present in those with atherosclerosis are known to express MPO [48]. It was reported that GM-CSF treatment resulted in a significant amount of MPO in activated macrophages. These findings strongly suggested that GM-CSF activates both neutrophils and macrophages to aggravate inflammatory responses.

Activity of GM-CSF is mediated by the specific GM-CSF receptor. Metcalf et al. precisely examined the clearance of GM-CSF from the bloodstream and found that its uptake by the specific receptor is very high, thus only a limited percentage of GM-CSF could be detected in the blood in a very short period [49]. In the present study, splenomegaly characterized by increased numbers of neutrophils and macrophages was induced in both strains of mice, but was severer in DBA/2. A major cytokine responsible for splenomegaly would be GM-CSF and in the experiments in vitro, GM-CSF was only detected in DBA/2. Considering Metcalf's observation, this does not mean that C3H/HeN mice did not produce GM-CSF at all, rather, the quantity is not great enough to register in culture supernatant. As shown in Fig. 3, splenic cell numbers increased in both strains in response to CAWS and were maintained for more than 5 weeks. The splenomegaly would be due, at least in part to GM-CSF acting as a growth factor for both strains. It is also suggested that DBA/2 might be produced higher concentration of GM-CSF, and thus excessive GM-CSF has an additional function for example in the differentiation, maturation and activation of leukocytes, and enhanced production of IFN-γ, [50,51] resulting in augmented cytokine production.

As shown in Figs. 4 and 6, the production of IFN-γ and release of MPO by splenocytes was most intense in the mice just after the administration of CAWS. This observation would also be related to the production of GM-CSF. From the results of the experiment in vitro shown in Fig. 8, IFN-γ production was significantly enhanced in the presence of GM-CSF. Harada et al reported that IFN-γ synthesis is induced by cell–cell contact between the adherent and non-adherent populations [35,36]. In addition, this reaction did not occur in nude mice, strongly suggesting a crucial role for the mature T cell populations. In a normal host, the T lymphocyte represents the major cellular source of IFN-γ. All $CD8^+$ T cell populations and certain subsets of $CD4^+$ T cells can produce the protein in response to antigenic stimulation mediated by antigen presenting cells [50]. Frucht et al. proposed the "jump start model" for production of IFN-γ by various types of antigen presenting cells such as resting peritoneal, peritoneal exudates, alveolar, bone-marrow-derived and splenic macrophages, and both $CD8^+$ and $CD8^-$ DCs [51]. Even though the type of cell synthesizing IFN-γ in this study was not identified and should soon be clarified, IFN-γ is a key cytokine for the maturation and activation of a variety of lymphocytes, and is closely involved in the induction of arteritis.

Previously, we have shown that CBA/j mice were resistant to CAWS-induced vasculitis and the resistance is strongly related to high levels of IL-10 [33]. In the present study, we showed that GM-CSF is the factor exacerbating the arteritis. IL-10 is known to be immunosuppressive. Hashimoto et al reported that IL-10 suppressed macrophage survival promoted by GM-CSF [52]. Some other reports have shown that IL-10 competes with GM-CSF [53–55]. In pulmonary alveolar proteinosis, IL-10 inhibited the synthesis of GM-CSF [56]. All these findings strongly support the underlying mechanism of CAWS-induced arteritis.

The identification of candidate genes involved in disease susceptibility is important for understanding diseases at a molecular level. It is also important for medical treatment and public health, such as for predicting the effects of medicines and side effects, and controlling the intake of foods for preventing diabetes, hyperlipidemia and cancer. The identification of such genes in experimental animals gives indispensable information for understanding human diseases. The major histocompatilibity complex is one of the most important genes for immune responses and several autoimmune diseases are known to be associated with a specific MHC. It is well recognized that collagen-induced rheumatoid arthritis (CIA) is specifically induced in DBA/1 mice which carry H2-q [57,58]. In the case of CAWS-induced coronary arteritis, we have already found it to be induced in a variety of inbred strains, such as A/J (H2-a), C57Bl (H2-b), BALB/c (H2-d), DBA/2 (H2-d), C3H (H2-k) and DBA/1 (H2-q), with the vasculitis in DBA/2 being lethal (unpublished results). These findings strongly suggested that MHC is not the major gene for determining susceptibility to CAWS-induced vasculitis. The pathogenesis of heart failure is well known to be complex, polygenic and multifactorial, thus the identification of candidate genes is difficult. Suzuki et al. analyzed genes for murine dilated cardiomyopathy, by establishing transgenic mice that develop severe dilated cardiomyopathy due to the cardiac-specific overexpression of calsequestrin [59]. A reciprocal backcross strategy was employed using two inbred strains, DBA/2 and C57Bl/6, showing distinct differences in survival and cardiac function and identified two loci significantly linked to survival on chromosomes 2 and 3, and one locus significantly linked to cardiac function on chromosome 3.

Matsumori and co-workers have developed a murine model of viral myocarditis induced by the encephalomyocarditis (EMC) virus in which severe myocarditis, congestive heart failure and dilated cardiomyopathy occur with high incidence [60–62]. When various inbred strains were tested, myocardial lesions developed in BALB/c (48.7%), C3H/He (61.8%) and DBA/2 (66.1%). In C3H/He and DBA/2 mice, dilatation and hypertrophy of the heart accompanying myocardial lesions persisted up to the 8th month after the virus' inoculation. In contrast, no pathologic findings were noted in A/J and C57BL/6 mice. Compared with CAWS-induced vasculitis, the susceptibility to this viral infection-triggered model has a narrow strain specificity. Considering these findings, CAWS-induced vasculitis might also be regulated by a polygene.

In the present study, we have identified GM-CSF as the principal cytokine responsible for exacerbating coronary

arteritis. The arteritis induced by CAWS in DBA/2 mice is lethal, but not only local inflammatory reactions but also systemic inflammation would be important. We have already described the biological activities of CAWS including, activation of complement, down-regulation of thrombomoduline synthesis, activation of neutrophils and macrophages, and so on [30,33]. The severity of arteritis might be regulated by each of these factors and related modulators. Research is in progress to clarify the molecular mechanism.

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Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen *in vivo*

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Silencing of gene expression by small interfering RNAs (siRNAs) is rapidly becoming a powerful tool for genetic analysis and represents a potential strategy for therapeutic product development. However, there are no reports of systemic delivery for siRNAs toward treatment of bone-metastatic cancer. Accordingly, we report here that i.v. injection of GL3 luciferase siRNA complexed with atelocollagen showed effective reduction of luciferase expression from bone-metastatic prostate tumor cells developed in mouse thorax, jaws, and/or legs. We also show that the siRNA/atelocollagen complex can be efficiently delivered to tumors 24 h after injection and can exist intact at least for 3 days. Furthermore, atelocollagen-mediated systemic administration of siRNAs such as enhancer of zeste homolog 2 and phosphoinositide 3′-hydroxykinase p110-α-subunit, which were selected as candidate targets for inhibition of bone metastasis, resulted in an efficient inhibition of metastatic tumor growth in bone tissues. In addition, up-regulation of serum IL-12 and IFN-α levels was not associated with the *in vivo* administration of the siRNA/atelocollagen complex. Thus, for treatment of bone metastasis of prostate cancer, an atelocollagen-mediated systemic delivery method could be a reliable and safe approach to the achievement of maximal function of siRNA.

bone metastasis | prostate cancer

RNA interference (RNAi) induced by small interfering RNA (siRNA) has recently emerged as a powerful technique that is capable of suppressing expression of individual genes with a high degree of specificity (1). The technique has been used for studies of gene function *in vivo*, primarily in mice. The first demonstration of RNAi-mediated repression in an adult animal showed effective repression of a luciferase reporter gene after hydrodynamic transfection of siRNA expression plasmids into mouse liver (2, 3). Subsequent studies have delivered siRNA by various methods, including viral vector-mediated delivery (4, 5) and lipid-based delivery (6, 7). A more recent study showed that chemically modified siRNAs can silence an endogenous gene after i.v. injection in mice (8). These findings provide hope for using RNAi technology in disease control.

Many studies have used siRNAs as an experimental tool to dissect the cellular pathways that lead to uncontrolled cell proliferation and cancer. To develop siRNAs for cancer therapy, several researchers have investigated them in animal models (9–13). However, reports of RNAi-delivery strategies for bone-metastatic cancer are very limited. For example, in advanced prostate cancer, the sites most frequently affected by metastasis are the bones and regional lymph nodes. Patients with these metastases suffer pain and low limb edema, making it extremely important to explore avenues of treating such bone metastases.

We previously demonstrated the efficacy of atelocollagen for delivery of nucleotides, such as plasmid DNA and antisense oligonucleotides, *in vitro* and *in vivo* (14–19). Recently, we also reported that atelocollagen complexed with siRNA is resistant to nucleases and is efficiently transduced into cells, thereby allowing long-term gene silencing (20). Furthermore, intratumor injection of atelocollagen complexed with siRNA against fibroblast growth factor 4 mRNA showed efficient inhibition of tumor growth in an orthotopic xenograft model of a human nonseminomatous germ cell tumor (20). Another group reported that radiolabeled siRNA mixed with atelocollagen existed in the tumors for at least a week and remained intact and that the vascular endothelial growth factor siRNA with atelocollagen dramatically suppressed tumor angiogenesis and tumor growth in a PC-3 s.c. xenograft model (21). Thus, for local administration of siRNA, an atelocollagen-based nonviral delivery method could be a reliable approach to achieve the maximal function of siRNA *in vivo*. In addition, an atelocollagen complex can be delivered for i.v. injection as nanoparticles, making systemic delivery of siRNA possible. A recent report showed the potential for atelocollagen-mediated systemic antisense therapeutics for treating inflammatory disease (19).

In this study, noninvasive optical imaging technologies were used to facilitate the detection of metastatic lesions and the effects of synthetic siRNAs on tumor regression. The results indicate that systemic administration of atelocollagen complexed with siRNA into a mouse model of bone metastasis demonstrated effective gene silencing and tumor regression in bone-metastatic lesions. Furthermore, we also showed that atelocollagen-mediated systemic delivery of siRNA did not cause any side effects. Thus, systemic delivery of a siRNA/atelocollagen complex may have therapeutic potential in the treatment of advanced prostate cancer with bone metastasis.

Materials and Methods

Atelocollagen. Atelocollagen is a highly purified type I collagen of calf dermis with pepsin treatment (Koken, Tokyo). A collagen molecule has an amino acid sequence called a telopeptide at both N and C termini, which confers most of the collagen's antigenicity. Atelocollagen obtained by pepsin treatment is low in immunogenicity because it is free from telopeptides (22), and it is used clinically for a wide range of purposes, including wound healing and vessel prosthesis and as a bone cartilage substitute and haemostatic agent (16).

Cell Lines. The bioluminescent human prostate carcinoma cell line PC-3M-luc-C6 (Xenogen, Alameda, CA) was cultured in Eagle's minimum essential medium (Invitrogen) supplemented

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Abbreviations: siRNA, small interfering RNA; RNAi, RNA interference; EZH2, zeste homolog 2; p110-α, phosphoinositide 3′-hydroxykinase p110-α-subunit.

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with 10% heat-inactivated FBS (Equitech-Bio, Kerrville, TX), nonessential amino acids (Sigma-Aldrich), L-glutamine (ICN), 1 mM sodium pyruvate (Sigma-Aldrich), MEM vitamin solution (Sigma-Aldrich), and 200 μg/ml zeocin (Invitrogen). The cells were maintained *in vitro* at 37°C in a humidified atmosphere of 5% CO_2.

siRNA Preparation. Synthetic 21-nt RNAs were purchased from Dharmacon Research (Lafayette, CO) in deprotected, desalted, and annealed form. The sequence for GL3 siRNA is reported in ref. 23. The sequence of human enhancer of zeste homolog 2 (EZH2) siRNA was 5′-GGA AAG AAC GGA AAU CUU AdTdT-3′ and 3′-dTdTC CUU UCU UGC CUU UAG AAU-5′, and human phosphoinositide 3′-hydroxykinase p110-α-subunit (p110-α) siRNA was 5′-GGU UAA AGA UCC AGA AGU AdTdT-3′ and 3′-dTdTC CAA UUU CUA GGU CUU CAU-5′. The nonspecific control siRNA duplex was also purchased from Dharmacon Research.

***In Vivo* Imaging of siRNA Delivery in Mice with Bone-Metastatic Tumors.** Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research at the National Cancer Center Research Institute. Eight- to 9-week-old male athymic nude mice (CLEA Japan, Osaka) were anesthetized by exposure to 1–3% isoflurane on day 0 and subsequent days. On day 0 of the experiments, to generate an experimental metastasis model, the anesthetized animals were injected with 3×10^6 PC-3M-luc-C6 cells suspended in 100 μl of sterile Dulbecco's PBS into the left heart ventricle (24, 25). For *in vivo* imaging, the mice were administered D-luciferin (150 mg/kg, Promega) by i.p. injection. Ten minutes later, photons from animal whole bodies were counted by using the IVIS imaging system (Xenogen) according to the manufacturer's instructions. Data were analyzed by using LIVINGIMAGE 2.50 software (Xenogen). A successful intracardiac injection was indicated by day 0 images that showed a systemic bioluminescence distributed throughout the animal, and only those mice evidencing a satisfactory injection were continued in the experiment. The development of subsequent metastasis was monitored twice a week *in vivo* by bioluminescent imaging.

For preparing the siRNA/atelocollagen complex, equal volumes of atelocollagen (0.1% in PBS at pH 7.4) and siRNA solution were combined and mixed by rotating for 20 min at 4°C. The final concentration of atelocollagen was 0.05%. Four weeks after tumor injection, individual mice (from cohorts containing five animals) were injected with 200 μl of atelocollagen containing 25 μg of luciferase GL3 siRNA, atelocollagen alone, siRNA alone, or nonspecific siRNA/atelocollagen by i.v. tail vein injection. Tumor growth was not affected by these treatments. To control for mouse-to-mouse variability, the bioluminescence ratio for each mouse was normalized by dividing by the 1-day-posttreatment/pretreatment ratio of luciferase intensity for that mouse.

Detection of siRNA in Tumor Tissues or Normal Tissues by RNase Protection Assay. To show siRNA delivery in tumor tissues, 10-week-old male athymic nude mice were inoculated s.c. with 3×10^6 PC-3M-luc-C6 cells suspended in 50 μl of sterile Dulbecco's PBS. After 8 days, when a tumor reached a volume of 50–100 mm^3, tumor-bearing mice (from cohorts containing three animals) were injected with 200 μl of 0.05% atelocollagen containing 25 μg of luciferase GL3 siRNA, atelocollagen alone, or siRNA alone by i.v. tail vein injection. The mice were killed 1 and 3 days after treatment of siRNA/atelocollagen complexes, and total RNA was extracted from a tumor and selected mice tissues by using ISOGEN (Nippon Gene, Tokyo). The RNase protection probe was made with a mirVana microRNA Probe Construction Kit (Ambion, Austin, TX). The cRNA probe specific for the antisense strand of GL3 siRNA was generated by using T7 RNA polymerase and ^{32}P-labeled UTP. Total RNAs were used in an RNase protection assay using the mirVana miRNA Detection Kit (Ambion) per the manufacturer's protocol. Protected fragments were separated by electrophoresis in 15% polyacrylamide 8 M urea gels. The gels were exposed to x-ray films for 30 min, and the films were then scanned and analyzed by using NIH IMAGE software. GL3 siRNA levels were corrected for wet tissue weights.

Atelocollagen-Mediated siRNA Transfection and Tumor Growth Assay *in Vitro*. The EZH2 siRNA or p110-α siRNA complexed with atelocollagen (final concentration = 0.008%) was prefixed to a six-well plate (37.5 pmol of siRNA/250 μl per well) according to the method described in refs. 15 and 20. The cultured PC-3M-luc-C6 cells were plated into the complex-prefixed plate at 5×10^4 cells per well. Bioluminescence from PC-3M-luc-C6 cells highly correlated to the total number of cells (26). For monitoring the inhibition of cell growth, the cells were lysed ($n = 3$) on days 2, 4, and 6 and then analyzed for luciferase activity (Bright-Glo Luciferase Assay System, Promega). Inhibition of luciferase production was normalized to the level of vehicle-treated cells.

Quantitative RT-PCR. Total RNA was extracted from PC-3M-luc-C6 cells by using ISOGEN and treated with DNase I (Takara Shuzo, Otsu, Japan). Five micrograms of total RNA was used to produce cDNAs with oligo(dT) 12 primer by superscript III RNA polymerase (Invitrogen). cDNA was diluted 5-fold and used for quantitative PCR. For quantitation, aliquots of 5 μl of cDNA samples were subjected to quantitative PCR in 50-μl reactions by using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and Assays-on-Demand TaqMan primers/probe sets (Applied Biosystems) specific for human EZH2, p110-α, and GAPDH. Reactions were carried out by using the Applied Biosystems PRISM 7700 Sequence Detection System. The reactions were incubated at 50°C for 2 min and then heated to 95°C for 2 min, followed by 45 cycles of 30 s at 95°C, 15 s at 60°C, and 20 s at 72°C. Human EZH2 and p110-α expression levels were normalized to GAPDH levels.

Analysis of siRNA/Atelocollagen Treatment for Bone-Metastatic Prostate Cancer. Mice were inoculated with PC-3M-luc-C6 cells into the left cardiac ventricle on day 0 as described above. The EZH2, p110-α, and nonspecific control siRNA (50 μg) with or without 0.05% atelocollagen in a 200-μl volume were injected into the mouse tail vein on days 3, 6, and 9 postinoculation. Each experimental condition included eight animals per group. The development of subsequent metastasis was monitored twice a week *in vivo* by bioluminescent imaging for 4 weeks. To control for mouse-to-mouse variability, the bioluminescence ratio for each mouse was normalized by dividing by the before/after treatment ratio of luciferase intensity for that mouse. At the end of the experiment on day 28, to confirm the presence of neoplastic cells, selected tissues were excised from the mice at necropsy. Tissues were fixed in 4% formaldehyde-PBS(−), embedded in paraffin, cut into 5-mm sections, and stained with hematoxylin/eosin.

Monitoring of IFN Induction in Mice Treated with Atelocollagen-Mediated siRNA. Eight-week-old male athymic nude mice were injected with nonspecific control siRNA (50 μg) with 0.05% atelocollagen in a 200-μl volume by i.v. tail vein injection. Each experimental condition included four animals per group. The positive control group was injected with poly(I:C) (Amersham Pharmacia Biosciences). To measure serum cytokine levels, blood was harvested from mice 2 h after injection by cardiac puncture.

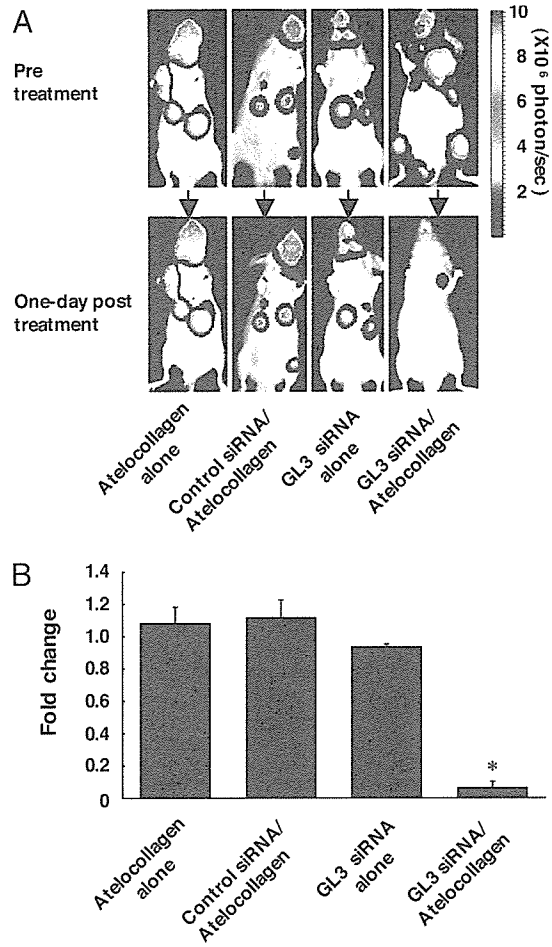

Fig. 1. Monitoring luciferase inhibition *in vivo* with bioluminescent imaging. (*A*) Representative images of nude mice injected with 3×10^6 PC-3M-luc-C6 cells suspended in 100 μl of sterile Dulbecco's PBS into the left ventricle of the heart. Four weeks after tumor injection, each animal was administered i.v. with 200 μl of 0.05% atelocollagen solution, 25 μg of luciferase GL3 siRNA, GL3 siRNA/atelocollagen complex, or nonspecific siRNA/atelocollagen complex. (*B*) Normalized fold change (1 day posttreatment/pretreatment) of bioluminescence emitted from whole body of mice. Data represent the mean ± SD ($n = 4$). *, $P < 0.001$ versus other experimental groups.

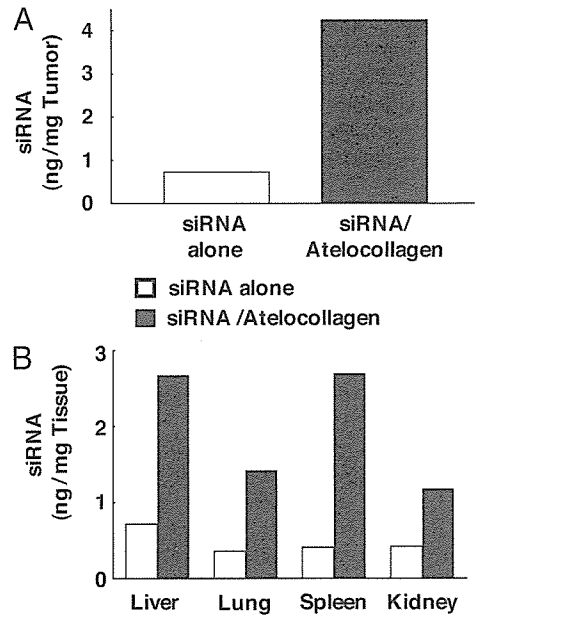

Fig. 2. Distribution of siRNA delivered with atelocollagen in tumor tissues and normal tissues. The nude mice were inoculated s.c. with 3×10^6 PC-3M-luc-C6 cells. Once tumors had reached 50–100 mm^3, tumor-bearing mice were injected with 200 μl of 0.05% atelocollagen containing 25 μg of luciferase GL3 siRNA or siRNA alone by i.v. tail vein injection. The mice were killed 1 day after treatment with siRNA/atelocollagen complexes, and total RNA was extracted from a tumor (*A*) and selected tissues (*B*). Detection of luciferase GL3 siRNA was performed by RNase protection assay. GL3 siRNA levels were corrected for wet tissue weights.

IL-12 (p40) and IFN-α levels (R & D Systems) were measured by ELISA according to the manufacturer's instructions.

Statistical Analysis. The results are given as mean ± SD. Statistical analysis was conducted by using the analysis of variance with the Bonferroni correction for multiple comparisons. $P \leq 0.05$ was considered a significant difference.

Results

Efficient Delivery of Atelocollagen-Mediated Luciferase siRNA in Bone-Metastatic Regions. To increase the potential for bone metastasis from PC-3M-luc-C6 cells, we injected the cells into the left ventricle of the heart (25). Mice with successful intracardiac injection of PC-3M-luc-C6 cells on day 0 were imaged twice a week for up to 4 weeks. In all mice, early indications of metastasis to various tissues were observed within 1 week after cell injection (data not shown). Four weeks after tumor injection, the observed patterns of metastasis indicated lesions developing in the thorax, jaws, and/or legs of the mice (Fig. 1*A*). To test whether atelocollagen-mediated siRNA systemic delivery is valid for a gene silencing effect on the metastatic sites, the animals were treated with atelocollagen alone, a nonspecific control siRNA/atelocollagen complex, a luciferase GL3 siRNA alone, or a luciferase GL3 siRNA/atelocollagen complex i.v. In mice receiving the luciferase siRNA/atelocollagen complex, bioluminescence was inhibited by 80–90% in the whole body, including the bone metastases, when compared with before treatment (Fig. 1). In contrast, the bioluminescent signals of most of the metastatic sites in the mice treated with atelocollagen alone or the control siRNA/atelocollagen complex had increased. Treatment with luciferase siRNA alone either had no effect or slightly suppressed photon emission from the tumor cells. After the imaging analysis, tissues expressing bioluminescence were excised from the mice at necropsy. Subsequent histopathology analysis confirmed micrometastases in the lung, dental pulp, tibia, femur, and other soft tissues (data not shown). Thus, our results indicate that siRNA can be delivered by using atelocollagen and can thereby inhibit gene expression in a specific manner in metastatic sites, including bone metastases.

Enhanced Delivery of siRNA into Tumors by Atelocollagen. The efficacy of delivery of siRNA into tumors was evaluated. Athymic nude mice were inoculated s.c. with 3×10^6 PC-3M-luc-C6 cells and injected i.v. with luciferase siRNA/atelocollagen, luciferase siRNA alone, or atelocollagen alone. We assessed the delivery of siRNA 1 day after the i.v. administration. As shown in Fig. 2*A* and also in Fig. 7, which is published as supporting information on the PNAS web site, a significant amount of siRNA was detected in tumors with atelocollagen-mediated delivery (4.3 ng of siRNA/mg of tumor weight). In contrast, i.v. injection of siRNA alone (0.7 ng/mg of tumor weight) was less efficient compared with atelocollagen-mediated delivery. We also assessed the delivery of luciferase siRNA in several tissues, such as liver, lung, spleen, and kidney. As shown in Fig. 2*B*, a relatively high amount of siRNA was detected in tissues from mice administered with the siRNA/atelocollagen complex com-

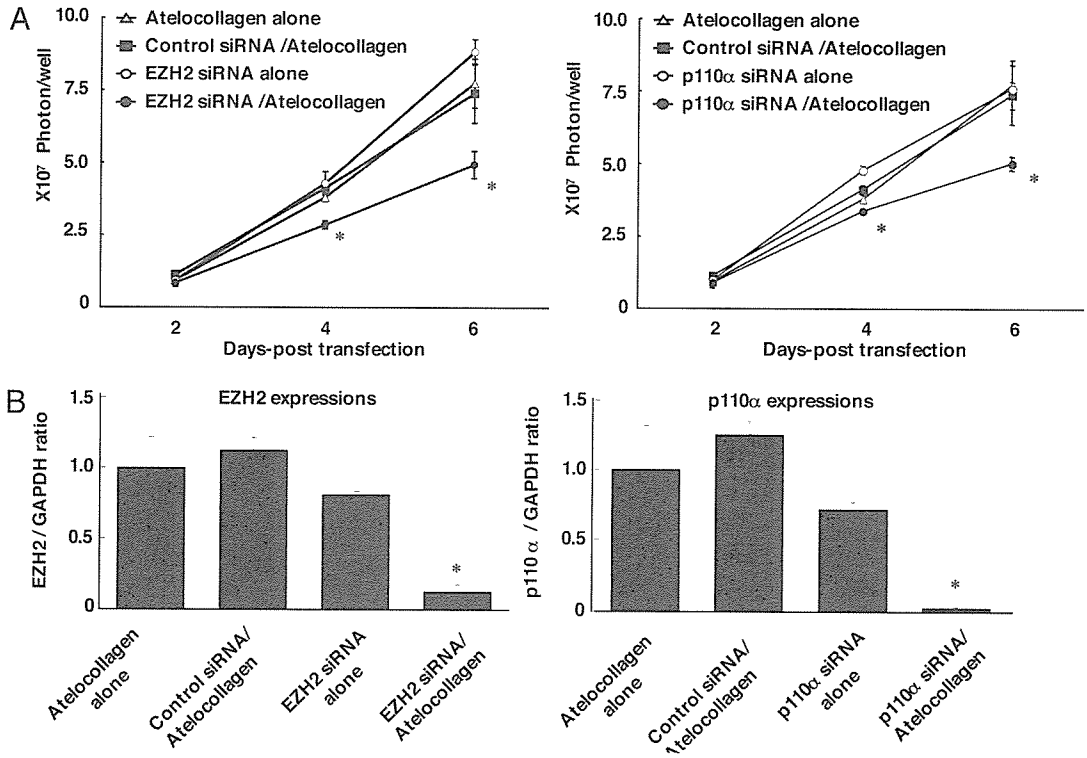

Fig. 3. The EZH2 and p110-α siRNA inhibit PC-3M-luc-C6 cell proliferation and suppress EZH2 and p110-α expression. (*A*) Inhibition of PC-3M-luc-C6 cell proliferation. For monitoring the inhibition of cell growth, cells were lysed on days 2, 4, and 6 and then analyzed for luciferase activity. (*B*) The effects of siRNA transfection on expression of EZH2 and p110-α mRNA. EZH2 and p110-α mRNA expression levels were measured by quantitative PCR. Data represent the mean ± SD ($n = 3$). *, $P < 0.05$ versus cells treated with atelocollagen alone.

pared with mice with siRNA alone. In addition, siRNA delivered with atelocollagen existed intact for at least 3 days (data not shown). Taken together, these results suggest that the systemic injection of the siRNA/atelocollagen complex allows a more efficient delivery of siRNA into tumors than siRNA alone and causes siRNA to be retained for a longer period therein.

Atelocollagen-Mediated siRNA Transfer Allows Efficient Inhibition of PC-3M-luc Cell Growth *in Vitro*. To screen target genes for showing growth inhibition of PC-3M-luc cells, EZH2 and p110-α were selected as target genes. The atelocollagen-mediated siRNA reverse cell transfection method was used. The cultured PC-3M-luc-C6 cells were plated into a siRNA/atelocollagen complex-prefixed plate. For monitoring cell growth, we analyzed luciferase activity. Inhibition of cell growth was observed on PC-3M-luc cells treated with EZH2 and p110-α siRNA/atelocollagen complexes (Fig. 3*A*). Inhibition of mRNA levels of targets was also shown (Fig. 3*B*). These results revealed that EZH2 and p110-α may be the target of inhibition of the metastasis of PC-3M-luc cells.

Inhibition of Metastatic Tumor Growth in Bone Tissues in Animals with Atelocollagen Complex. To assess the inhibition of bone metastasis by the atelocollagen-mediated siRNA delivery system, EZH2 and p110-α siRNA/atelocollagen complexes were administered i.v. into mice on days 3, 6, and 9 of postintracardiac ventricle injection of PC-3M-luc cells. The development of bone metastasis was monitored *in vivo* by bioluminescent imaging. At the end of the experiment on day 28, mice treated with atelocollagen alone and the control siRNA/atelocollagen complex-treated group showed high metastasis in the thorax, jaws, and/or legs (Figs. 4*A* and 5*A*). Total luminescence from all tumors was determined at different times posttreatment for each mouse. As seen in Fig. 4*B*, there was an increase in luminescence in mice treated with atelocollagen alone, the control siRNA/atelocollagen complex, EZH2 siRNA alone, and p110-α siRNA alone, whereas the EZH2 siRNA/atelocollagen-treated and p110-α siRNA/atelocollagen-treated groups had no increase in luminescence during the same observation period. There were significant differences between the EZH2- and p110-α siRNA/atelocollagen-treated groups and the other three experimental groups on day 28 ($P < 0.05$). Histopathological analysis revealed that metastasis of PC-3M-luc-C6 cells in the dental pulp was significantly inhibited by the EZH2 and p110-α siRNA/atelocollagen complexes (Fig. 5*B*). Therefore, the atelocollagen-mediated systemic delivery of siRNA could be a unique strategy for inhibition of bone-metastatic prostate tumor growth *in vivo*.

Absence of IFN Response to Atelocollagen-Mediated siRNA Delivery System. To test whether the atelocollagen-mediated siRNA systemic delivery has the possibility of inducing IFN responses in mice, the plasma levels of IFN-α and IL-12 in mice exposed to the siRNA/atelocollagen complex or poly(I:C) by i.v. injection were measured by ELISA (Fig. 6). As observed with IFN-α and IL-12, the siRNA/atelocollagen complex failed to elicit IFN-α and IL-12 responses, whereas poly(I:C) induced a strong response. These results show that it is possible to administer a siRNA/atelocollagen complex without inducing nonspecific turning on of genes, leading to an immune response.

Discussion

Our findings indicate that siRNA can be delivered to bone-metastatic lesions by atelocollagen-mediated systemic injection. Furthermore, we also showed that an atelocollagen-mediated siRNA delivery system can be used to silence endogenous genes involved in metastatic tumor cell growth.

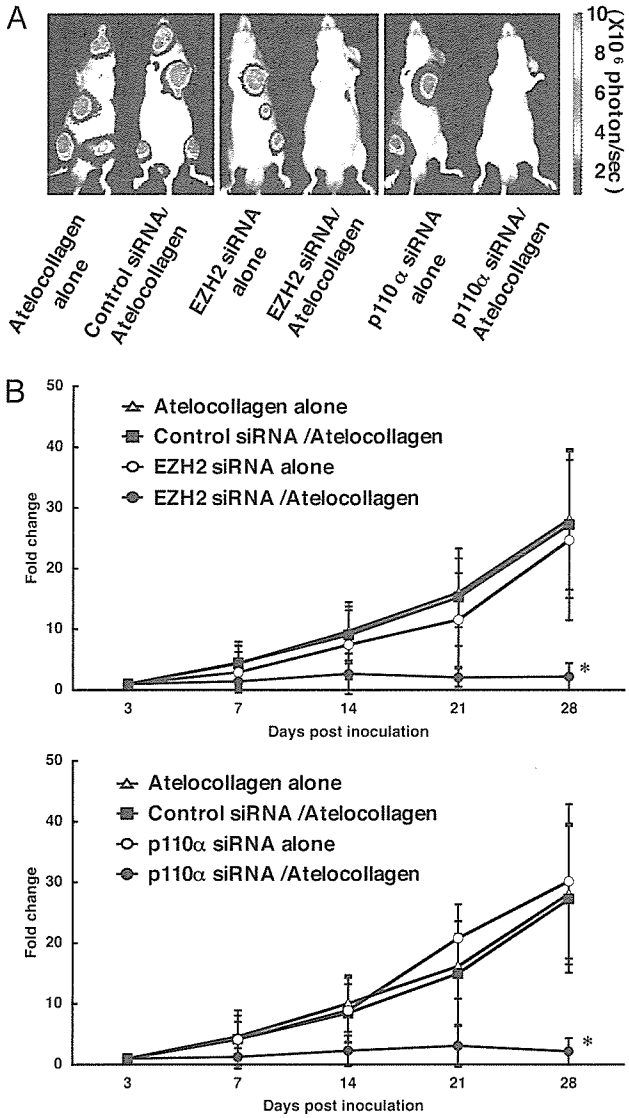

Fig. 4. Inhibition of metastatic tumor growth in bone tissues by the atelocollagen-mediated siRNA delivery system. Mice were inoculated with PC-3M-luc-C6 cells into the left cardiac ventricle on day 0. The EZH2, p110-α, and nonspecific control siRNAs (50 μg) with or without 0.05% atelocollagen in a 200-μl volume were injected into the mouse tail vein on days 3, 6, and 9 postinoculation. Each experimental regimen comprised eight animals. (*A*) Representative images of nude mice at the end of the experiment on day 28. (*B*) Normalized fold change (posttreatment/pretreatment) of bioluminescence emitted from whole body of mice. Data represent the mean ± SD (n = 8). *, $P < 0.05$ versus other experimental groups.

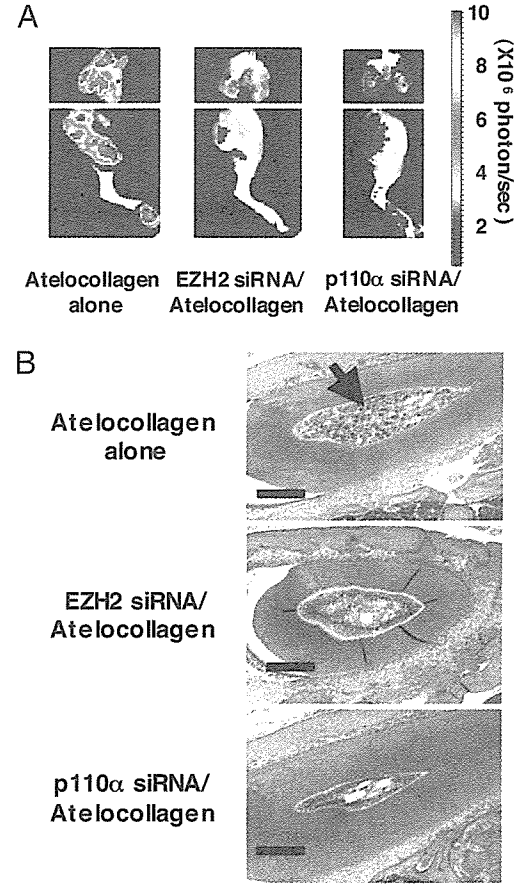

Fig. 5. Confirmation of prostate cancer bone metastasis by *ex vivo* imaging and histopathology. (*A*) The isolated organs from mice (Fig. 4) were reimaged. The bioluminescent signals were detected from the bone region in the jaw and leg of mice treated with atelocollagen alone, EZH2 siRNA/atelocollagen, and p110-α siRNA/atelocollagen, respectively. (*B*) Hematoxylin/eosin-stained sections of the dental pulp from the same mice that were imaged in Fig. 4. Arrow marks the carcinomatous micrometastasis. (Scale bars, 100 μm.)

At present, the main obstacle to the development of therapeutic products using RNAi technologies is a suitable delivery method. Viral delivery systems are efficient but cause concerns over serious side effects (27). Cationic lipid complexes also can be effective siRNA delivery agents (6). However, lipid delivery of synthetic siRNAs can reportedly induce immune activation *in vivo* (28). An important consideration for siRNA-mediated inhibition of gene expression is whether the observed effects are specific and not due to nonspecific "off-target" effects (29) and are free from potential IFN responses (30). Heidel *et al.* (31) showed that it is possible to administer naked, synthetic siRNAs to mice and down-regulate an endogenous or exogenous target without inducing an IFN response. In our experiments, in agreement with Heidel *et al.* (31), injection of siRNA/atelocollagen did not induce an IFN response or IL-12. Therefore, our atelocollagen-based siRNA delivery method can be varied to minimize the potential for an off-target effect of siRNA.

Successful application of RNAi as a therapeutic method requires an efficient and suitable delivery system that can target a restricted cell population *in vivo*. The prolonged circulation time of high-molecular-weight macromolecules enables them to use the vascular abnormalities of solid tumor tissues, a phenomenon called the enhanced permeability and retention (EPR) effect (32, 33). This EPR effect is attributed to anatomical and pathophysiological alterations such as increased vascular density due to neoangiogenesis, impaired lymphatic recovery, and lack of smooth muscle layer in solid tumor vessels. The EPR effect facilitates extravasation of polymeric drugs more selectively at tumor tissues, and this selective targeting to solid tumor tissues may lead to superior therapeutic benefits with fewer systemic adverse effects. In our experiments, siRNA/atelocollagen complexes showed greater selective accumulation in tumor tissues, compared with normal tissues, possibly due to an EPR mechanism. Although further analysis is required, our atelocollagen-mediated siRNA delivery method could possess the potential for selective targeting to tumor tissues.

The major risk faced by patients with prostate cancer is the development of metastatic disease. Although genes associated with metastatic prostate cancer can be identified readily by

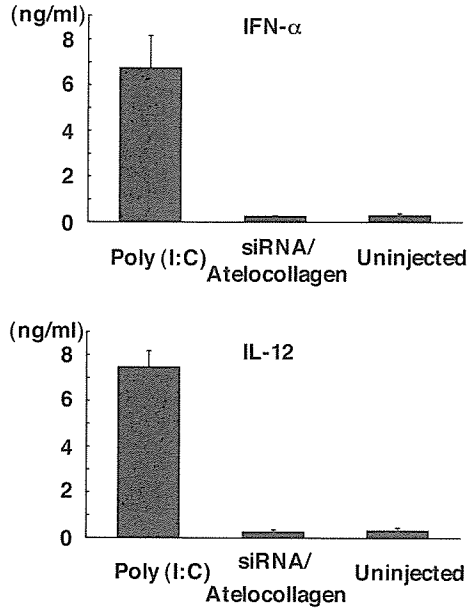

Fig. 6. Absence of IFN response to the atelocollagen-mediated siRNA delivery system. Nude mice were injected with nonspecific control siRNA (50 μg) with 0.05% atelocollagen in a 200-μl volume by i.v. tail vein injection. The positive control group was injected with poly(I:C). Serum was collected 2 h postinjection; IFN-α and IL-12 levels were determined by ELISA. Data represent the mean ± SD (n = 4).

screening techniques [e.g., gene arrays (34)], the validation and characterization of these genes will require sophisticated animal models and gene delivery systems. Expression microarray studies identified EZH2 as a gene overexpressed in hormone-refractory metastatic prostate cancer, and it has been found that patients with clinically localized prostate cancers that express EZH2 have a worse prognosis than those who do not express the protein (34). In addition, knocking down the EZH2 protein in PC3 invasive prostate cancer cells by using RNAi technology inhibited proliferation of cells *in vitro*. In contrast, catalytic subunits of the phosphatidylinositol 3-kinase p110-α regulate a variety of cellular responses such as survival, proliferation, and cell migration (35). In this report, our data demonstrate that expression of EZH2 and p110-α are involved in tumor growth in metastatic osseous sites. It has been reported that EZH2 and p110-α protein are also elevated in breast cancer (36, 37). Therefore, EZH2 and p110-α siRNA/atelocollagen complexes may also have therapeutic potential for inhibiting the growth of breast cancer in bone-metastatic sites. Although EZH2 and p110-α siRNA efficiently inhibited proliferation of PC-3M-luc cells, further work will be required to develop a siRNA therapy that induces the cytocidal effect specific to prostate cancer cells.

In conclusion, we have developed a technique to efficiently and safely deliver siRNA to a bone-metastatic lesion by an atelocollagen-mediated systemic injection and demonstrated specific inhibition of target gene expression. To our knowledge, our results present the first evidence that gene silencing by means of systemic delivery of siRNA/atelocollagen complexes may have therapeutic potential in the treatment of advanced prostate cancer with bone metastasis.

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Review Article

Therapeutic potential of RNA interference against cancer

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One of the most dramatic events of the past 5 years in the field of molecular biology has been the discovery of RNA interference (RNAi). Although RNAi is an evolutionarily conserved phenomenon for sequence-specific gene silencing in mammalian cells, exogenous small interfering RNA (siRNA) and vector-based short hairpin RNA (shRNA) can also invoke RNAi responses. Both are now not only experimental tools for analyzing gene function but are expected to be excellent avenues for drug target discovery and the emerging class of gene medicine for targeting incurable diseases such as cancer. The success of cancer therapeutic use of RNAi relies on the development of safe and efficacious delivery systems that introduce siRNA and shRNA expression vectors into target tumor cells. For their delivery, a variety of strategies have been used, most of them based on traditional gene therapy delivery systems. In this review, we present siRNA delivery method strategies and discuss the potential of RNAi-based gene therapy in cancer treatment. (*Cancer Sci* 2006; 97: 689–696)

The completion of the sequencing of the human genome in 2003 and intense studies on genes that mediate cancer progression and therapeutic resistance have identified many therapeutic gene targets that regulate apoptosis, proliferation and cell signaling. Molecules that can inhibit expression of such genes are powerful tools in cancer research. Previous efforts focused on sequence-specific gene suppression strategies involving antisense oligonucleotides (AS-ODN) and ribozymes. These methodologies are still pursued, but adapting them as broadly applicable functional genomic and therapeutic tools has proven difficult.

Recently a novel strategy of gene silencing using RNA interference (RNAi) has been discovered. RNAi has rapidly become a powerful tool for drug discovery and target validation in cell culture, and now has largely displaced previous efforts involving AS-ODN and ribozymes.

The initial evidence of RNAi in nature came from work in petunia flowers in which overexpression of the gene responsible for purple pigmentation actually caused the flowers to lose their endogenous color.[1] This phenomenon was named 'cosuppression', because both the transgene and the endogenous gene were suppressed. It was also known that gene silencing occurred post-transcriptionally because transcripts from both genes were produced but were then degraded rapidly in the cytosol, hence the term 'post-transcriptional gene silencing'. RNAi was first described in animal cells by Fire and colleagues in the nematode *Caenorhabditis elegans* as a naturally occurring cellular mechanism that induces post-transcriptional gene silencing, in which double-stranded RNA (dsRNA) suppresses the expression of a target gene by triggering specific degradation of the complementary mRNA sequence.[2] The natural role of RNAi is thought to be that of a cellular defense against viral infection or potentially harmful destabilizing genomic intruders such as transposons. RNAi can also be induced in mammalian cells by the introduction of synthetic small interfering RNA (siRNA) 21–23 base pairs in length[3] or by plasmid[4–6] and viral vector systems[7] that express short hairpin RNAs (shRNA) that are subsequently processed to siRNA by the cellular machinery. The attractiveness of RNAi in contrast to other methods of manipulation arises from its extremely high inhibitory activity and the fact that the inhibition is very specific. RNAi has become the tool of choice to analyze the loss-of-function of individual genes and has been exploited to identify complex regulatory pathways following genomic screening, and is currently the most widely used gene-silencing technique in functional genomics. Furthermore, as many diseases are rooted in the inappropriate activity of specific genes, RNAi has been heralded as a great therapeutic intervention for gene medicine against a wide range of human diseases, such as infection, respiratory disease and cancer. The pace of siRNA-based drug development has been rapid, and some companies have already started clinical trials for an RNAi therapeutic for age-related macular degeneration (AMD). AMD is caused by the abnormal growth of blood vessels behind the retina. Treatment strategies are inhibition of the vascular endothelial growth factor pathway by siRNA. These RNAi therapeutics are designed to be administered directly to sites of disease in the eye. However, like other forms of gene medicine, the clinical utility of systemic therapeutic siRNA will depend on the development of safe and efficacious delivery systems. Strategies for the inhibition of cellular proliferation by systemic treatment of tumor-bearing animals with siRNA are beginning to emerge and are becoming suitable for evaluation of systemic delivery of siRNA as a means for cancer treatment. In this article, we will review and assess the use of RNAi delivery reagents for their potential in cancer therapeutics.

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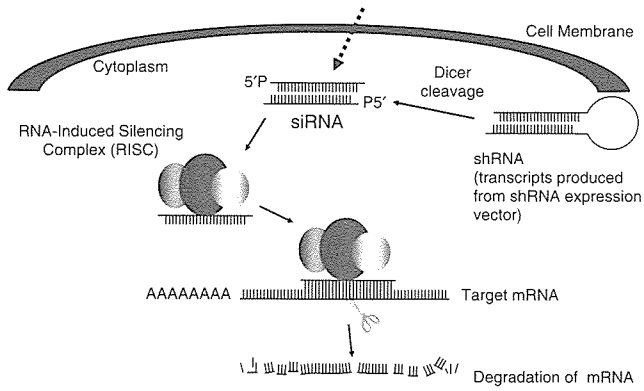

Fig. 1. Mechanisms of RNA interference. Vector-based short hairpin RNA (shRNA) and endogenous micro RNA are processed by Dicer, a multienzyme complex, into 21–23-bp small interfering RNA (siRNA) duplexes. These siRNA are incorporated into the RNA-induced silencing complex (RISC), which contains a helicase that unwinds the duplex. The antisense strand of the duplex guides the active RISC to the complementary mRNA, which is then cleaved by a nuclease. The cleaved mRNA is degraded rapidly and the protein that it encodes is not produced.

Mechanism of RNA interference

RNA interference is a conserved biological process among multicellular organisms as diverse as plants, worms, yeast and humans, in which dsRNA suppresses the expression of a target gene by triggering specific degradation of the complementary mRNA sequence.[2] Whereas initial studies utilized the introduction of exogenous dsRNA, it is now clear that higher eukaryotes contain a large number of genes that encode small RNA referred to as micro RNAs (miRNA).[8] The mechanism for miRNA appears to be similar but not identical to that of siRNA. These miRNA generally have only incomplete sequence homology to their targets, often recognizing sequences in the untranslated 3′ end of a gene, and usually work by blocking the translation of mRNA into protein rather than by destroying the mRNA transcript. The naturally occurring miRNA are synthesized in the nucleus in large precursor forms, which are processed in the nucleus by Drosha, an RNase III enzyme, into pre-miRNA (60–80 nucleotides). Subsequent to transport to the cytoplasm and processing by Dicer, mature miRNA (22 nucleotides) are taken up into a multisubunit ribonucleoprotein complex called RNA-induced silencing complex (RISC). RISC incorporates the antisense strand of the unwound siRNA and defines the target region of the mRNA via sequence complementarity to promote its specific cleavage. These endogenous miRNA are proving to regulate processes such as proliferation, apoptosis and differentiation.[9] Dicer is responsible for cleaving double-stranded molecules, including those derived from vector-based shRNA (Fig. 1). The siRNA derived from vector-based shRNA and synthetic siRNA are also incorporated into the RISC, and are able to induce the sequence-specific and effective silencing of genes by mimicking the RNAi pathway. The silencing by siRNA is highly efficient,[10] presumably because the guide strand RNA is protected from degradation by RISC and can repeat the cleavage of many mRNA molecules.

Key requirements for siRNA therapeutic development

For the therapeutic use of siRNA in cancer, although the efficacy of siRNA has to be validated in animal models, evaluation in cultured cancer cells is required before any *in vivo* study. At first, candidate target genes for RNAi-mediated knockdown are identified. These genes might be selected from several key oncogenes, antiapoptotic genes or tumor promoting genes, including growth and angiogenic factors or their receptors. As a matter of course, cancer-specific genes that are ideally mutated or translocated are chosen. Because siRNA effects are extremely specific, initial *in vitro* studies have demonstrated effective silencing of a wide variety of mutated oncogenes such as *K-Ras*,[7] mutated *p53*,[11] *Her2/neu*[12] and *bcr-abl*.[13] When target genes are selected, optimal design of the siRNA sequences are required. In general, it appears that specificity can be attained depending on the position and sequence of a given siRNA. Now that many siRNA companies have developed siRNA design software, it can be downloaded by researchers who can design effective siRNA sequences more easily. Screening candidate siRNA for homology with available sequence databases can, in principle, predict and avoid many off-target effects. An off-target effect is the silencing of an unintended target gene. After two or three different sequences for a siRNA target site are synthesized, the most specific and effective siRNA sequence must be validated by measuring levels of target mRNA or protein *in vitro*. Furthermore, some functional analyses (cell morphology, proliferation, apoptosis, etc.) are required to understand the mechanism of the antitumor effect. In subsequent *in vivo* studies, the appropriate cancer model must be developed for the evaluation of siRNA effects on tumors. How to evaluate the effect of siRNA is also important. Furthermore, the key challenges for the development of siRNA as human therapeutics are largely dependent on the development of suitable delivery systems. A transition from *in vitro* success to *in vivo* systems is emerging, but further improvement remains a critical need for the application of siRNA to drug target research, and potential clinical application. In the next section, recent advances in siRNA delivery methods are discussed.

siRNA delivery into animals

The key to a successful *in vivo* application is a delivery system that transports the siRNA into the target tissue and into the cell cytoplasm, or shRNA expression cassette to the nucleus much like the dependence of gene therapy on appropriate delivery methods. Several studies have demonstrated efficient *in vivo* delivery of siRNA and its therapeutic benefit in rodents. These have been mostly based on delivery systems developed previously for plasmid DNA or AS-ODN. The first direct delivery of siRNA *in vivo* was carried out using the 'hydrodynamic' technique of administering naked siRNA in a large volume of a physiological solution under

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high pressure into the tail vein of mice.[14,15] To evaluate the efficacy of this delivery method, mice were coinjected with plasmids expressing the luciferase gene as a reporter gene along with synthetically prepared siRNA targeted to the luciferase mRNA. Suppression of luciferase expression was observed in such varied tissues as liver, spleen, lung, kidney and pancreas; observations that influenced other groups to investigate the potential of siRNA for treatment of liver failure. The *in vivo* silencing effect of siRNA directed against the gene encoding the Fas receptor was tested in murine liver for its potential to protect mice from liver failure and fibrosis in models of autoimmune hepatitis.[16] There is a unique report from another group that hydrodynamic injection of naked siRNA into a distal vein of a limb transiently isolated by tourniquet or blood pressure cuff was able to show an efficient and repeatable delivery of nucleic acids to muscle cells (myofibres) throughout the limb muscle of rats.[17] However, although effective in rodents, hydrodynamic delivery is unlikely to be applicable to human therapy. However, it was demonstrated that intranasal administration of naked siRNA targeting the organ-protecting enzyme heme oxygenase-1 led to effective gene silencing and consequently an increase in ischemia-reperfusion injury.[18] This intranasal administration may be suitable for lung-specific siRNA delivery and treatment of respiratory infections in humans.

In the case of systemic injection, tissue-targeting technologies are required. One interesting study reported that liver-targeted delivery of siRNA may be enhanced using chemical modification of the oligonucleotide, for example, with cholesterol conjugates. These conjugates are more resistant to nuclease degradation, the cholesterol attachment stabilizing the siRNA molecules in the blood by increasing binding to human serum albumin and increasing the uptake of siRNA molecules by the liver.[19]

RNAi therapeutic studies in cancer models

To develop siRNA for cancer therapy, several researchers have investigated siRNA in animal models. Studies on the inhibition of cellular proliferation by treatment of tumor-bearing animals with siRNA are summarized in Table 1. To obtain efficient and long-lived gene silencing using RNAi, several groups have incorporated the siRNA expression cassettes into a variety of viral vectors. An adenovirus, despite its disadvantage in that immunogenicity of viral vector has precluded multiple administrations and resulted in toxicity limitations, is one of the most well-known viral vectors for gene delivery. Zhang *et al.* showed that intratumoral injection of an adenovirus encoding the hypoxia-inducible factor-1 (HIF-1)-targeted siRNA had a significant effect on tumor growth when combined with ionizing radiation.[20] Although past experience with AS-ODN and ribozymes suggest that most cationic lipid (liposome) delivery systems are too toxic when used *in vivo*, some companies (e.g. Nippon Shinyaku, Kyoto, Japan) have developed novel cationic liposomes that can be administered safely *in vivo*. Yano *et al.* have used such a liposome to demonstrate that anti-bcl-2 siRNA complexed with liposome had a strong antitumor activity when administered intravenously in the mouse model of liver metastasis.[21] In addition, Nogawa *et al.* reported that intravesical injection of polo-like kinase-1 (PLK-1) siRNA/liposomes successfully prevented the growth of bladder cancer in an orthotopic mouse model.[22] One attractive method is through delivery of siRNA using cancer cell-specific antibody. Song *et al.* showed that an antibody against ErbB2 fused to a protamine fragment specifically and effectively delivers siRNA only to ErbB2-expressing breast cancer cells.[23] We recently developed an atelocollagen-mediated siRNA delivery system *in vivo*. In the next section, advances using atelocollagen-mediated gene delivery methods are introduced.

Atelocollagen

Atelocollagen was the first biomaterial with the potential for use as a carrier for gene delivery.[47] Atelocollagen is obtained from type I collagen of calf dermis by pepsin treatment.[48,49] At the N- and C-terminals of the collagen molecules is an amino acid sequence called a telopeptide that contains most of collagen's antigenicity. Atelocollagen obtained by pepsin treatment is low in immunogenicity because it is free from telopeptides, and it is used clinically for a wide range of purposes. Atelocollagen is liquid at low temperature, making admixing of nucleic acid solutions easy. Because the surface of atelocollagen molecules is positively charged, the molecules can bond electrostatically with negatively charged nucleic acid molecules. The size of the complex particles can be changed by altering the ratio of nucleic acid to atelocollagen. When the concentration of atelocollagen is high, the complex persists locally for a long time, which is advantageous for a sustained release carrier. On the other hand, if the concentration of atelocollagen is low, the diameter of the complex particles is 100–300 nm, which is considered adequate for systemic treatment. In this system, the siRNA–atelocollagen complexes also can be precoated on a microwell plate into which the cells are seeded.[45,50] Using this method, cells take up the siRNA–atelocollagen complex and siRNA exerts a gene silencing effect. One problem for systemic treatment *in vivo* is that, although most endogenous RNases are inactive against dsRNA, some serum RNases can degrade siRNA. However, siRNA complexed with atelocollagen is resistant to nucleases and is transduced efficiently into cells, thereby allowing long-term gene silencing.[45] We previously demonstrated the efficacy of atelocollagen for nucleotide delivery. Minipellets prepared with atelocollagen containing plasmid DNA encoded with fibroblast growth factor-4 (FGF-4, known as HST-1) were administered to the femoral muscle of mice. In the Minipellet-administered mice, an increase in serum FGF-4 levels remained for at least 60 days.[47] In contrast, mice injected with plasmid DNA alone showed transient high FGF-4 protein levels, with barely detectable levels at day 30 after injection. Adenovirus vectors suffer under repeated administration *in vivo*. However, it was demonstrated that a complex of adenoviral vector and atelocollagen can be used for repeated administration to animals that have neutralizing antibodies to the adenovirus.[48] The application of these delivery systems has expanded the utility of AS-ODN. To evaluate the effect of AS-ODN and atelocollagen complexes, an orthotopic xenograft model of a human non-seminomatous germ cell tumor was developed. The growth of these cancer cells is

Table 1. Delivery of small interfering RNA (siRNA) in cancer models

Carriers	Routes	Type of cancer (cell line)	Implanted site (target organ)	Target gene	Reference
Naked siRNA	i.p., i.v., s.c., i.t.	Fibrosarcoma (JT8)	s.c.	VEGF	24
Naked siRNA + gemcitabine	i.v.	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3)	Orthotopic pancreas	FAK	25
Naked siRNA	i.v.	Pancreatic adenocarcinoma (BxPC3)	s.c., Orthotopic pancreas	CEACAM6	26
Naked siRNA	i.v.	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3, Capan2)	s.c., Orthotopic pancreas, liver metastasis	EphA2	27
Naked siRNA + gemcitabine	i.v.	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3, Capan2)	s.c., Liver metastasis	RRM2	28
Naked siRNA	i.v.	Breast cancer (MDA-MB-231)	Lung metastasis	CXCR4	29
Liposome	i.p.	Colon cancer (HTC116)	s.c., i.p.	β-Catenin	30
Liposome	i.v.	Liver metastatic spleen cancer (A549)	Liver	bcl-2	21
Liposome	i.t.	Bladder cancer (UM-UC-3-LUC)	Bladder	PLK-1	22
Liposome	i.t.	Pancreatic carcinoma (Capan-1)	s.c.	Somatostatin	31
CCLA (NeoPhectin-AT)	i.v.	Prostate cancer (PC-3)	s.c.	Raf-1	32
CCLA	i.v.	Breast cancer (MDA-MB-231)	s.c.	c-*raf*	33
shRNA plasmid + pegylated immunoliposome	i.v.	Glioma (U87)	Brain	EGFR	34
PEI	i.p.	Ovarian carcinoma cells (SKOV-3)	s.c.	HER-2	35
shRNA plasmid + PEI	i.t.	Ewing's sarcoma (TC71)	s.c.	VEGF	36
Adenovirus vector	i.t.	Cervical adenocarcinoma, colon cancer (HeLa, HTC116)	s.c.	HIF-1α	20
Adenovirus vector	i.t.	Lung cancer (ACC-LC-172)	s.c.	Skp-2	37
shRNA plasmid	i.t.	Glioblastoma (SNB19)	Brain	MMP-9 + cathepsin B	38
shRNA plasmid	i.t.	Glioma (SNB19)	Brain	Cathepsin B, uPA	39
shRNA plasmid + ATA	i.v.	Cervical adenocarcinoma, lung cancer (HeLa S3, A549)	s.c.	PLK1	40
PEI-PEG-RGD	i.v.	Neuroblastoma (N2A)	s.c.	VEGF-R2	41
CDP-AD-PEG-transferrin	i.v.	Ewing's sarcoma (TC71)	Multiple organ metastasis	EWS-FLI1	42
HVJ envelope vector + cisplatin	i.t.	Cervical adenocarcinoma (HeLa)	Intradermally	Rad51	43
ErbB2-protamine fusion protein	i.t., i.v.	Melanoma (B16)	s.c.	c-*myc* MDM2 VEGF (mix)	23
Atelocollagen	i.t.	Prostate cancer (PC-3)	s.c.	VEGF	44
Atelocollagen	i.t.	Germ-cell tumor (NEC8)	Testis	FGF-4	45
Atelocollagen	i.v.	Prostate cancer (PC-3M-Luc)	Bone metastasis	EZH2, p110α	46

AD-PEG, adamantane-PEG5000; ATA, aurintricarboxylic acid, nuclease inhibitor; CCLA, cationic cardiolipin analog-based liposome; CDP, cyclodextrin-containing polycations; i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous; PEG, polyethylene glycol; PEI, polyethylenimine; RGD, Arg-Gly-Asp; s.c., subcutaneous.

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dependent on FGF-4 production. After orthotopic implantation of tumor cells, direct injection of AS-ODN against HST-1/FGF-4 and atelocollagen inhibited the growth of testicular tumors significantly as well as the incidence of lymph node metastasis.[51] Takei *et al.* demonstrated that subcutaneous injection of atelocollagen with AS-ODN against midkine suppressed cell growth of mouse rectal carcinoma cells inoculated into nude mice.[52,53] Furthermore, Hanai *et al.* reported that systemic injection of atelocollagen with AS-ODN against intracellular adhesion molecule-1 (ICAM-1) inhibited inflammation of the ear in an allergic dermatitis mouse model.[54] Thus, these data show that atelocollagen is useful for both local and systemic delivery of AS-ODN *in vivo*. In addition, Nakamura *et al.* used atelocollagen for *in vivo* conversion of the transthyretin gene by single-stranded oligonucleotides, delivered with atelocollagen, designed to promote endogenous repair of genomic DNA.[55] These results led us to expect that atelocollagen may be applicable for siRNA delivery *in vivo*.

Atelocollagen-mediated synthetic siRNA delivery *in vivo*

To test whether atelocollagen-mediated siRNA transfer is valid for gene silencing *in vivo* (Fig. 2), we used nude mice bearing luciferase-producing tumor cells. Non-invasive *in vivo* bioluminescence imaging analysis can be utilized to evaluate the efficiency of delivery of siRNA against luciferase mRNA (luc-siRNA) into tumor cells by suppression of luciferase expression and production of photons from tumor cell-inoculated mice. With this strategy, subcutaneous injection of the luc-siRNA–atelocollagen complex showed a sustained inhibition of luciferase expression from tumor cells xenografted back into mice.[45] As another group showed, radiolabeled siRNA mixed with atelocollagen existed in the tumors for at least 1 week and remained intact.[44] In the case of inhibition studies of tumor growth, intratumoral injection of a HST-1/FGF-4-siRNA–atelocollagen complex presented efficient inhibition of tumor growth in an orthotopic xenograft model of a human testicular cancer (Fig. 3).[45] Takei *et al.* reported that treatment of the vascular endothelial growth factor (VEGF)-siRNA–atelocollagen complex dramatically suppressed tumor angiogenesis and tumor growth in a PC-3 s.c. xenograft model.[44] Thus, for local administration of siRNA, an atelocollagen-based delivery method could be a reliable approach to treatment for achieving the maximal function of siRNA *in vivo*.

An atelocollagen complex also can be delivered by intravenous injection as nanoparticles, making systemic delivery of siRNA possible. In recent reports, in order to estimate the effectiveness of systemic delivery of siRNA, we prepared a mouse model of bone metastatic human prostate cancer (Figs 2 and 4).[46,56] In this model, bone metastases developing in the jaws and/or legs of the mice were detected by non-invasive *in vivo* bioluminescence imaging analysis. In mice receiving the luc-siRNA–atelocollagen complex, bioluminescence at 1 day post treatment was inhibited by 80–90% in the whole body, including the bone metastases, when compared with before treatment (Fig. 4).[46] In contrast, the bioluminescent signals from the mice treated with atelocollagen alone increased, and treatment with luciferase siRNA alone either had no effect or slightly suppressed luciferase expression. Furthermore, in order to assess the inhibition of tumor growth on bone metastasis by the atelocollagen-mediated siRNA delivery system, human enhancer of zeste homolog 2 (EZH2) and human phosphoinositide 3′-hydroxykinase p110α subunit (p110α) siRNA–atelocollagen complexes were administered intravenously into mice on days 3, 6 and 9 post injection of luciferase-producing human prostate cancer cells.[46] As a result, there was significant inhibition of tumor growth in bone tissues on EZH2 and p110α siRNA–atelocollagen-treated groups at experimental day 28. In addition, upregulation of serum interleukin-12 and interferon-α levels was not associated with systemic injection of the siRNA–atelocollagen complex. Thus, for treatment of bone metastasis of prostate cancer, a new atelocollagen-mediated systemic delivery method could be a reliable and safe approach to the achievement of maximal function of siRNA *in vivo*.

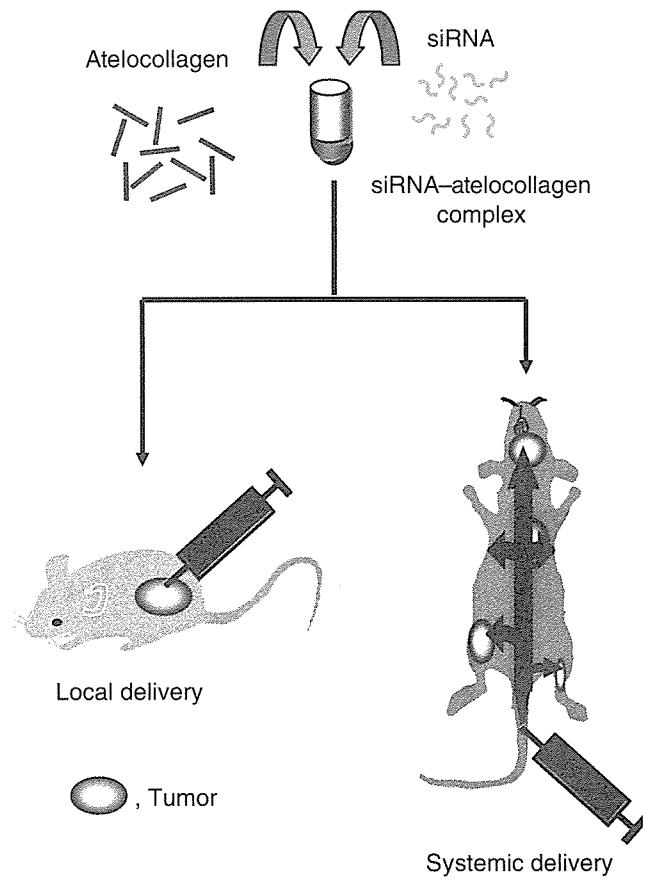

Fig. 2. Schematic representation of the atelocollagen-mediated *in vivo* delivery of small interfering RNA (siRNA). Atelocollagen is useful for both local and systemic delivery of siRNA, as the siRNA–atelocollagen complex is stable *in vivo*. For the evaluation of systemic treatment of siRNA–atelocollagen, a mouse model of bone metastatic human prostate cancer was prepared. In this model, bone metastases developing in the jaws and/or legs of the mice were detected by non-invasive *in vivo* bioluminescence imaging analysis.

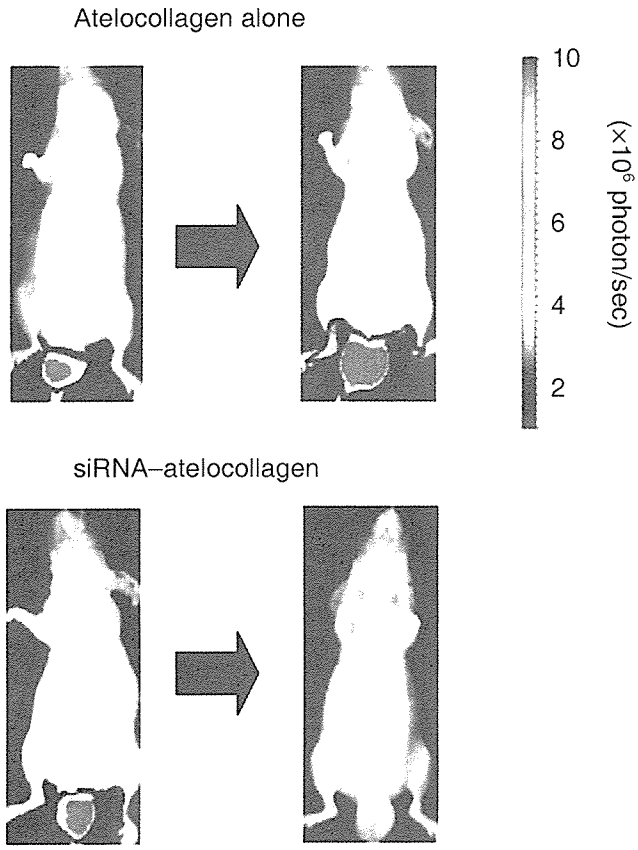

Fig. 3. Effect of the small interfering RNA (siRNA)–atelocollagen complex on the growth of a xenograft tumor. Human fibroblast growth factor-4 (FGF-4, known as HST-1) siRNA (2.5 μg) complexed with 0.5% atelocollagen (lower) or atelocollagen alone (upper) were transduced into an orthotopic germ cell tumor of NEC8 cells expressing the luciferase gene. The images on pre and 11 days post siRNA treatment are shown. The tumor volume in the mouse treated with siRNA complexed with atelocollagen was smaller than that in the control mouse treated with atelocollagen alone.

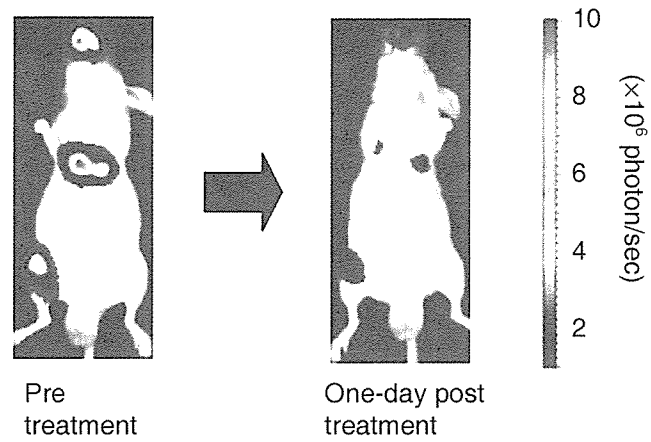

Fig. 4. Monitoring luciferase inhibition *in vivo* using bioluminescent imaging. The images of nude mice injected with 3×10^6 PC-3M-luc-C6 cells into the left ventricle of the heart. Four weeks after tumor injection, each animal was administered with luciferase GL3 small interfering mRNA (25 μg) complexed with 0.05% atelocollagen. On the next day, the bioluminescent signals of most of the metastatic sites were inhibited by 80–90% in the whole body when compared with before treatment.

Conclusions

The fact that siRNA molecules can inhibit target genes through sequence-specific cleavage of the cognate mRNA is currently serving mainly as a research tool in functional genomics and as a proof of principle for potential RNAi therapeutics. The effectiveness of RNAi reagents will improve as more is gleaned about the biology of RNAi in mammalian systems and improvements in the stability, delivery and reduction of off-target and non-specific effects are made. In particular, as with any new compound, issues of delivery, distribution and clearance are major obstacles to be overcome before siRNA can be adapted to clinical trials. Although systemic siRNA delivery imposes several requirements and greater hurdles than local siRNA delivery, diseases like cancer are considered as systemic diseases, including metastatic distribution of microdisseminated cells, and thus require systemic treatment with siRNA. In the near future the systemic delivery of siRNA will be required, possibly using a tissue-specific or cell-specific gene promoter vector or specific antibody-conjugated carriers, thus reducing applied dose of siRNA and resulting in decreased side effects. For specific targeting, angiogenesis and metastasis can be exploited for the differences between cancerous cells and normal cells, which include uncontrolled proliferation, insensitivity to negative growth regulation and antigrowth signals. There is a growing tableau of unique cancer markers brought about by recent advances in proteomics and genomics, which form the basis of key interactions between the siRNA–carrier complexes and cancer cells.

This review has summarized the salient reports of RNAi applications in preclinical xenograft models. Recognition that human xenograft models in immunodeficient mice frequently overpredict activity and underpredict toxicity is important because the target antigen is tumor-specific in the mouse but tumor-associated in patients. This issue will need more extensive and careful research to bring about a better appreciation of the effects of the dose schedule and dose intensity for siRNA treatment.

Work to resolve these problems is ongoing, and, when fully developed, the RNAi approach will hopefully replace the more toxic conventional treatment modalities and lead to better tolerated but more effective anticancer therapeutics.

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Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing *in vitro* and *in vivo*

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ABSTRACT

Silencing gene expression by siRNAs is rapidly becoming a powerful tool for the genetic analysis of mammalian cells. However, the rapid degradation of siRNA and the limited duration of its action call for an efficient delivery technology. Accordingly, we describe here that Atelocollagen complexed with siRNA is resistant to nucleases and is efficiently transduced into cells, thereby allowing long-term gene silencing. Site-specific *in vivo* administration of an anti-luciferase siRNA/Atelocollagen complex reduced luciferase expression in a xenografted tumor. Furthermore, Atelocollagen-mediated transfer of siRNA *in vivo* showed efficient inhibition of tumor growth in an orthotopic xenograft model of a human non-seminomatous germ cell tumor. Thus, for clinical applications of siRNA, an Atelocollagen-based non-viral delivery method could be a reliable approach to achieve maximal function of siRNA *in vivo*.

INTRODUCTION

RNA interference (RNAi) as a protecting mechanism against invasion of foreign genes was first described in *Caenorhabditis elegans* (1) and has subsequently been demonstrated in diverse eukaryotes, such as insects, plants, fungi and vertebrates (2). In many eukaryotes, expression of nuclear-encoded mRNA can be strongly inhibited by the presence of a double-stranded RNA (dsRNA) corresponding to exon sequences in the mRNA. RNAi can be exploited in cultured mammalian cells by introducing shorter, synthetic duplex RNAs (~20 nt) through liposome transfection (3–5) and a peptide-based delivery (6). In mammalian cells, siRNAs have become a new and powerful alternative to other genetic knockdown methods for the analysis of loss-of-function phenotypes. In theory, the technique is simple and elegant. In practice, however, limited stability *in vivo* and the absence of a reliable delivery method hamper the utility of siRNA for therapeutic application. Reports have shown that liposomes (7,8), adenovirus (9), adeno-associated viral vectors (10) and lentivirus (11) can be considered as useful delivery systems. A virus vector-based siRNA delivery overcomes the problem of poor transfection efficiency of plasmid-based systems. However, viral vectors have several limitations when they are used *in vivo*.

Atelocollagen is a highly purified pepsin-treated type I collagen from calf dermis. Collagen is a fibrous protein in the connective tissue that plays an important role in the maintenance of the morphology of tissues and organs. A collagen molecule has an amino acid sequence called as telopeptide at both N- and C-terminals, which confers most of the collagen's antigenicity. Atelocollagen obtained by pepsin treatment is low in immunogenicity because it is free from telopeptides (12), and it is used clinically for a wide range of purposes, including wound-healing, vessel prosthesis and also as a bone cartilage substitute and hemostatic agent (13). We have demonstrated previously that Atelocollagen complexed with DNA molecules was efficiently transduced into mammalian cells (14) and allowed long-term gene expression (15). Since Atelocollagen allows increased cellular uptake, nuclease resistance and prolonged release of genes and oligonucleotides (13), an Atelocollagen complex is applicable for an efficient delivery of siRNA *in vitro*. Furthermore, Atelocollagen displays low-toxicity and low-immunogenicity when it is transplanted *in vivo* (13,16). Thus, our gene delivery method using an Atelocollagen implant should permit safe and efficient siRNA-mediated gene silencing in therapeutic applications.

MATERIALS AND METHODS

Atelocollagen

Atelocollagen is a highly purified type I collagen of calf dermis with pepsin treatment (Koken Co., Ltd, Tokyo, Japan).

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siRNA preparation

Synthetic 21-nt RNAs were purchased from Dharmacon (Lafayette, CO) in deprotected, desalted and annealed form. The sequence of our prepared human fibroblast growth factor (FGF)-4 (HST-1/FGF-4) siRNA was 5′-CGAUGAGUGCACGUUCAAGdTdT-3′; 3′-dTdTGCUACUCACGUGCAAGUUC-5′. Non-specific control siRNA duplex (VIII), luciferase GL3 siRNA duplex and luciferase GL2 siRNA were also purchased from Dharmacon, and were used as controls.

Formation of siRNA/Atelocollagen complex

The siRNAs and Atelocollagen complexes were prepared as follows. An equal volume of Atelocollagen (in PBS at pH 7.4) and siRNAs solution was combined and mixed by rotation at 4°C for 20 min. The complex was then kept at 4°C for 16 h before use. The final concentration of Atelocollagen *in vitro* and *in vivo* was 0.008 and 0.5%, respectively.

Stability of siRNA/Atelocollagen complex

An aliquot of 0.9 μg of siRNAs (luciferase GL3 duplex) and 0.5% Atelocollagen or cationic liposome (jetSI; Polyplus-transfection SAS, Illkirch Cedex, France) complexes were incubated in the presence of 0.1 μg/μl RNase A (NipponGene, Tokyo, Japan) for 0, 5, 15, 30, 45 and 60 min at 37°C. The solutions were extracted with phenol and phenol/chloroform/isoamyl alcohol (25:24:1). The siRNAs were precipitated with ethanol and agarose gel electrophoresed (3.5%) and visualized by ethidium bromide staining.

Cell lines

NEC8 cells (American Type Culture Collection, Rockville, MD) derived from human testicular tumor were maintained in DMEM with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2. Increased expression of the *HST-1/FGF-4* gene in this cell line has been reported previously (17). B16-F10 melanoma cells continuously express luciferase (B16-F10-luc-G5; Xenogen Corp., Alameda, CA) and were maintained in DMEM with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2.

Atelocollagen or liposome-mediated siRNA transfer

The siRNA/Atelocollagen (0.008%) complexes were prefixed to a 24-well plate (0.1–1.4 μg siRNA/50 μl/well) according to the method described previously (14). The cultured cells were plated into the complex-prefixed 24-well plate at 3.5×10^4 cells/well and the effects of siRNA transfer were then observed. The cationic liposome-mediated transfer of siRNA was performed as described by the manufacturer.

Inhibition of cell growth

For monitoring the inhibition of cell growth, the TetraColor One cell proliferation assay reagent (Seikagaku Co., Tokyo, Japan) was used according to the recommended method. The color reaction was assessed by measuring the absorbance at 450 nm with an UVmicroplate reader.

Biochemical analysis

Protein levels of human HST-1/FGF-4 in the culture supernatant and tumors were determined by using enzyme-linked immunosorbent assay (ELISA) using anti-human FGF-4 monoclonal antibody (R&D Systems, Minneapolis, MN). Absorbance was measured at a wavelength of 492 nm with a kinetic microplate reader (model 3550; Biorad, Richmond, CA).

Luciferase assays

For luciferase-based reporter gene assays, 24 μg pGL3 control vector (Promega, Madison, WI) was introduced into HEK 293 cells at 90% confluency in 10 cm dishes using LipofectAMINE™ 2000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. After transfection for 4 h, the cells were collected by trypsinization and plated in the 24-well dishes for siRNA transfection. Atelocollagen-mediated or conventional transfection of siRNAs into 293 cells was performed as detailed above. Cells were lysed (n = 4) on day 2 and analyzed for luciferase activity (Bright™-Glo Luciferase Assay System; Promega). Inhibition of luciferase production was normalized to the level of vehicle-treated cells. GL2 siRNA was used as control.

Analysis of siRNA delivery using *in vivo* imaging

B16-F10-luc-G5 cells were subcutaneously injected (1×10^5 cells per site) into athymic nude mice. Two days later, luciferase GL3 siRNA alone, siRNA mixed with liposome, siRNA complexed with Atelocollagen and Atelocollagen alone were injected into the tumors. For preparing the siRNA/Atelocollagen complex, an equal volume of Atelocollagen (1.0% in PBS at pH 7.4) and siRNA solution was combined and mixed by rotating for 20 min at 4°C. The siRNAs and their complexes were directly injected into the tumor (2.5 μg siRNA/50 μl/50 mm^3 tumor). The final concentration of Atelocollagen was 0.5%. The siRNA concentration used in the liposome experiments was 2.5 μg/tumor equivalent to that used in the Atelocollagen experiments. Each group contains four animals. *In vivo* bioimaging was conducted on a cryogenically cooled IVIS system (Xenogen Corp.) using LivingImage acquisition and analysis software (18). Tumor growth was not affected by these treatments. As a control for GL3 siRNA, GL2 siRNA was used.

Reporter gene labeling of tumor cells

NEC8 cells were transfected with a complex of 2 μg pEGFPLuc plasmid DNA (Clontech, Palo Alto, CA) and 30 μl lipofection reagent (LipofectAMINE™ 2000; Invitrogen). Stable transfectants were selected in geneticin (400 μg/ml; Invitrogen) and bioluminescence was used to screen transfected clones for luciferase gene expression using the IVIS system. Clones expressing the luciferase gene were named NEC8-luc.

In vivo imaging study for orthotopic xenografts model

A total of 1.0×10^6 NEC8-luc cells were injected into mice intratesticularly. Cells were suspended in 50 μl of a serum-free medium and injected using a 26-gauge needle into both testes of 8-week-old athymic nude mice obtained from CLEA Japan (Shizuoka, Japan). Ten days after the injection of cells, tumor cell-bearing nude mice were randomly divided into four treatment groups (FGF-4 siRNA alone, FGF-4 siRNA complexed with Atelocollagen, control siRNA complexed with Atelocollagen and Atelocollagen alone). Each group consisted of four animals. The siRNAs and their complexes were injected directly into the testes (2.5 μg siRNA/50 μl/testis). The final

concentration of Atelocollagen was 0.5%. Tumor growth was monitored by measuring light emission from individual mice 21 days after siRNA administration. Three days after siRNA administration, tumors were harvested and subjected to ELISA analysis for the detection of FGF-4 protein. Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Statistical analysis

The results are given as means ± SE. Statistical analysis was conducted using the analysis of variance with the Bonferroni correction for multiple comparisons. A *P*-value of 0.05 or less was considered to indicate a significant difference.

RESULTS

Atelocollagen-based delivery of siRNA into cells

To develop a method for more efficient siRNA delivery into cells, we have developed a new method for condensing and delivering siRNA using Atelocollagen. Atelocollagen, which is positively charged interacts with the negatively charged siRNA duplex to form an siRNA/Atelocollagen complex (Figure 1),

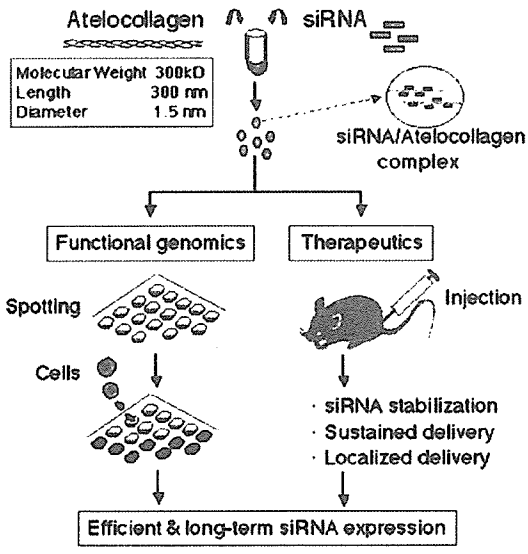

Figure 1. Schematic representation of Atelcollagen-mediated transfer of siRNA duplex for functional genomics and therapeutics. Atelocollagen is a decomposition product of type I collagen derived from the dermis of cattle with a molecular weight of 300 kDa. It is a rod-like molecule with a length and diameter of 300 and 1.5 nm, respectively. Atelocollagen, which is positively charged interacts with the negatively charged siRNA duplex to form an siRNA/Atelocollagen complex, a nanosize particle with a diameter of 100–300 nm. The siRNA/Atelocollagen complex spotted onto the well of a microplate was stable for a long period and allowed the cells to transduce and express siRNAs. The present method using Atelocollagen-based siRNA transfer is also applicable to *in vivo* siRNA transfer, since the siRNA/Atelocollagen complex is stable *in vivo*. Atelocollagen is soluble at a lower temperature but solidifies to refibrillation at a temparature over 30°C. Therefore, the siRNA/Atelocollagen complexes can be injected locally for tissue-targeting siRNA delivery. Once introduced into animals, the complex becomes a solid state and the siRNA is controlled-released for a defined period due to the biodegradable nature of Atelocollagen.

a nanosize particle with a diameter of 100–300 nm. In this system, the siRNA/Atelocollagen complexes are pre-coated on a micro-well plate on which the cells are then seeded (16) (Figure 1). Using this method, cells take up the siRNA/Atelocollagen complex and siRNA exerts a gene silencing effect. To examine whether Atelocollagen blocks degradation of siRNA from nuclease, naked siRNA, siRNA/liposome complex and siRNA/Atelocollagen complex were incubated in the presence of RNase (0.1 μg/μl) for 0, 5, 15, 30, 45 and 60 min at 37°C followed by agarose gel electrophoresis. The results indicated that the siRNA/Atelocollagen complex showed partial resistance to degradation of siRNA in the presence of nuclease (Figure 2). In addition, ~50% of the siRNA were incorporated into the Atelocollagen, which suggests non-incorporated siRNAs are degraded (data not shown). Furthermore, Atelocollagen demonstrated 40–60% efficiency of cellular uptake of FITC-labeled siRNAs 24 h after transfection (data not shown). To evaluate the efficiency of Atelocollagen-mediated transfer technology using well-characterized siRNA, we employed a luciferase reporter

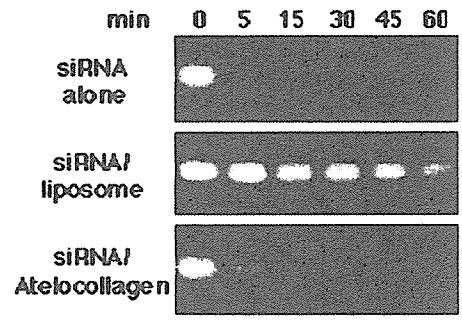

Figure 2. Atelocollagen blocks degradation of siRNA by RNase A. Naked siRNA, siRNA/liposome and siRNA/Atelocollagen complexes were incubated in the presence of RNase A for 0, 5, 15, 30, 45 and 60 min at 37°C and then agarose gel electrophoresed. The presence of siRNA was revealed by ethidium bromide staining.

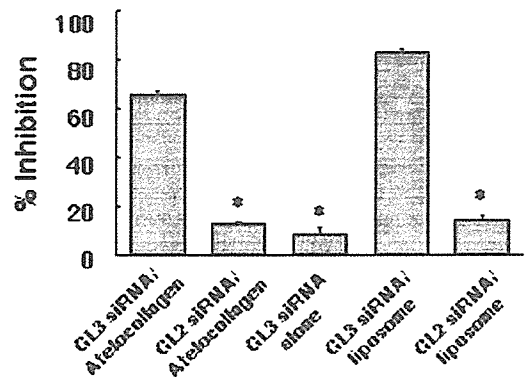

Figure 3. Characteristics of Atelocollagen-mediated siRNA transfer technology. Inhibitory effect of luciferase production in 293 cells. The GL3 siRNA duplexes were transfected into pGL3 control plasmid transfected 293 cells by polycation-reagent or complexed with Atelocollagen. Luciferase activity was measured on day 2 ($n = 4$, mean ± SE). *, $P < 0.001$ versus GL3 siRNA/Atelocollagen and GL3 siRNA liposome-treated cells. As a control for GL3 siRNA, GL2 siRNA was used.