A Nanoparticle System Specifically Designed to Deliver Short Interfering RNA Inhibits Tumor Growth In vivo

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Abstract
Use of short interfering RNA (siRNA) is a promising new approach thought to have a strong potential to lead to rapid development of gene-oriented therapies. Here, we describe a newly developed, systemically injectable siRNA vehicle, the “wrapsome” (WS), which contains siRNA and a cationic lipofection complex in a core that is fully enveloped by a neutral lipid bilayer and hydrophilic polymers. WS protected siRNA from enzymatic digestion, providing a long half-life in the systemic circulation. Moreover, siRNA/WS leaked from blood vessels within tumors into the tumor tissue, where it accumulated and was subsequently transsected into the tumor cells. Because the transcription factor KLF5 is known to play a role in tumor angiogenesis, we designed KLF5-siRNA to test the antitumor activity of siRNA/WS. KLF5-siRNA/WS exhibited significant antitumor activity, although neither WS containing control scrambled-siRNA nor saline containing KLF5-siRNA had significant antitumor activity, although neither WS containing control scrambled-siRNA nor saline containing KLF5-siRNA affected tumor growth. KLF5-siRNA/WS inhibited KLF5 expression within tumors at both mRNA and protein levels, significantly reducing angiogenesis, and we detected no significant acute or long-term toxicity. Our findings support the idea that siRNA/WS can be used to knock down specific genes within tumors and thereby exert therapeutic effects against cancers. [Cancer Res 2009;69(16):6531–8]

Introduction
Short interfering RNA (siRNA) is an attractive technology that has the potential to effectively suppress expression of selected genes, including genes with products that are not considered to be practical drug targets (e.g., transcription factors). However, achievement of effective gene knockdown in vivo requires highly efficient delivery of siRNA to target tissues (1). For efficient delivery of siRNA and therapeutic gene knockdown, a drug delivery system needs to (a) protect the siRNA from degradation, (b) suppress nonspecific uptake by nontarget tissues, and (c) mediate accumulation of siRNA within the target tissues and cells.

Cationic lipids and polymers are two major classes of nonviral siRNA delivery carriers that have been tested; both are positively charged and form complexes with negatively charged siRNA (1, 2).

In general, these carriers have a high electric charge density on their surface, which causes nonspecific interactions with proteins in the blood and endothelium, resulting in their rapid removal from the systemic circulation (3). In addition, many carriers, the siRNA is exposed to the outside and is therefore susceptible to degradation by serum proteins. Furthermore, positively charged complexes may induce embolization by forming aggregates with blood proteins (4). To overcome the problems associated with existing cationic carriers, a delivery system that provides physical containment of the siRNA and inhibits nonspecific electrostatic interactions with blood proteins and the endothelium would be desirable.

Assuming that its half-life could be lengthened, another important hurdle would be transporting the siRNA to the target tissue. It is well documented that the higher permeability of tumor blood vessels, coupled with poor lymphatic drainage, enhances retention of macromolecules such as nanoparticles within tumors (the EPR effect; refs. 5–7). This could potentially enable selective accumulation of drugs and other bioactive molecules within tumors; however, the EPR effect is only observed with stabilized macromolecules, and efficient tumor uptake requires long systemic residence times (8). The size of macromolecules is also known to greatly affect the efficacy of the EPR effect (9).

In the present study, we describe the “wrapsome” (WS), which is based on our previously developed nanoparticle technology (10) and was developed with the aim of making use of the EPR effect to transport native siRNA to target tumors following systemic administration. We found that, when systemically administered via the tail vein, wrapsomal siRNA (siRNA/WS) accumulated in s.c. tumors, and the siRNA was taken up by the tumor cells. The resultant siRNA/WS-mediated knockdown of the transcription factor KLF5 suppressed both angiogenesis within tumors and tumor growth.

Materials and Methods

siRNA preparation. KLF5-siRNA and fluorescence-labeled KLF5-siRNA was synthesized and purified by Hokkaido System Science. The sequences of siRNA were KLF5-siRNA: AAGCUACCUGGACUCAUTT (sense) and UGGUCCUGCCAUUGCCUUTT (antisense; ref. 11), KLF5-siRNA#2: GCAUCAGAAGGCUCUU and AAGACGUCAUACUGGCUUG, secreted human alkaline phosphatase gene (12)-siRNA: AGGGCAACUUCCAGACCAUTT and AUGCUGCAUUGGCCACUCCUTT, scrambled-siRNA: GGUCACAGAUGUGCACACTT and GUGUGUGUGUGACACUGTT, and luciferase-siRNA: CUUACGUGAUCUGAGGATT and UCGAGUACUCUGUAAGTT (13).

Preparation of wrapsomes. Sixty milligrams of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 24 mg of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol-2000) (PEG-DSE) were...
hydrated in 2.5 mL distilled water and extruded 20 times through two polycarbonate membrane filters with pore sizes of 0.4 and 0.1 μm (Liposofast; Avestin). The resultant suspensions were mixed with 1.25 mL distilled water containing 10 μg siRNA, which formed complexes with the DOTAP and PEG-DSPE. An aliquot of the solution containing the complexes (0.75 mL) was then diluted with 1 mL ethanol and added to 0.25 mL of an ethanol solution containing 96 μg/mL egg phosphatidylcholine (egg-PC) and 20 μg/mL PEG-DSPE. In the resultant suspension, the concentration of ethanol was 62.5% (v/v); siRNA, 1.0 mg/mL; DOTAP, 6.0 mg/mL; PEG-DSPE, 2.4 mg/mL; egg-PC, 12 mg/mL; and PEG-DSPE, 2.5 mg/mL. Twenty-three milliliters of distilled water were then slowly added to this suspension while stirring to dilute the ethanol to 5% (v/v), after which the suspension was centrifuged for 1 h at 110,000 × g, and the supernatant was removed to eliminate the ethanol completely. Less than 10% of the siRNA was detected in the supernatant. The pellet was resuspended in PBS to provide a preparation containing 24 mg/mL egg-PC, and PEG-DSPE in ethanol (4%, v/v) was added to a final egg-PC/PEG-DSPE ratio of 2:14.8 (w/w). The mixture was then heated for 2 min at 70°C, and the particle size of the resultant wrapsomes was measured based on dynamic light scattering.

Cell culture. Mouse Lewis lung carcinoma cells (LL/2; American Type Culture Collection) and human prostate carcinoma cells (PC-3; American Type Culture Collection) were cultured in DMEM supplemented with 10% fetal bovine serum without antibiotics.

Evaluation of siRNA stability against nucleases. One nanomole of siRNA/WS or siRNA in saline solution was incubated with 0.2 IU RNase A (Qiagen). The siRNA was then extracted and separated on 15% TBE-urea gels (Invitrogen).

Animal experiments. All experiments were approved by Ethics Committees for Animal Experiments of the University of Tokyo and Kyowa Hakko Kirin and strictly adhered to the guidelines for animal experiments.

Measurement of blood clearance. Male 5-week-old C57BL/6Jc mice (CLEA Japan) were administered a single injection of FAM-labeled KLF5-siRNA/WS (50 μg siRNA/100 μL; n = 3), and their plasma was collected at various time points thereafter. FAM fluorescence intensities were measured in the presence of 1% Triton X-100 using an ARVO system (Perkin-Elmer), after which blood concentrations of siRNA and its half-life in the circulation were calculated (WinNonlin; Pharsight).

Cy5 dye was synthesized from Cy5-amidite (Pharmacia Biotech). The protection groups on Cy5-amidite were removed, after which the chemical structure was identified using 1H nuclear magnetic resonance and mass spectrometry. Cy5 dye was mixed with the same lipid components that comprise the WS envelope and then extruded through a polycarbonate membrane (100 nm pore) 20 times in saline. Liposome-encapsulated Cy5 was isolated and administered to mice as described above.

Whole-body near-infrared imaging of Cy5-siRNA. The distribution of Cy5-siRNA in the bodies of mice and its accumulation in tumors following systemic administration was analyzed noninvasively using an IVIS imaging system 200 (Xenogen). Male 5-week-old BALB/cJcl mice were purchased from CLEA Japan. LL/2 cells (1 × 10⁶) or PC-3 cells (1 × 10⁶) in 100 μL DMEM were inoculated s.c. into the backs of mice for each mouse; Vector Laboratories). Thereafter, the tumor was allowed to grow to a volume of 200 mm³. The mice were thengivenasingleinjectionofsalinesolutionorWSformulationcontaining10μgCy5-labeledKLF5-siRNAin100μLviathevertailvein.Cy5fluorescencewasytmonitoredforupto72h(LL/2)or2weeks(PC-3)afteradministration. Total photon flux in the tumor tissue was quantified by analyzing regions of interest using image analysis software (Xenogen).

Fluorescence confocal microscopy. A confocal laser scanning microscope (LSM510 Meta; Carl Zeiss) equipped with ×10 dry and ×100 oil objectives was used to collect tissue images. Mice were given a single injection of saline solution or WS formulation containing 10 μg Cy5-labeled KLF5-siRNA in 100 μL volume via the tail vein. To visualize the vasculature 24 h later, the mice were sacrificed by cervical dislocation 30 min after intravascular injection of FITC-conjugated Grifonia simplicifolia isoelectin GS-B4 (50 μg for each mouse; Vector Laboratories). Thereafter, the tumor and surrounding s.c. tissues were removed, washed, and incubated with Hoechst 33342 (40 μmol/L; Molecular Probes) for 15 min at room temperature. The tissue was then excited using multiple color laser lines (405 nm for Hoechst, 488 nm for isoelectin with FITC, and 633 nm for Cy5), and the emission was collected through appropriate narrow band-pass filters.

Fluorescence-activated cell sorting analysis. Flow cytometry (Cytomics FC 500; Beckman Coulter) was used to evaluate the incorporation of siRNA into cells. Mice bearing LL/2 tumors were administered a single injection of saline solution or WS formulation containing 150 μg Cy5-labeled KLF5-siRNA in 100 μL volume via the tail vein. Twenty-four hours later, the tumor tissues were harvested and treated with 0.2% (w/v) collagenase D in HEPES-buffered saline (pH 7.4) for 30 min at 37°C. After removing the cell aggregates by filtration, isolated cells were analyzed for Cy5-siRNA fluorescence using a flow cytometer equipped with a 488 nm argon ion laser. Mice administered saline served as a negative control.

Analysis of tumor growth. In the syngeneic model, LL/2 cells (1 × 10⁶) in 100 μL DMEM were inoculated s.c. into the backs of male 5-week-old mice.
C57Bl/6j/cjcl mice on day 0. In the PC-3 xenograft model, PC-3 cells ($1 \times 10^6$) were inoculated s.c. into the backs of the male 5-week-old BALB/cJcl-nu/nu mice. In both models, tumor size was measured every other day using calipers, and tumor volume was calculated using the formula $V = \frac{1}{2} \times a \times b^2$, where $a$ and $b$ represent the larger and smaller tumor diameters, respectively (14). Two days after the inoculation (day 2), the LL/2 tumor volume was $< 20 \text{mm}^3$. Mice that did not have a clear tumor mass at that time were excluded from the following experiments. For LL/2 tumors, WS particles containing KLF5-siRNA or scrambled-siRNA (50 μg/head) were administered into the tail veins daily from days 2 to 8. For PC-3 tumors, WS particles containing KLF5-siRNA or control luciferase-siRNA (150 μg/head) were administered into the tail veins daily from days 7 to 16. The sizes of PC-3 xenografts were

Figure 2. Whole-body distribution of siRNA/WS. Whole-body in vivo near-infrared imaging of Cy5-labeled siRNA administered systemically via the tail vein. A, mice bearing LL/2 tumors were injected with saline solution (left), Cy5-siRNA/saline (center), or Cy5-siRNA/WS (right). B, tumor accumulation of siRNA fluorescence was quantified based on the photon count determined from regions of interest analysis. Mean ± SE. *, $P < 0.05$ (Aspin-Welch test). C, long-term retention of Cy5-siRNA/WS (top) and Cy5-siRNA/saline (bottom) by PC-3 tumors. Whole-body in vivo near-infrared imaging of mice bearing PC-3 tumors.
followed until they reached 1,000 mm³, which was the ethically allowable criterion for sacrifice (n = 10 in each group). This criterion was selected to comply with institutional animal care committee protocols, as PC-3 xenografts were regarded as lethal when the tumor mass reached 1,000 mm³. For real-time PCR analysis, LL/2 tumors were collected 36 h after a single administration of siRNA. For Western analysis, LL/2 tumors were collected 24 h after a twice of daily administration of siRNA.

**Immunohistochemistry.** Tumor samples were fixed in 95% methanol and embedded in paraffin. CD31 was immunolabeled with anti-CD31 antibody (Pharmingen), after which the samples were incubated for 60 min at ambient temperature with anti-rat immunoglobulin biotinylated antibody (1:200 dilution; Dako). Immunolocalization was accomplished using a Vector Elite ABC kit (Vector Laboratories).

**Western blotting.** Cells were lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 0.1% NP-40, 0.1% sodium lauryl sulfate, 0.5% sodium deoxycholate, 0.1 mmol/L DTT, and Complete Protease Inhibitor Cocktail (Roche)], after which 10 μg aliquots of protein were subjected to SDS-PAGE as described previously (16).

**Statistical analysis.** Differences in tumor growth were analyzed using repeated-measures two-way ANOVA followed by a post hoc Tukey-Kramer test. Comparisons between multiple growths were analyzed using ANOVA followed by a post hoc Tukey-Kramer test, and comparisons between two groups were made using Student's t test. Survival among tumor-bearing mice was analyzed using the log-rank test. *P* values < 0.05 were considered significant.

**Results**

**WS improves the stability and prolongs the systemic half-life of siRNA.** For introduction of siRNA into tumors, a WS was designed with a core composed of a cationic lipid bilayer and siRNA/transfection reagent complex enveloped in a neutral lipid bilayer with PEG on the surface (Fig. 1A). We anticipated that this design would physically contain the siRNA within the particle and protect it from degradation by RNase and rapid dissociation from the particle. As expected, WS protected siRNA from RNase A–dependent degradation, and the incorporated siRNA remained in its intact double-stranded form for 24 h (Fig. 1B; Supplementary Fig. S1). In sharp contrast, unprotected siRNA was degraded within 15 min.

WS particles were designed to be 100 nm in diameter to maximize the EPR effect (9). The WS particles appeared translucent in solution, and their size distribution was Gaussian, with an average diameter of ~100 nm (Supplementary Fig. S2A and Supplementary Table 2). Moreover, in the process of generating WS particles, almost all of the added siRNA molecules are incorporated into the inside of the outer lipid bilayer independently of the siRNA sequence (data not shown).

The mean zeta potential of WS particles was 0 mV, indicating that they primarily carried a neutral surface charge (Supplementary Fig. S2B). As expected from the protective effects of WS in *vivo*, 29% of the input dosage of siRNA was detected in the systemic circulation 24 h after venous injection of siRNA/WS into mice, and the plasma half-life of the siRNA/WS was calculated to be 17.6 h. By contrast, siRNA administered in saline solution rapidly disappeared from the circulation; its initial elimination half-life was <4 min (Fig. 1C).

**WS efficiently mediates accumulation of siRNA in tumors.** We next used near-infrared whole-body imaging to analyze the distribution of Cy5-labeled siRNA after systemic administration. We first eliminated the possibility that WS would itself stabilize Cy5 dye in systemic circulation. We found that the fluorescent signal from Cy5 dye incorporated into WS particles disappeared from the systemic circulation within 10 min (Supplementary Fig. S3), confirming that WS stabilizes siRNA molecules but not Cy5.

Mice bearing LL/2 tumors in their backs were administered a single injection of Cy5-labeled siRNA/WS or naked siRNA into their tumors to analyze Cy5 fluorescence (Fig. 3A–C). As expected, the Cy5 signal (red) was observed in tumors from mice administered siRNA/saline. By contrast, in tumors from mice administered siRNA/WS, the Cy5 signal was observed in tumors outside of the blood vessels; no Cy5 signal was observed in the blood vessels. A higher-magnification image of tumor tissue from the mouse administered Cy5-siRNA/WS and shown in A. In unfixed tumor tissues, nuclei were labeled with Hoechst (green). Note that the Cy5 signal (red) came from within the cells. B, higher-magnification image of Cy5-labeled siRNA fluorescence in cells isolated from the tumor mass. Whereas cells from mice administered Cy5-siRNA/WS showed clearly increased levels of Cy5 fluorescence, cells from mice administered Cy5-siRNA/saline showed no more fluorescence than those from mice administered saline alone.
Figure 4. Effects of KLF5-siRNA/WS on tumor growth in vivo. A and B, effects of systemic administration of KLF5-siRNA/WS on LL/2 tumor growth. Mice were i.v. injected daily on days 2 to 8 (arrows) with 100 μL siRNA/WS or siRNA/saline containing 50 μg siRNA or with saline alone (n = 10 in each group). Time-dependent changes in tumor volume (A) and body weights of the tumor-bearing mice (B). C, effects of systemic administration of KLF5-siRNA/WS on PC-3 tumor growth. On days 7 to 16 (arrows), KLF5-siRNA/WS (150 μg) was i.v. injected daily into mice bearing PC-3 xenografts (n = 10 in each group). *, P < 0.05 versus saline controls on day 10 (LL/2) and day 18 (PC-3). D, Kaplan-Meier analysis of survival to ethically allowable maximal PC-3 tumor burden. The survival endpoint for this study was a tumor mass of 1,000 mm³. *, P = 0.0002 (log-rank test).

tail vein. Soon after the injection, fluorescence from both naked Cy5-siRNA and Cy5-siRNA/WS was detected throughout the animals' bodies (Fig. 2A). After 12 h, however, the naked Cy5-siRNA was mainly restricted to the kidneys and liver, and no accumulation was detected within the tumor mass. At the same time point, Cy5-siRNA/WS was still detected throughout the entire body of the mice, and accumulation within the tumor was apparent. Twenty-four hours after the injection, mice injected with naked Cy5-siRNA showed fluorescence in the liver, bladder, and kidneys, whereas fluorescence was found in a much broader area in mice injected with Cy5-siRNA/WS, and the siRNA/WS had continued to accumulate within the tumor mass (Fig. 2A). We found that the accumulation of siRNA/WS peaked 24 h after its administration and then gradually declined (Fig. 2B), although significant fluorescence continued to be detected within the tumors of siRNA/WS-injected mice 72 h after administration. Because the rapid growth of LL/2 tumors did not allow for longer observations, to further test the ability of WS to deliver siRNA to tumors and to analyze the Cy5 signal for a longer period, Cy5-siRNA/WS was injected into mice harboring s.c. tumors composed of PC-3 human prostate adenocarcinoma cells, which grow more slowly than LL/2 tumors. We detected Cy5 fluorescence from PC-3 tumors, even 11 days after the injection (Fig. 2C).

We then confirmed the selective accumulation of siRNA/WS in tumors by using radiolabeled siRNA to visualize its whole-body distribution autoradiographically. Radioactivity was mainly detected in the tumors, kidneys, and livers of mice injected with siRNA/WS (Supplementary Fig. S4), but no accumulation of radioactivity was found in the tumors of mice injected with naked siRNA.

To assess the distribution of Cy5-siRNA/WS within the tumor mass in more detail, we next examined tumors using laser confocal microscopy. siRNA was clearly detected outside of the blood vessels, within the tumor tissue, following systemic administration of WS/siRNA, whereas no siRNA signal was observed in the tumors of mice administered naked Cy5-siRNA (Fig. 3A). High-magnification images showed that fluorescence was present within the majority of cells, most of which were considered to be tumor cells, judging from their morphologies (Fig. 3B). Moreover, flow cytometric analysis confirmed that cells of the tumor mass from mice receiving Cy5-siRNA/WS contained higher levels of siRNA fluorescence than cells from the mice receiving naked Cy5-siRNA in saline (Fig. 3C).

KLF5-siRNA/WS exhibits antitumor activity. We recently showed that angiogenesis was diminished in heterozygous knockout mice of the Krippel-like zinc finger transcription factor Klf5 (Klf5+/-) using tumor implantation and hind-limb ischemia models (17). Notably, Klf5+/- mice still expressed KLF5 at levels approximately half those seen in wild-type animals, suggesting that moderate suppression of Klf5 expression is sufficient to inhibit angiogenesis. As expected, knocking down Klf5 using siRNA inhibited angiogenesis in Matrigel plug assays (Supplementary Fig. S5). To further test whether KLF5-siRNA might inhibit angiogenesis within tumors, we administered daily injections of saline solution containing either control scrambled-siRNA or KLF5-siRNA into tissues surrounding LL/2 Lewis lung carcinoma tumors implanted s.c. in the backs of mice. On histologic examination, tumors treated with KLF5-siRNA tended to have fewer blood vessels, particularly near the edge of the tumor mass (Supplementary Fig. S6), which suggests that KLF5-siRNA has the potential to inhibit angiogenesis in vivo but that passive diffusion of siRNA from surrounding tissues did not deliver sufficient numbers of molecules into the tumor mass.
We then evaluated the antitumor effects of KLF5-siRNA/WS. Beginning 2 days after inoculation of LL/2 cells, KLF5-siRNA/WS or scrambled-siRNA/WS (50 μg) was injected daily into the tail veins of mice for 7 days. Alternatively, saline solutions of KLF5-siRNA or scrambled-siRNA, or saline alone, were injected as a control. Subsequent whole-body imaging confirmed that siRNA/WS accumulated during the early stages of LL/2 tumor development (~2 mm in diameter; Supplementary Fig. S7). Moreover, only KLF5-siRNA/WS exhibited significant antitumor activity; neither scrambled-siRNA/WS nor saline containing KLF5-siRNA affected tumor growth (Fig. 4A). There were no significant differences in body weight among the mice given the various siRNA formulations (Fig. 4B), and no abnormalities of the liver, lung, or spleen were detected on examination of their gross morphology and histology (data not shown). We observed similar antitumor activity when we tested a second siRNA sequence targeting Klf5 (Supplementary Fig. S8), which lends further support the idea that tumor growth can be suppressed using RNA interference against Klf5. Similarly, KLF5-siRNA/WS also significantly inhibited the growth of PC-3 cell tumors (Fig. 4C) and prolonged the time to tumor size of 1,000 mm3 (Fig. 4D), indicating that the antitumor effects of KLF5-siRNA/WS are not limited to LL/2.

When we analyzed the acute and chronic toxicities of KLF5-siRNA/WS, we detected no signs of toxic effects on body weight or the gross morphology of the major organs and hematologic examination (Supplementary Table 1). By contrast, systemic administration of the same dose of the unprotected complex of transfection reagent (DOTAP) and siRNA was lethal (data not shown), indicating that incorporation of the molecules into WS also reduced their systemic toxicity. Only the total cholesterol level was increased (KLF5-siRNA/WS 318 mg/dL versus control 146 mg/dL) in the mice administered with 20 mg/kg KLF5-siRNA/WS once a day for 7 days. However, 4 mg/kg KLF5-siRNA/WS, which is higher than the therapeutic dose used in the present study (~2.5 mg/kg), did not increase the total cholesterol level. KLF5-siRNA/WS down-regulates Klf5 expression and inhibits tumor angiogenesis. Finally, we tested whether the antitumor effect of KLF5-siRNA/WS could be attributable to inhibition of Klf5 expression in vivo. Among all the formulations administered, only KLF5-siRNA/WS inhibited Klf5 expression within tumors at the mRNA (Fig. 5A) and protein (Fig. 5B and C) levels. Moreover, tumors from mice receiving KLF5-siRNA/WS showed substantially reduced levels of angiogenesis on day 10 after tumor implantation (Fig. 6A). The numbers of CD31+ cells were significantly lower in KLF5-siRNA/WS-treated animals than in those treated with saline solution or scrambled-siRNA/WS (Fig. 6B and C). Thus, inhibition of KLF5 expression through WS-mediated delivery of siRNA reduces tumor angiogenesis in vivo.

Discussion

It is now widely recognized that efficient intracellular delivery of siRNA to target sites in the body following systemic administration is the most important hurdle that must be cleared before there can be widespread use of siRNA in the clinic (2). The results of the present study clearly show that incorporation into WS nanoparticles can markedly stabilize siRNA in the circulation following systemic administration and enable its accumulation within tumors, where it can exert RNA interference effects.

WS was specifically designed to enable systemic delivery of siRNA. To inhibit the nonspecific interaction with the negatively charged endothelial cell membrane and plasma proteins, and to avoid entrapment by the reticuloendothelial system, the outer layer is composed of neutral phospholipids and is PEGylated. PEGylation of the outer layer was essential for accumulation of the WS particles in tumors in vivo,7 suggesting that the long half-life in

7 N. Yagi, unpublished observations.
circulation is one of the critical characteristics of WS for the overall efficacy of target gene silencing in vivo. Moreover, the outer lipid bilayer protects siRNA from degradation by enzymes in serum (Fig. 1B). Consequently, the half-life of siRNA/WS was markedly longer than that of naked siRNA (Fig. 1C). To our knowledge, WS is the first liposomal siRNA delivery system that involves enveloping siRNA with electrically neutral lipid molecules. To facilitate accumulation within tumors via the EPR effect and to suppress renal excretion and reticuloendothelial uptake, the diameter of WS was set to be 100 nm (2,18). With these properties, WS was capable of delivering siRNA to target tumors following systemic administration in vivo (Fig. 2; Supplementary Fig. S4). The siRNA delivered by WS was taken up by the tumors (Fig. 3), where it exerted RNA interference effects (Fig. 5) that inhibited tumor growth and prolonged the time-to-progression (Fig. 4). Moreover, WS suppressed adverse effects otherwise seen with complexes of cationic lipid and siRNA. The imaging methodologies used in the present study cannot differentiate between intact and degraded forms of siRNA molecules. However, the observed improvement in the stability of the intact siRNA duplex in the WS formulation in vitro and the knockdown of Klf5 within the tumor mass in vivo strongly suggest that at least a part of duplex remained active for RNA interference when delivered to the tumor using WS particles. Moreover, liquid chromatography-mass spectrometric analysis detected intact siRNA molecules in PC-3 tumors from mice receiving siRNA/WS.8

The tolerability of a delivery system is also critical for its clinical application. Mice given KLF5-siRNA/WS exhibited no signs of significant toxicity in acute and long-term toxicologic tests. By contrast, the siRNA-DOTAP complex was lethal at a dose that showed no toxicity when incorporated into WS particles. Thus, the WS formulation appears to inhibit nonspecific interactions with cellular membranes and plasma proteins, thereby suppressing the adverse effects of cationic lipids and polymers (e.g., acute embolization and immune responses; ref. 4). Moreover, WS is composed of constituents that are already being used clinically in humans, which should facilitate introduction and trial of siRNA/WS for clinical use.

In conclusion, our results show that the chemical and biological characteristics of WS provide both a longer half-life in the systemic circulation and retention within target tumors. Our finding that systemic delivery of KLF5-siRNA using WS nanoparticles efficiently inhibits tumor growth in vivo supports the idea that siRNA-containing WS can be used knock down specific genes within tumors, thereby exerting a therapeutic effect against a variety of cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 6. KLF5-siRNA/WS inhibited tumor angiogenesis. A, photomicrographs of tumor sections obtained from mice treated with saline or KLF5-siRNA/WS. Magnification, ×40 or ×200. Quantification of CD31+ cells per field in peripheral (B) and central (C) regions of tumor tissues. CD31+ cells that exhibited vessel-like structures were counted in five random fields in each tumor section (n = 5). Mean ± SE. *, P < 0.05.

8 Unpublished observations.
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