Published online July 22, 2004

Nucleic Acids Research, 2004, Vol. 32, No. 13 ***e109***
doi:10.1093/nar/gnh093

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

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Received April 8, 2004; Revised May 26, 2004; Accepted June 7, 2004

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^{TM} 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^{TM}-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 pEGFP-PLuc plasmid DNA (Clontech, Palo Alto, CA) and 30 μl lipofection reagent (LipofectAMINE^{TM} 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), 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

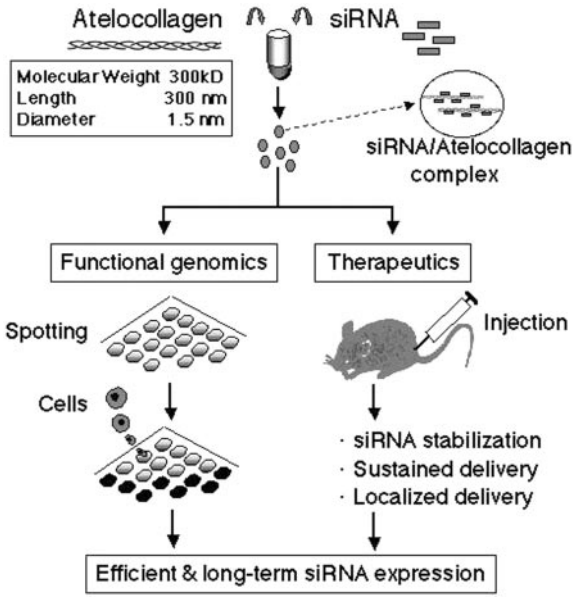

Figure 1. Schematic representation of Atelcollagen-mediated transfer of siRNA duplex for functional genomics and therapeutics. Atelocollagen is a decomposition product of type I collagen derived from the dermis of cattle with a molecular weight of 300 kDa. It is a rod-like molecule with a length and 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.

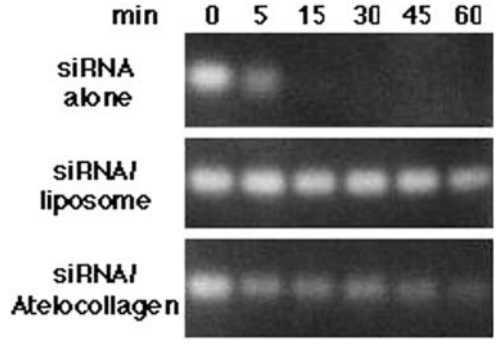

Figure 2. Atelocollagen blocks degradation of siRNA by RNase A. Naked siRNA, siRNA/liposome and siRNA/Atelocollagen complexes were incubated in the presence of RNase A for 0, 5, 15, 30, 45 and 60 min at 37°C and then agarose gel electrophoresed. The presence of siRNA was revealed by ethidium bromide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

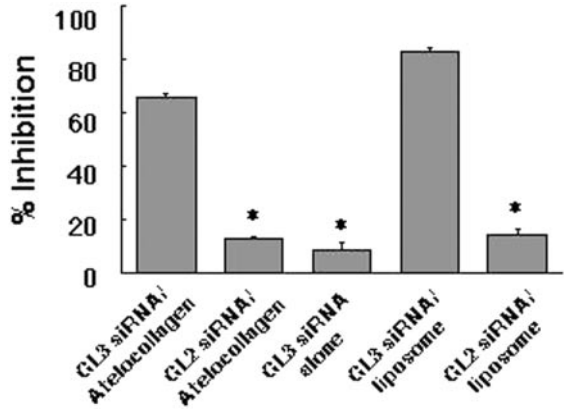

Figure 3. Characteristics of Atelocollagen-mediated siRNA transfer technology. Inhibitory effect of luciferase production in 293 cells. The GL3 siRNA duplexes were transfected into pGL3 control plasmid transfected 293 cells by polycation-reagent or complexed with Atelocollagen. Luciferase activity was 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.

gene system in 293 cells. As shown in Figure 3, our Atelocollagen-mediated siRNA delivery technology exhibited an inhibitory effect as efficient as that in the conventional liposome transfer method.

In the next experiment, we employed human testicular tumor cells, NEC8, which showed high levels of HST-1/FGF-4 mRNA expression (17) and specifically inhibited cell growth by suppression of HST-1/FGF-4 (19). An Atelocollagen-mediated delivery of human HST-1/FGF-4 siRNA was performed to inhibit NEC8 cell growth. The inhibitory effect of HST-1/FGF-4 siRNA was dose-dependent and 1.4 μg per 3.5×10^4 cells produced maximum inhibition (Figure 4A). At a dose of 1.4 μg per 3.5×10^4 cells showed ~10% toxicity by the trypan blue exclusion. Therefore, we used human HST-1/FGF-4 siRNA at a submaximal dose of 0.7 μg per 3.5×10^4 NEC8 cells for further studies. The NEC8 cells transfected with siRNA plus polycation reagent showed an inhibitory effect for maximum of 4 days post-transfection and there was no inhibition of cell growth thereafter (Figure 4B). In addition, siRNA alone and liposome alone showed no significant inhibitory effect (data not shown). In contrast, HST-1/FGF-4 siRNA complexed with Atelocollagen displayed inhibition of cell growth for at least 7 days in culture. To verify further that cell growth inhibition reflected a gene-specific silencing event, HST-1/FGF-4 protein production in NEC8 cells was investigated by ELISA (20). As shown in Figure 5, HST-1/FGF-4 protein levels were significantly inhibited when cells were transfected with the siRNA/Atelocollagen complex. Taken together, these data show that the Atelocollagen stabilized siRNA and thereby siRNA/Atelocollagen complex was able to produce an efficient and a long-term gene silencing effect *in vitro*.

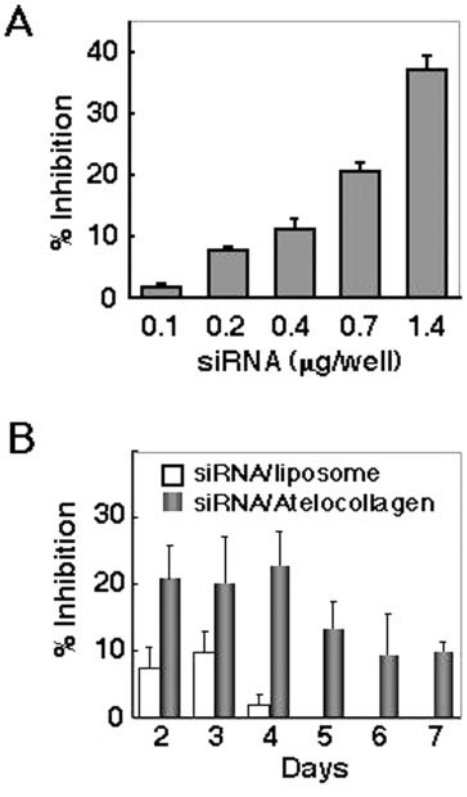

Figure 4. Inhibition of human testicular tumor cell growth by siRNA/Atelocollagen complex. (**A**) Dose-dependent inhibition of NEC8 cell growth. Human HST-1/FGF-4 siRNAs (0.1–1.4 μg) complexed with 0.008% Atelocollagen were transduced into NEC8 cells. Cell proliferation was measured at 2 days after treatment ($n = 4$, mean ± SE). (**B**) Long-term inhibition of NEC8 cell growth by siRNA/Atelocollagen complex. HST-1/FGF-4 siRNA (0.7 μg) was transfected into NEC8 cells by polycation-reagent and complexed with 0.008% Atelocollagen ($n = 6$, mean ± SE).

Enhanced gene silencing by siRNA/Atelocollagen complex *in vivo*

To test whether Atelocollagen-mediated siRNA transfer is valid for gene silencing *in vivo* (Figure 1), animal experiments were performed on mice bearing a luciferase-producing melanoma. Non-invasive *in vivo* bioluminescence imaging analysis showed that luciferase expressions in the tumor of mice injected with GL3 siRNA alone and liposome-complexed siRNA were maximally inhibited at 2–3 days after injection, and increased thereafter. In contrast, mice administered with the siRNA/Atelocollagen complex showed a relatively strong and sustained inhibition of luciferase expression *in vivo* (Figures 6A and B). As previously shown, radiolabeled siRNA mixed with Atelocollagen existed in the tumors for at least a week and remained intact (21). These results suggest that an Atelocollagen-mediated *in vivo* transfer of siRNA could be a powerful and simple method to study loss-of-function of genes in animals.

Inhibition of tumor growth by siRNA/Atelocollagen complex

Testicular injections of NEC8 cell lines in Balb/c nude mice demonstrated relevant tumor biology (19). In this study, the NEC8 cell line was labeled through expression of a stable integrant of the luciferase gene. Athymic nude mice laden

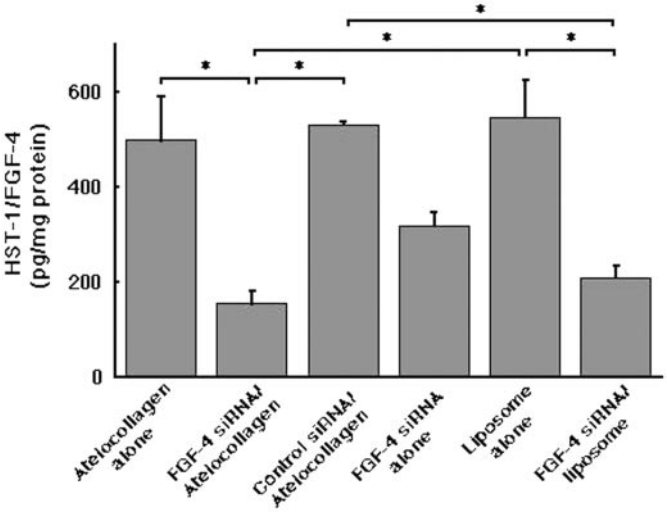

Figure 5. Silencing effect on HST-1/FGF-4 protein production in NEC8 cells. HST-1/FGF-4 siRNA (0.7 μg) complexed with 0.008% Atelocollagen was transduced into NEC8 cells. As a control, an Atelocollagen complex with non-specific control siRNA duplex that shows no silencing effect on human HST-1/FGF-4 was used (control-siRNA/Atelocollagen). Production of HST-1/FGF-4 protein was measured by ELISA 3 days after the transfer of siRNA ($n = 3$, mean ± SE). *, $P < 0.05$.

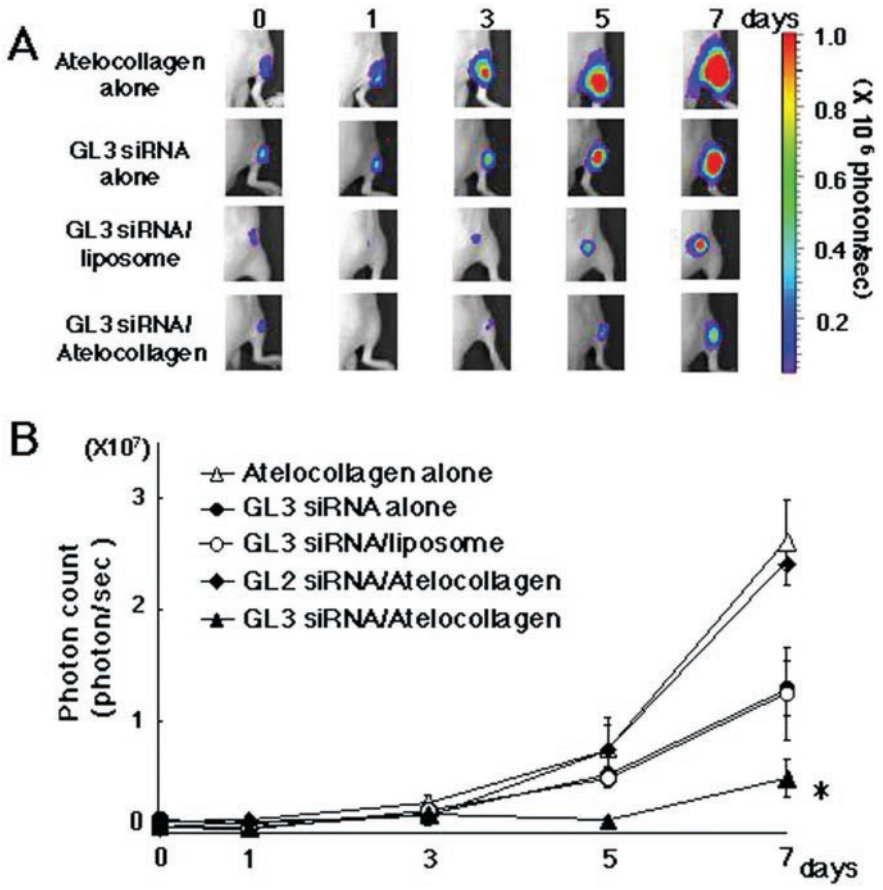

Figure 6. *In vivo* imaging of gene silencing effect of siRNA/Atelocollagen complex. (**A**) Luciferase GL3 siRNA (2.5 μg) complexed with 0.5% Atelocollagen was administered into mice and luciferase expression of xenografted tumors was monitored by *in vivo* imaging analysis. As a control, mice administered with siRNA alone, siRNA complexed with liposome and Atelocollagen alone were investigated. Color bar represents signal intensity code over body surface area. (**B**) Luciferase gene expression was measured periodically and is represented as photon/s. Number of tumors at each time point is 4. As a control for GL3 siRNA, GL2 siRNA was used. Data represent the mean ± SE. *, $P < 0.05$ versus Atelocollagen treatment.

with a testicular injection of NEC8-luc cells were randomly selected for treatment with HST-1/FGF-4 siRNA alone, siRNA complexed with Atelocollagen or Atelocollagen alone. Previously, bioluminescence imaging of orthotopic xenografts in mice demonstrated a linear correlation between tumor bioluminescence and tumor volume (18,22). Tumor growth was inhibited by treatment with human HST-1/FGF-4 siRNA complexed with Atelocollagen. At 21 days following treatment, tumor volume in mice treated with siRNA complexed with Atelocollagen was smaller than that in the control mice treated with Atelocollagen alone (Figure 7A and B). In contrast, tumors treated with siRNA alone and control siRNA/Atelocollagen showed no significant volume reduction. Furthermore, the FGF-4 siRNA/Atelocollagen complex significantly inhibited the production of FGF-4 in the tumors (Figure 7C) and this inhibition lasted for 20 days. Therefore, the Atelocollagen-mediated siRNA transfer is a significant novel method for inhibition of tumor growth *in vivo*.

DISCUSSION

Silencing of gene expression by siRNAs is rapidly becoming a powerful tool for the genetic analysis of a wide variety of mammalian cells. Although in the original studies, the expression of siRNA in mammalian cells was achieved via the transfection of double-stranded oligonucleotides, subsequent studies described the limited duration of the gene silencing effect. To overcome this problem, the use of plasmids to achieve a long-term and stable expression of siRNA was established (23–25). In addition, several groups have described the use of adenoviral vectors (9), retroviral vectors (26) and self-inactivating lentiviral vectors (27) for siRNA delivery. However, viral vectors suffer from the problem of severe side effects. Although the 'hydrodynamic transfection method' and a liposome transfection method were recently reported for siRNA delivery into animals (8,28), none is suitable for clinical use. Therefore, the development of safe non-vector-based siRNA delivery systems is critical for the future of siRNA-based therapies. Here, we used an Atelocollagen-mediated siRNA transfer in an *in vitro* and *in vivo* germ cell tumor-suppression model. Because Atelocollagen allowed increased cellular uptake, nuclease resistance and prolonged release of siRNAs, Atelocollagen complexed with siRNA rather than siRNA alone or a polycation transfer method resulted in stronger gene silencing effects over other methods. It is known that Atelocollagen has the ability to transfer genes to both dividing and non-dividing

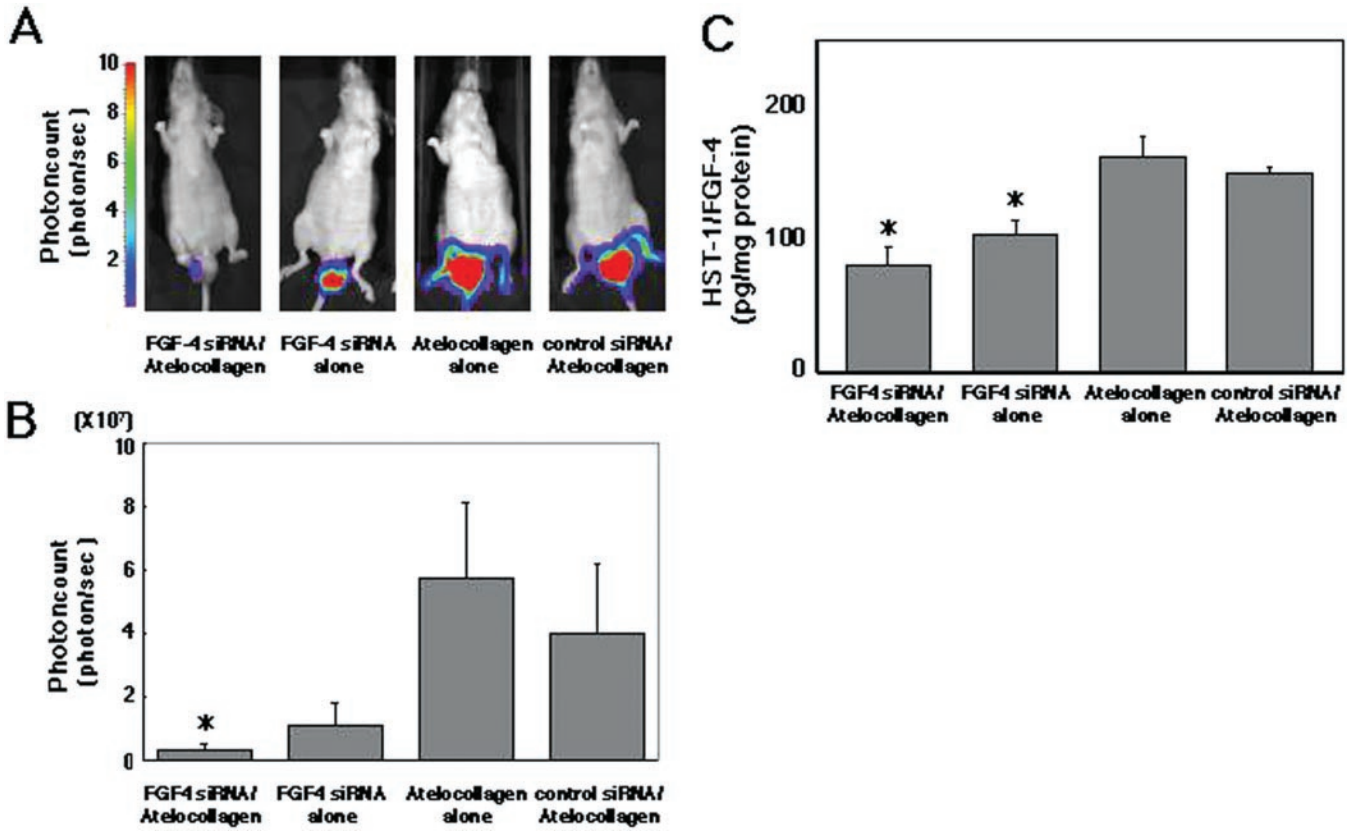

Figure 7. Effect of siRNA/Atelocollagen complex on the growth of a xenograft tumor. (**A**) Human HST-1/FGF-4 siRNA (2.5 μg) complexed with 0.5% Atelocollagen was transduced into an orthotopic germ cell tumor of NEC8 cells expressing the luciferase gene. Representative images at 21 days after treatment are shown. As a control, an Atelocollagen complex with non-specific control siRNA duplex that shows no silencing effect on human HST-1/FGF-4 was used (control siRNA/Atelocollagen). (**B**) Measurements of a xenograft tumor bioluminescence at 21 days after treatment. Data represent the mean ± SE. *, $P < 0.05$ versus Atelocollagen alone treatment. (**C**) Evaluation of HST-1/FGF-4 protein expression in tumor tissue extracts 3 days after treatment. Protein levels were quantified by ELISA. Data represent the mean ($n = 4$) ± SE. *, $P < 0.05$ versus Atelocollagen alone and control siRNA/Atelocollagen treatment.

cells. Thus, for clinical applications in RNAi therapy, an Atelocollagen-based siRNA transfer system represents an attractive method to achieve maximal function of siRNA-based gene silencing *in vivo*.

One technical problem associated with siRNA transfer *in vivo* is the targeting of siRNA delivery to a specific tissue. For this purpose, our Atelocollagen-based transfer method has great potential for site-specific transportation of target siRNAs because the complex of siRNA/Atelocollagen becomes solid when transplanted and remains so for a defined period *in vivo*. In addition, an Atelocollagen complex can be delivered as micro-particles for intravenous injection, making systemic delivery of siRNA possible. A recent report showed the potential for Atelocollagen-mediated systemic antisense therapeutics for inflammatory disease (29). Following *in vivo* administration, the incorporated siRNAs are slowly released over an extended period of time. This eliminates the need for multiple injections of siRNA and siRNA vectors, in lessened side effects.

Although siRNAs are thought to be too short to induce interferon expression, recent reports has shown that siRNA sequences and their method of delivery may trigger an interferon response (30,31). Therefore, alternative strategies are needed to reduce the induction of non-specific side effects. In this regard, our Atelocollagen-mediated non-vector transfer method is an attractive strategy to deliver siRNAs *in vivo*, since our Atelocollagen has low-toxicity and is low-immunogenic, and hence unlikely to stimulate interferon expression *in vivo*.

Finally, based on the ability of Atelocollagen to achieve the sustained release of siRNA and to enhance the stability of siRNA *in vivo*, our novel delivery method demonstrates potential for use as a therapeutic tool for the delivery of siRNA.

ACKNOWLEDGEMENTS

We thank Ms Kazumi Kimura, Ms Masako Hosoda and Ms Ayako Inoue for their excellent technical work. This work was supported in part by a Grant-in-Aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control, Health Science Research Grants for the Research on the Human Genome and Gene Therapy from the Ministry of Health, Labour and Welfare of Japan, a Grant-in-Aid for Scientific Research on Priority Areas Cancer from the Ministry of Education, Culture, Sports, Science and Technology, and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan.

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