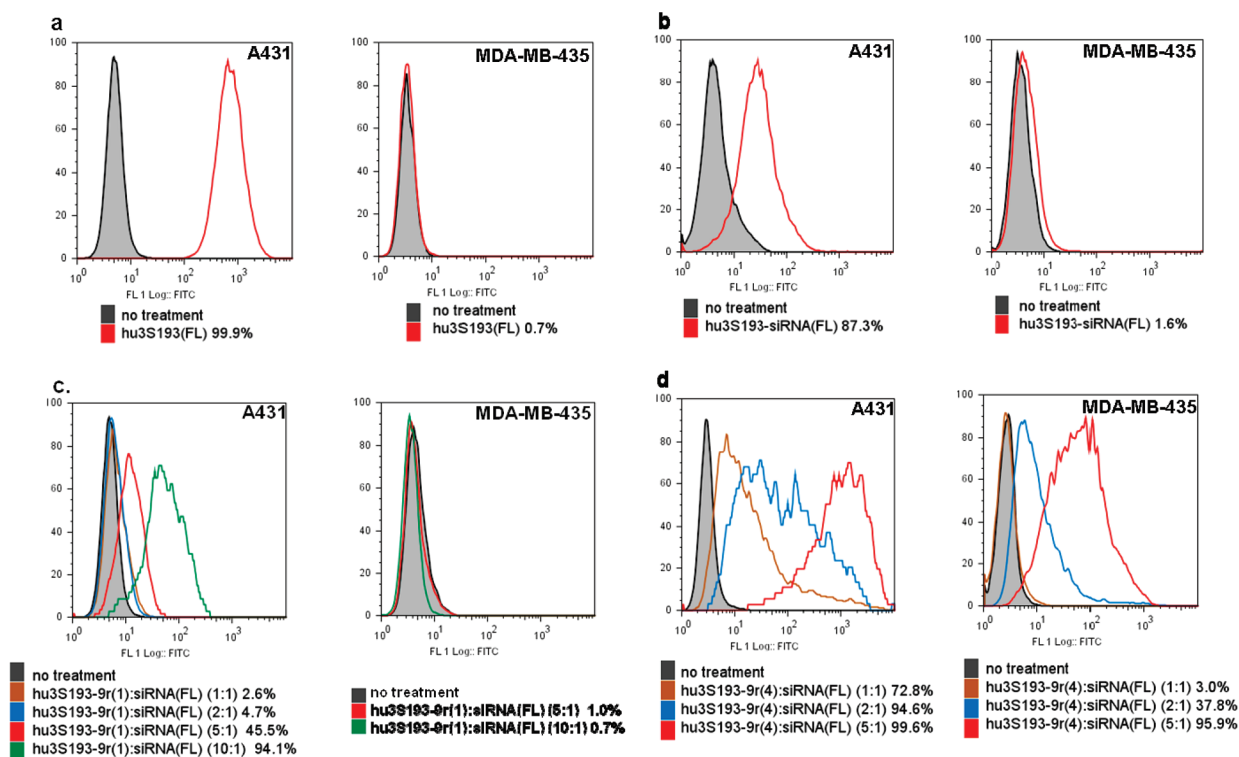


Figure 3. Flow cytometry analysis of cell binding. (a) Le^y expression in A431 and MDA-MB-435. The cells were stained with hu3S193(FL). (b) Cell binding of hu3S193-siRNA(FL). Cell binding of (c) hu3S193-9r(1):siRNA(FL) and (d) hu3S193-9r(4):siRNA(FL). The constructs hu3S193-9r(1) or hu3S193-9r(4) were incubated with siRNA(FL) at various molar ratios. The mixtures were then used to stain the cells. The siRNA concentration was 300 nM for all binding studies. The percentage of fluorescein-positive cells is indicated for each treatment.

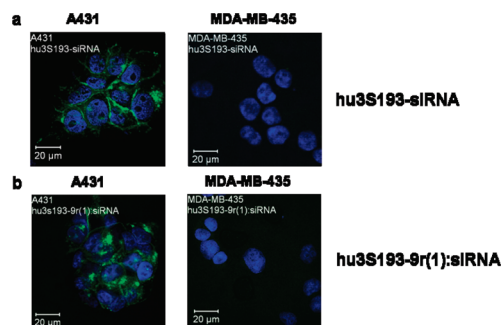


Figure 4. Confocal microscopy. Le^y expressing A431 and Le^y -negative MDA-MB-435 cells were treated for 5 h with (a) hu3S193-siRNA(FL) or (b) hu3S193-9r(1):siRNA(FL) (5:1). The siRNA concentration was 600 nM for all experiments.

cell lines were treated with both covalent and noncovalent constructs, and *STAT3* expression levels were determined by quantitative PCR using *GAPDH* as reference gene. Surprisingly, the covalent construct hu3S193-siRNA did not show any significant gene silencing effects, even when used at a concentration of 1500 nM (data not shown). Since significant binding and internalization was observed by flow cytometry and confocal imaging, we speculated that the siRNA was stuck in the endosomes and inefficient endosome release is the reason for the lack of gene silencing. To test this hypothesis, we treated A431 and MDA-MB-435 cells with hu3S193-siRNA in the presence of chloroquine,^{48–50} a known endosome disrupting reagent. At a concentration of 100 μ M chloroquine *STAT3* expression levels

were decreased by $\sim 60\%$ in the Le^y -positive cell line A431 but not in the Le^y -negative cell line MDA-MB-435 (Figure 5a and 5d). *STAT3* levels did not decrease after adding chloroquine to A431 cells treated with the negative control siRNA (siLuci) (Figure 5a).

Unfortunately, the cytotoxicity of chloroquine prohibits its broad clinical application for siRNA delivery.⁵¹ So instead, our attention turned toward arginine-rich peptides, which also have been suggested to promote siRNA delivery by facilitating the escape of siRNAs from endosomes.^{43,44} Moreover, no significant toxicity has been reported with arginine peptide administration *in vivo*.^{16,41,42,44} When the covalent construct hu3S193-siRNA was preincubated with 9r peptide, a significant improvement in gene knockdown was observed. Optimal gene silencing was achieved when hu3S193-siRNA was mixed with 9r at a ratio of 1:2 (Figure 5b). The decrease in knockdown efficiency at higher hu3S193-siRNA:9r ratios could be attributed to an inefficient release of the siRNA from the peptide complex.⁴⁴ *STAT3* expression in the Le^y -negative MDA-MB-435 cell line was not influenced by the same treatment (Figure 5d).

We also tested the knockdown efficiency of the noncovalent system, hu3S193-9r(1):siRNA. This construct effectively decreases *STAT3* expression under optimized conditions. The molar ratio of vehicle-to-siRNA is critical for achieving efficient knockdowns. The best gene silencing ($>70\%$) was observed at a molar ratio of hu3S193-9r(1) to siRNA of 5:1 (Figure 5c). The decrease in knockdown efficiency that was observed at higher vehicle-to-siRNA ratios might be due to the inefficient release of siRNA from the 9r peptide.⁴⁴ We also tested the influence of the peptide length on the gene knockdown efficiency by comparing a

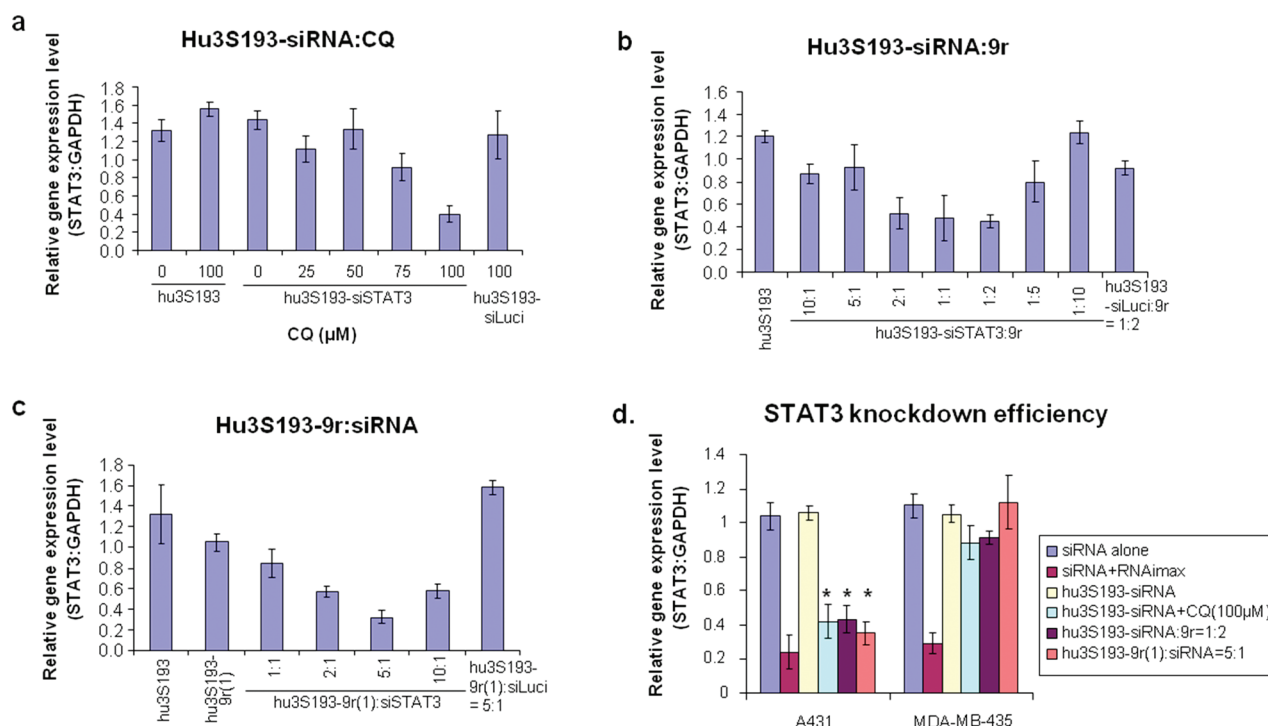


Figure 5. Knockdown efficiency. Cells were treated with the covalent or noncovalent construct for 24 h. The effect of the treatment on *STAT3* expression levels was measured by quantitative PCR. The data were normalized to the *GAPDH* expression. (a) Influence of chloroquine (CQ) on the knockdown efficiency of the covalent construct. A431 cells were treated with hu3S193-siSTAT3 and CQ at the indicated concentrations. (b) Influence of 9r peptide on the knockdown efficiency of the covalent construct. A431 cells were treated with hu3S193-siSTAT3 and 9r peptide at the indicated molar ratios. Cells treated with noncovalent constructs containing luciferase siRNA (hu3S193-siLuci:9r, molar ratio 1:2) were used as a negative control. (c) Knockdown efficiency of the noncovalent construct. A431 cells were treated with hu3S193-9r(1):siSTAT3 at the indicated molar ratios. Cells treated with hu3S193-9r(1):siLuci at a molar ratio of 5:1 were used as a negative control. (d) Comparison of the knockdown efficiency in *Le^y*-positive and *Le^y*-negative cells. A431 (*Le^y*-positive) and MDA-MB-435 (*Le^y*-negative) cells were treated with siSTAT3, hu3S193-siSTAT3 alone, hu3S193-siSTAT3+CQ (100 μM), hu3S193-siSTAT3:9r(1:2), or hu3S193-9r(1):siSTAT3 (5:1) or transfected with 50 nM siRNA using RNAiMAX as transfection reagent. The siRNA concentration was 300 nM for all experiments (except for the transfection reaction). **P*-values compared with siRNA alone treatment on A431 cell line were <0.0006.

15r peptide modified hu3S193 (hu3S193-15r(1)) with hu3S193-9r(1). Similar knockdown efficiencies were observed, suggesting that increasing the peptide length from 9 to 15 amino acids does not significantly alter the siRNA delivery efficiency for the noncovalent system (Supplementary Figure S3a).

To determine the siRNA concentration necessary to achieve optimal silencing, A431 cells were treated with various siRNA concentrations. The ratio of hu3S193-9r(1) to siRNA was kept at 5:1. Maximal silencing was observed at a siRNA concentration of 300 nM. Increasing this siRNA concentration did not lead to improved gene silencing (Supplementary Figure S3b).

To confirm the specificity of siRNA delivery into *Le^y*-positive cells we treated MDA-MB-468 cells, a *Le^y*-positive cell line (with reduced *Le^y* expression levels compared to A431), and *Le^y*-negative MDA-MB-435 cells using the same conditions. *STAT3* expression levels were reduced in MDA-MB-468 by ~50% (data not shown). No significant gene knockdown was observed in MDA-MB-435 cells (Figure 5d). siRNA alone or the covalent conjugate hu3S193-siRNA alone also did not induce gene knockdown regardless of the cell type (Figure 5d). On the other hand, using RNAiMAX as transfection reagent (siRNA 50 nM) led to a 70–80% reduction in *STAT3* expression levels in all of the cell lines similarly (Figure 5d).

Taken together, our data indicate that the covalent construct hu3S193-siRNA requires chloroquine or 9r peptide for efficient

gene silencing. The noncovalent construct hu3S193-9r(1):siRNA shows promising, target-cell-specific gene knockdown. Both the covalent and the noncovalent system are capable of delivering siRNA into *Le^y*-positive cells. The 9r peptide in the noncovalent system not only mediates siRNA binding and delivery but also helps the siRNA escape from the endosomes and/or protects the siRNA from degradation.^{42–44} Since the hu3S193-9r(1):siRNA noncovalent delivery system has general applicability for delivering different siRNAs or multiple siRNAs without modification of the delivery vehicle, the following experiments were focused on this 9r modified noncovalent system.

To confirm the knockdown efficiency and specificity at the protein level, we treated cells with hu3S193-9r(1):siRNA (5:1) and determined the *STAT3* levels by Western blot analysis after 24, 48, and 72 h. A significant decrease in *STAT3* protein levels was observed at 48 and 72 h (Figure 6a). No decrease in *STAT3* was observed upon treatment with hu3S193-9r(1) alone or with hu3S193-9r(1):luciferase siRNA (siLuci) (Figure 6b). The same treatment did not change the *STAT3* expression in MDA-MB-435 cells (Figure 6b).

Cell Proliferation. Since a strong correlation exists between the overexpression of *STAT3* and cell proliferation,^{27–31} we further examined the effects of the treatment with hu3S193-9r(1):siSTAT3 on A431 cells *in vitro*. After 48 h, there is a slight but consistent reduction of cell proliferation with

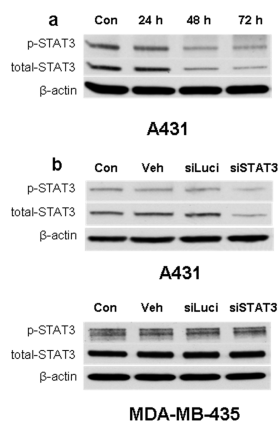


Figure 6. Western blot analysis of STAT3 expression. (a) Time course of STAT3 knockdown: A431 cells were treated with hu3S193-9r(1):siSTAT3(5:1) for 24, 48, and 72 h. (b) Specificity of STAT3 knockdown: Le^Y-positive A431 and Le^Y-negative MDA-MB-435 cells were treated with hu3S193-9r(1) alone (Veh), hu3S193-9r(1):siLuci(5:1) (siLuci) or hu3S193-9r(1):siSTAT3(5:1) (siSTAT3) for 72 h. Untreated cells were used as a control (Con). The siRNA concentration was 300 nM for all experiments.

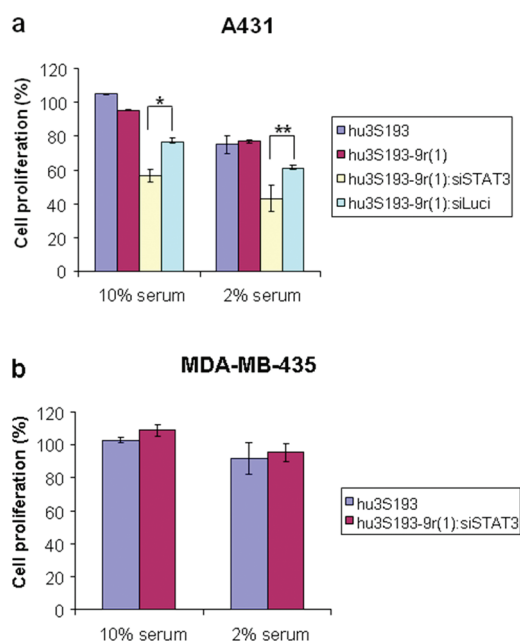


Figure 7. Cell proliferation assay. (a) A431 cells were treated with hu3S193, hu3S193-9r(1), hu3S193-9r(1):siSTAT3(5:1), or hu3S193-9r(1):siLuci(5:1) (b) MDA-MB-435 cells were treated with hu3S193 and hu3S193-9r(1):siSTAT3(5:1) for 48 h and then analyzed using the MTS assay. The siRNA concentration was 300 nM. *,**P-value between hu3S193-9r(1):siSTAT3(5:1) and hu3S193-9r(1):siLuci(5:1) treatment on A431 cell line with 10% serum is 0.0006*, with 2% serum is 0.008**.

hu3S193-9r(1):siSTAT3 treatment compared to vehicle alone or hu3S193-9r(1):siLuci (Figure 7a). Reducing the serum level from 10% to 2% resulted in greater levels of inhibition. These results are consistent with data previously reported by Bonner *et al.*, who used STAT3 shRNA for the treatment of A431 cells.⁵² The proliferation of MDA-MB-435 cells was not influenced by the treatment with hu3S193-9r(1):siSTAT3 (Figure 7b). These results

are consistent with our earlier observations on the cell-specific binding and gene knockdown in Le^Y-positive cells.

STAT3 has been suggested to influence not only the cell viability and proliferation but also, more importantly, the tumor cell microenvironment and/or angiogenesis *in vivo*.²⁸ *In vivo* experiments including biodistribution and antitumor activity studies are currently underway. In conclusion, our results suggest that hu3S193-9r(1) is a promising vehicle for efficient cell-specific delivery of siRNAs (*e.g.*, STAT3 or other tumor-related targets) into Le^Y-expressing tumor cells.

METHODS

Preparation of hu3S193-siRNA and hu3S193-9r:siRNA. D-siRNAs were designed according to published guidelines.^{7,53,54} An initial screening was performed using RNAiMAX (Invitrogen) as transfection reagent. The siRNAs (50 nM, final concentration) were transfected according to the manufacturer's protocol for reverse transfections. The siRNA with best STAT3 knockdown efficiency was used in the study (data not shown). Sequence of STAT3 D-siRNA: 5'-GGAAGCUGCA-GAAAGAUACGACUdGdA-3' (sense strand); 5'-UCAGUGGUUAU-CUUUCUGCAGCUUCCGU-3' (antisense strand). Sequence of luciferase siRNA: 5'-GGUUCUGGAACAAUUGCUUUUAdCdA-3' (sense strand); 5'-UGUAAAAGCAAUUGUCCAGGAACCAG-3' (antisense strand).

For the synthesis of the covalent construct, hu3S193 was allowed to react with S-HyNic (Solulink) and the average HyNic linker number per antibody was determined by reacting the resulting product with 2-sulfobenzaldehyde sodium salt according to manufacturer's protocol (Solulink). Hu3S193-HyNic batches with different linker numbers ($N = 1-5$) per mAb were obtained after purification *via* Zeba Desalt Spin Columns (Thermo Scientific). The antisense strand was 5'-amino modified during solid phase synthesis and further reacted with linker S-DSS-FB (Solulink). The linker modified antisense strand was purified by HPLC, annealed with the sense strand, and conjugated with hu3S193-HyNic. Products were purified by FPLC. The stoichiometry of hu3S193-siRNA was calculated on the basis of the BCA protein assay (Thermo Scientific) and A280, A260 measurements. The RNA component of hu3S193-siRNA conjugate was confirmed by disulfide reduction (β -mercaptoethanol, Thermo Scientific) and analyzed on 15% PAGE in 1X TBE. For the fluorescein modified covalent conjugate, 5'-fluorescein-labeled, amino modified antisense RNA was prepared during solid phase synthesis using 6-fluorescein phosphoramidite (Glen Research, cat. no. 10-1094-XX). The fluorescein-labeled hu3S193-siRNA (hu3S193-siRNA(FL)) conjugate was similarly synthesized according the aforementioned methods.

For the noncovalent construct, hu3S193 was thiolated using Traut's reagent (Thermo Scientific) and then modified with Cys(Npys)-(D-Arg)₉ (9r, AnaSpec). The stoichiometry of thiol modification on hu3S193 was calculated based on signals resulting from reaction with Ellman's reagent (Thermo Scientific). The hu3S193-9r:siRNA complex was obtained by mixing hu3S193-9r and siRNA in PBS at the indicated molar ratios and incubating the mixtures at RT for 20 min. Fluorescein-labeled siRNA (siRNA(FL) from IDT) was used instead of regular siRNA in the preparation of the noncovalent construct for flow cytometry and confocal imaging studies.

SYBR Gold (Invitrogen) was used for siRNA staining and SimplyBlue SafeStain (Invitrogen) was used for protein staining in all gel studies.

Gel Shift Assay. hu3S193, hu3S193-9r(1), or hu3S193-9r(4) were incubated with siRNA at different ratios for 20 min at RT. The binding of siRNA to vehicle was analyzed on 6% PAGE in 0.5X TBE.

Cell Lines. A431 and MDA-MB-435 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated FBS.

Flow Cytometry. For cell binding analysis, 5×10^5 cells suspended in PBS were incubated with hu3S193(FL), hu3S193-siRNA(FL), or hu3S193-9r:siRNA(FL) at 4 °C for 30 min, washed 3 times with PBS, and then fixed with 2% paraformaldehyde for 10 min. Fluorescence data were collected on a FACScalibur (Beckton Dickinson) and analyzed using FlowJo software (Tree Star).

Confocal Microscopy. Cells were grown on slides, incubated with hu3S193-siRNA(FL) or hu3S193-9r(1):siRNA(FL) at 37 °C for 5–24 h, washed with PBS, and fixed with 2% paraformaldehyde. The slides were mounted after staining the nuclei with DAPI (Vector) and analyzed by confocal microscopy (Zeiss Upright LSM510 2-Photon Microscope).

Quantitative PCR. Hu3S193-siRNA:9r or hu3S193-9r(1):siRNA were premixed at RT for 20 min at the indicated molar ratios. A431 or MDA-MB435 cells (3×10^4) were seeded on a 48-well plate and treated with hu3S193-siRNA, hu3S193-siRNA with chloroquine, hu3S193-siRNA:9r, or hu3S193-9r(1):siRNA for 24 h. Total RNA was extracted from cells using the RNeasy kit (Qiagen). The Express One-Step SuperScript qRT-PCR kit (Invitrogen) was used to reverse-transcribe and amplify 25 ng of total RNA per reaction according to the manufacturer's protocol. The ProbeFinder software (Roche Applied Science) was used to design the primer sets for *STAT3* and *GAPDH* and to select the respective probes from the Universal ProbeLibrary (Roche). Probe no. 17 and the following primers were used for the *STAT3* assay: 5' cgatggagctacgtgcagaaa and 5' tgagattctgtaatgcagttatcc. Probe no. 60 and the following primers were used for the *GAPDH* assay: 5' agccacatcgtcagacac and 5' gcccaatcagaccaatcc. All samples were run in triplicates. Amplifications were performed on a Bio-Rad iCycler iQ5 Multiple-Color Real-time PCR Detection System. The data were normalized to the *GAPDH* expression and the relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Western Blot Analysis. Cells were lysed with M-per buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Thermo Scientific). Twenty micrograms of protein was resolved in 4–15% SDS-PAGE (Bio-Rad) and transferred to NuPage nitrocellulose membranes (Invitrogen). The membranes were washed with PBS-0.1% Tween 20, blocked for 1 h at RT with 5% milk in PBS-0.1% Tween 20, and then incubated overnight at 4 °C with the indicated primary antibodies (phospho-STAT3 (Cell Signaling, cat. no. 9131), total STAT3 (Cell Signaling, cat. no. 9132), β -actin (Sigma-Aldrich, cat. no. A5441)). The membrane was then washed with PBS-0.1% Tween 20 and incubated for 1 h at RT with HRP-conjugated anti-rabbit (GE, NA9340) or anti-mouse (GE, NA931) secondary antibodies. The blots were developed using an enhanced chemiluminescence detection system (Thermo Scientific). For detection of total STAT3, the corresponding phospho blots were incubated with stripping buffer (Thermo Scientific) and reblotted.

MTS Assays. Cell proliferation assays were performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Each well of a 96-well plate was seeded with 5,000 cells in culture medium. After 16 h culture, the cells were treated with hu3S193-9r(1):siRNA. After 48 h of treatment, MTS was added to the cells according to the supplier's protocol, and the absorbance was measured at 490 nm using a 96-well plate reader (Synergy 4, Biotek).

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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