an intraperitoneal model of ovarian cancer, Ad/PEG/FGF2 mediated increased transgene expression in tumor tissue and reduced localization of the vectors to nontarget tissues compared with unmodified Ad vectors (Lanciotti *et al.*, 2003). Ogawara *et al.* reported PEGylated Ad vectors containing E-selectin-specific antibody at the tip of PEG, which target activated endothelial cells (Ogawara *et al.*, 2004). They showed that the systemic administration of PEGylated Ad vectors with anti-E-selectin antibody selectively targeted inflamed skin and mediated local transgene expression in mice with a delayed-type hypersensitivity (DTH) inflammation. As for modification with peptides, α_v integrin-specific RGD peptide-modified PEGylated Ad vectors have been developed (Eto *et al.*, 2004; Ogawara *et al.*, 2004).

Ad vectors coated with polymers other than PEG have also been developed. Seymour's group used a multivalent hydrophilic polymer based on poly[*N*-(2-hydroxypropyl)methacrylamide] to modify Ad vectors (Fisher *et al.*, 2001; Green *et al.*, 2004). Their vector showed an extended plasma circulation time and decreased toxicity, and evaded neutralizing antibodies.

Approaches by chemical modification with polymers are advantageous, in that many ligands, such as peptides, antibodies, and antigens, may be applied to the tips of the polymers. A great deal of knowledge and techniques about chemical modification have been acquired in the study of pharmaceutic preparations for drug delivery systems. Although improved pharmacokinetic properties of polymer (including PEG)-coated Ad vectors without ligands have been reported, those of polymer-coated Ad vectors with ligands have not been reported in detail. The exact nature of those vectors must be characterized further.

CONCLUSIONS

In this review, we have focused on the development of targeted Ad vectors based on specific virus entry mechanisms. These approaches are easily combined with transcriptional targeting, using tissue/cell-specific promoters. Ideally, combining a better targeted vector containing a modified capsid with a fully deleted Ad genome (i.e., helper-dependent Ad vectors) is desirable to reduce the innate and acquired immunogenicity of the vectors. These combined vectors should be carefully evaluated in terms of transgene expression profile, distribution of the vectors, and pharmacokinetics, including circulation half-life, interaction with blood components, and so on. Anatomical barriers, such as the tightness of endothelial cells, should also be taken into account, because the vectors must pass endothelial barriers to reach target tissues. Although progress still needs to be made in perfecting targeted Ad vectors, steady improvements have been achieved through comprehensive approaches. Targeted Ad vectors are a source of great promise for gene therapy in future, because they enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

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Brief Report

Adenovirus Vector-Mediated Doxycycline-Inducible RNA Interference

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ABSTRACT

RNA interference (RNAi) is a powerful tool for the knockdown of gene expression. Here, we report on the development of an adenovirus (Ad) vector-mediated doxycycline (Dox)-inducible small interfering RNA (siRNA) expression system. We used this siRNA system to control the expression of p53 and c-Myc in human cancer cells. Coinfection of Ad vectors containing the siRNA expression system under the control of the Dox-inducible H1 promoter and Ad vectors expressing a tetracycline repressor inhibited the expression levels of p53 and c-Myc in a dose-dependent manner with both Dox and viral dose. Regulated silencing of p53 and c-Myc expression was obtained. Because an Ad vector-mediated inducible RNAi system can efficiently transduce a variety of cell types *in vitro* and *in vivo*, and the degree of loss of gene expression can be modulated according to the dose of Dox, this expression system should be a useful tool for both basic research on the analysis of gene function and therapeutic applications of RNAi.

INTRODUCTION

RNA INTERFERENCE (RNAi) mediates the sequence-specific suppression of gene expression in a wide variety of eukaryotes by double-stranded RNA homologies to the target gene (McManus and Sharp, 2002). In mammalian cells, small interfering RNA (siRNA; 19- to 29-nucleotide RNA) leads to the inhibition of target gene expression in a sequence-specific manner (Elbashir *et al.*, 2001). Vector-based siRNA systems have also been developed with RNA polymerase III (Pol III) promoters, such as the small nuclear RNA U6 promoter or the human RNase P RNA H1 promoter, to express siRNA (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Miyagishi and Taira, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). Because Pol III promoters, however, are constitutive and ubiquitous, knockdown of the target gene in an inducible or cell-specific manner is more difficult than with the RNA polymerase II (Pol II) promoters. An inducible RNAi system becomes a more powerful tool for the analysis of gene function, because the loss-of-function or phenotype change can be analyzed according to the degree of loss of gene expression.

In the present study, we developed a doxycycline (Dox)-inducible siRNA expression system utilizing the H1 promoter containing a tetracycline operator (*tetO*) sequence. This vector system was constructed by modifying the Pol II promoter-based gene regulation system, using the tetracycline repressor (TetR), which was developed by Yao *et al.* (1998). In the absence of Dox, TetR binds the *tetO* sequence in the modified H1 promoter, thus preventing transcription. In contrast, TetR does not bind the *tetO* sequence in the presence of Dox, thus allowing transcription. Therefore, target gene expression is turned off in the absence of Dox, but is turned on in its presence.

As a delivery system for the inducible-siRNA expression cassette, the adenovirus (Ad) vector was employed because of its

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numerous attractive characteristics. Recombinant Ad vector has been extensively used to deliver foreign genes to a variety of cell types and tissues both *in vitro* and *in vivo*. Ad vector can be easily grown to high titer, and can efficiently transfer genes into both dividing and nondividing cells. Furthermore, several types of improved Ad vector systems, such as tropism-modified vectors, have been developed (Curiel, 1999; Wickham, 2000; Koizumi *et al.*, 2003a,b). An Ad vector-mediated inducible siRNA expression system would be an effective strategy to use for the basic analysis of gene function and have potential for therapeutic use. In the present study, we demonstrate the efficiency of Ad vector-mediated inducible RNAi against two endogenous genes: *p53* and c-*myc*.

MATERIALS AND METHODS

Cells

293 cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). A549 cells were cultured with F12-K nutrient mixture (Kaighn's modification) medium supplemented with 10% FCS. HepG2 cells were cultured with minimum essential medium supplemented with 10% FCS.

Plasmid and virus

H1 promoter was amplified from human genomic DNA (BD Biosciences Clontech, Palo Alto, CA), using the following primers: 5′-ccatggaattcgaacgctgacgtc-3′ and 5′-gcaagcttagatctgtgtctcatacagaacttataaattccc-3′. The amplified polymerase chain reaction (PCR) product was inserted into the *Eco*RI–*Bgl*II site of pHM5 (Mizuguchi and Kay, 1999), generating pHM5-H1. H1 promoter containing the *tetO* sequence was amplified from pHM5-H1, using the following primers: 5′-tttgccagaattcgaacgctgacgtcatcaacccg-3′ and 5′-ttggaagatctctatcactgatagggacttataagattcccaaatccaaagacatttcacgtttatg-3′ (*tetO* sequence is underlined). The amplified PCR product was inserted into the *Eco*RI–*Bgl*II site of pHM5-H1, generating pHM5-H1tetO. pHM5-H1 and pHM5-H1tetO are designed to express short hairpin RNA (shRNA) on the insertion of an appropriate sequence into the *Bgl*II–*Xba*I site. To insert the target sequence that encodes *p53* and c-*myc* shRNA, oligonucleotides for *p53* (5′-gatccccgactccagtggtaatctacttcaagagagtagattaccactggagtctttttggaaat-3′ and 5′-ctagatttccaaaaagactccagtggtaatctactcttcaagagtagattaccactggagtcggg-3′) (Brummelkamp *et al.*, 2002) and c-*myc* (5′-gatccccgatgaggaagaaatcgatgttcaagagacatcgatttcttcctcatcttttggaaat-3′ and 5′-ctagatttccaaaaagatgaggaagaaatcgatgtctcttgaacatcgatttcttcctcatcggg-3′) (loop sequences are underlined) (van de Wetering *et al.*, 2003) were synthesized, annealed, and cloned into the *Bgl*II and *Xba*I sites of pHM5-H1 and pHM5-H1tetO, generating pHM5-H1-p53, pHM5-H1-Myc, pHM5-H1tetO-p53, and pHM5-H1tetO-Myc, respectively. The sequence was verified with a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Ad vectors were constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999). Briefly, pHM5-H1-p53, pHM5-H1-Myc, pHM5-H1tetO-p53, and pHM5-H1tetO-Myc were digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I- and PI-*Sce*I-digested pAdHM15-RGD (Mizuguchi *et al.*, 2001). The resulting plasmids were digested with *Pac*I and transfected into 293 cells plated in a 60-mm dish with SuperFect (Qiagen, Valencia, CA), according to the manufacturer's instructions. Viruses (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, and Ad-H1tetO-Myc) were prepared as described previously (Mizuguchi and Kay, 1998). Ad vectors containing only the H1 promoter sequence (without a target sequence) (Ad-H1 and Ad-H1tetO) were similarly prepared. Ad-TR, the Ad vector expressing TetR, had been previously prepared (Xu *et al.*, 2003a). Ad-null contains no transgene in the E1 deletion region. Virus was purified by $CsCl_2$ gradient centrifugation, dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, and 10% glycerol, and stored in aliquots at −70°C. Determination of virus particle titer and infectious titer (plaque-forming units; PFU) was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and by the method of Kanegae *et al.* (1994), respectively. The PFU-to-particle ratio was 1:56 for Ad-H1-p53, 1:58 for Ad-H1-Myc, 1:56 for Ad-H1tetO-p53, 1:65 for Ad-H1tetO-Myc, 1:36 for Ad-H1, 1:50 for Ad-H1tetO, 1:24 for Ad-TR, and 1:57 for Ad-null.

Adenovirus-mediated gene transduction

A549 and HepG2 cells (2×10^5 cells) were seeded into a 12-well dish. The next day, they were transduced with the Ad vectors for 1.5 hr. The cells were cultured with medium containing various concentrations of Dox (BD Biosciences Clontech), a derivative of tetracycline. Tet system-approved FCS (BD Biosciences Clontech), a tetracycline-free serum that has been determined to be optimal for the tetracycline-controllable expression system, was used as the FCS.

Western blotting for p53 and c-Myc proteins

Cell extracts were prepared in lysis buffer (25 m*M* Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 m*M* EDTA, 150 m*M* NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured with an assay kit from Bio-Rad (Hercules, CA), using bovine serum albumin as the standard. Protein samples (10 μg) were electrophoresed on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA). After blocking in Block Ace (Dainippon Pharmaceuticals, Osaka, Japan), the filters were incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA), c-Myc (Santa Cruz Biotechnology), and actin (Oncogene Research Products, San Diego, CA), followed by incubation in the presence of peroxidase-labeled goat anti-mouse IgG antibody (American Qualex Antibodies, San Clemente, CA) or peroxidase-labeled goat anti-mouse IgM antibody (Oncogene Research Products). The filters were developed using chemiluminescence (ECL Western blotting detection system; Amersham Biosciences, Piscataway, NJ). Signals were read with an LAS-3000 (Fujifilm, Tokyo, Japan), and quantified by Image Gauge software (Fujifilm).

Northern blot for p53 *and* c-myc *siRNAs*

Total RNA was isolated with ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruc-

tions. To determine the levels of *p53* and c-*myc* siRNAs, 20 μg of total RNA, which was denatured with formamide, was separated on 15% polyacrylamide gels containing 7 *M* urea and electrotransferred to Hybond-N+ membrane (Amersham Biosciences). Loading was checked by ethidium bromide staining. Hybridization was performed with Rapid-Hyb buffer (Amersham Biosciences). Probes which were antisense oligonucleotide (19 bp) of target sequence, were labeled with a MEGALABEL DNA 5′-end labeling kit (TaKaRa Bio, Shiga, Japan). Signals were read with a BAS-2500 (Fujifilm).

RESULTS AND DISCUSSION

Using a combination of Ad vectors and an siRNA expression system is clearly an advantage in gene transfer experiments and therapeutic applications. An inducible siRNA expression system is more desirable, because the degree of gene silencing can be controlled by adjusting the dose or concentration of the inducer. In this study, we developed Ad vectors containing a Dox-inducible siRNA expression system. For inducible siRNA expression, the *tetO* sequence was placed between the TATA box and transcription start site of the H1 promoter (the sequence is described in Materials and Methods). Various Ad vectors, in which target sequences were inserted to express shRNA under the control of the H1 promoter and a mutant H1 promoter containing the *tetO* sequence, were constructed and are shown in Fig. 1. For proof of concept, the expression of endogenous genes *p53* and c-*myc* was silenced.

First, to examine the feasibility of the Ad vector-mediated siRNA expression system, p53 and c-Myc expression was constitutively knocked down by infection with Ad vectors containing the normal H1 promoter or a mutant H1 promoter containing the *tetO* sequence. A549 and HepG2 cells were infected with various concentrations of Ad vector (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, Ad-H1tetO-Myc, Ad-H1, Ad-H1tetO, or Ad-null), and cultured without Dox for 3 days. Levels of p53 and c-Myc protein expression were examined by Western blotting (Fig. 2). Expression of actin was also measured as an internal control. Expression of p53 and c-Myc in

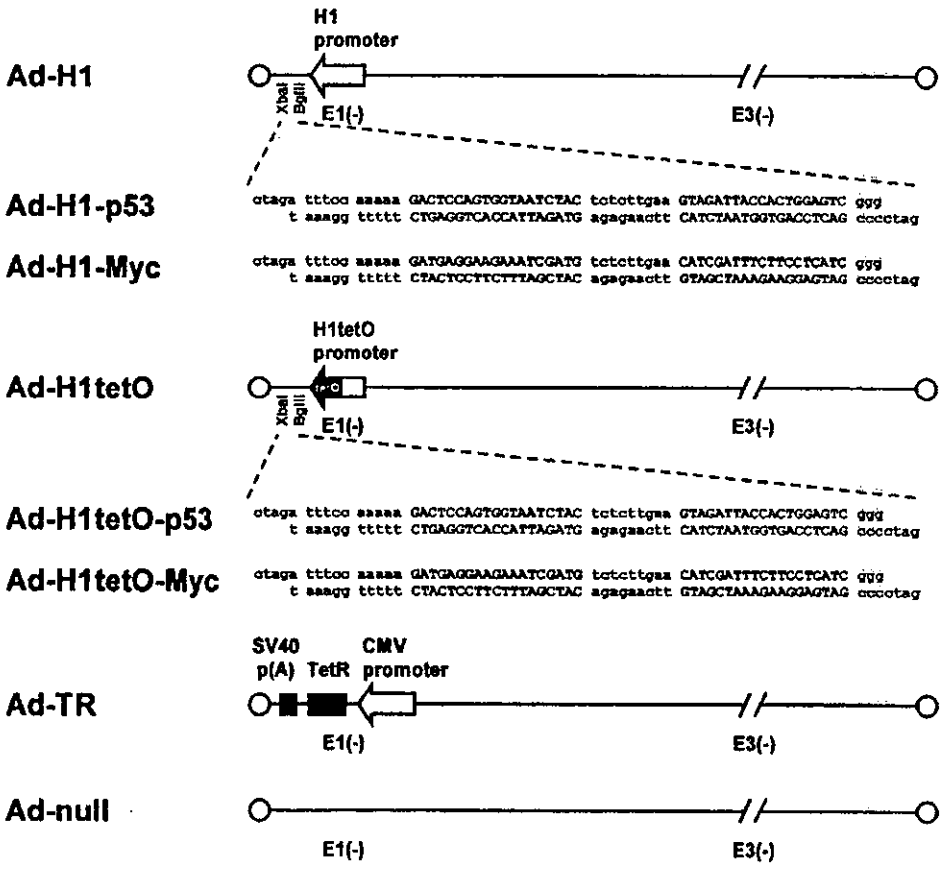

FIG. 1. Structure of Ad vectors used in the present study. The H1 promoter-based siRNA expression cassette was inserted into the E1 deletion region of the Ad genome. For inducible siRNA expression, a tetracycline operator (*tetO*) sequence was introduced downstream of the TATA box in the H1 promoter, as described in Materials and Methods. Target sequences against *p53* and c-*myc* genes are shown in upper case letters. Ad-TR is Ad vector containing a tetracycline repressor sequence under the control of the CMV promoter-enhancer. Ad-null is Ad vector without foreign genes in the E1 deletion region.

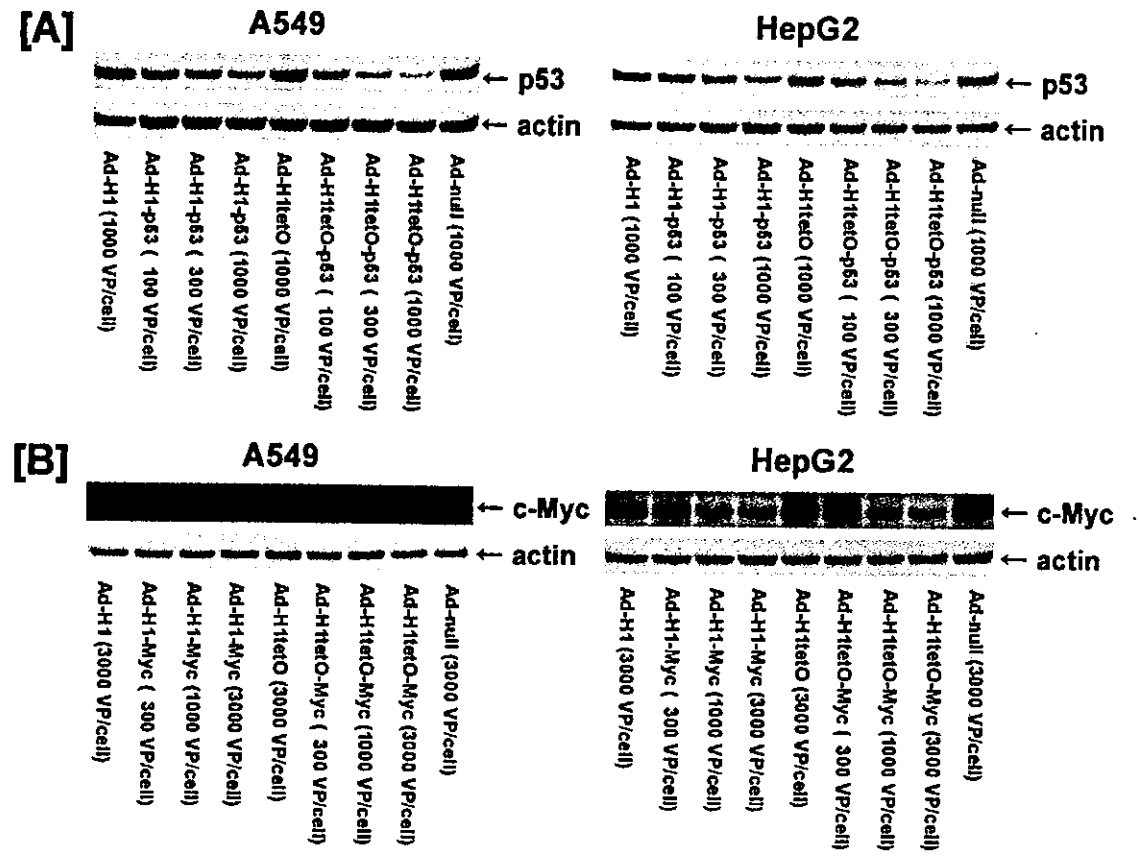

FIG. 2. Dose-dependent suppression of p53 and c-Myc protein expression by Ad vector-delivered siRNA. A549 and HepG2 cells were infected with each Ad vector for 1.5 hr, and then cultured for 3 days. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

A549 and HepG2 cells decreased in a dose-dependent manner with Ad vector carrying the siRNA expression cassette for p53 or c-Myc (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, or Ad-H1tetO-Myc). In the case of p53, a viral concentration of 1000 virus particles (VP)/cell seemed to be enough to knock down expression. Levels of p53 expression in cells treated with Ad-H1-p53 (1000 VP/cell) or Ad-H1tetO-p53 (1000 VP/cell) were decreased to 35–37 or 14–23%, respectively (Fig. 2A), relative to cells treated with Ad-null (1000 VP/cell), according to Image Gauge software (Fujifilm). In the case of c-Myc, a viral concentration of 3000 VP/cell was required to completely knock down expression, although a viral concentration of 1000 VP/cell enabled a moderate knockdown of expression. c-Myc protein expression in cells treated with Ad-H1-Myc (3000 VP/cell) or Ad-H1tetO-Myc (3000 VP/cell) was decreased to 14–44 or 16–35%, respectively (Fig. 2B), relative to cells treated with Ad-null and Ad-H1 (3000 VP/cell). The difference in degree of gene silencing may reflect the effectiveness of the siRNA sequence against each target gene. Compared with Ad-null, Ad-H1 and Ad-H1tetO did not show any effect on gene expression. These results indicate that the *tetO* sequence, placed between the TATA box and the transcription start site of the H1 promoter, does not interfere with promoter activity, and that Ad vectors containing the mutant H1 promoter, as well as the normal H1 promoter-mediated siRNA expression cassette, efficiently silence target gene expression.

Next, we examined whether regulated gene silencing is obtained by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR, the Ad vector expressing TetR, into A549 cells cultured with or without Dox (10 μg/ml). As shown in Fig. 3, in the presence of Dox the silencing effect on p53 expression decreased in proportion to the dose of Ad-TR. Efficient release of gene silencing was obtained with a 1:6 molar ratio of Ad-H1tetO-p53 to Ad-TR, although more Ad-TR might be required to completely release gene silencing. These results suggest that increased amounts of TetR are required to block transcription from the mutant H1 promoter, which contains the *tetO* sequence, in the presence of Dox (Fig. 3A). In the absence of Dox, p53 expression in cells was silenced by coinfection with Ad-H1tetO-p53 and Ad-TR. Therefore, for the regulated silencing of p53 expression, increased amounts of Ad-TR, compared with Ad-H1tetO-p53, were required. A similar result was observed in experiments on c-Myc expression (Fig. 3B), and also in HepG2 cells (both *p53* and c-*myc* genes; data not shown).

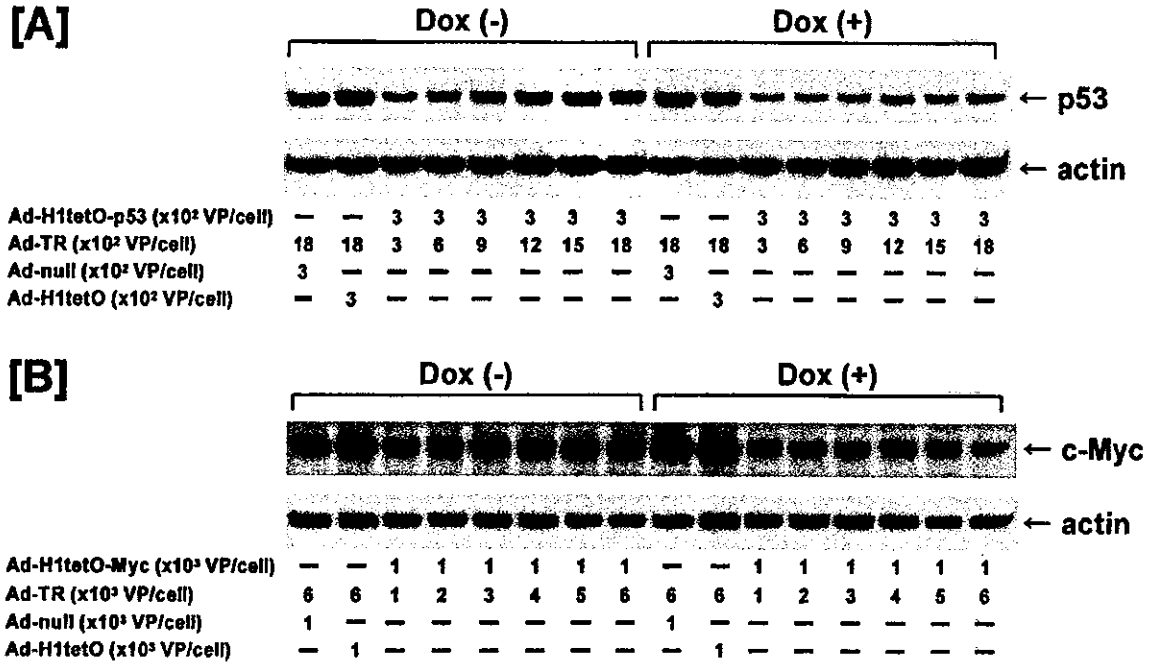

FIG. 3. Regulated suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with the indicated amounts of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR for 1.5 hr, and then cultured with or without Dox (10 μg/ml) for 3 days. The cells were also infected with Ad-null or Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

Ad-TR expresses TetR from the conventional cytomegalovirus (CMV) promoter-enhancer (Xu *et al.*, 2003a). Addition of the intron A sequence to the CMV promoter-enhancer, or the use of a stronger promoter such as the hybrid promoter of the β-actin promoter and CMV enhancer (Niwa *et al.*, 1991; Xu *et al.*, 2001, 2003b), would result in higher expression of TetR, thus decreasing the concentration of Ad vector expressing TetR needed to obtain inducible gene silencing efficiently. These modifications for TetR expression would make the system more effective, and would therefore enable more widespread use of this system.

We then examined the Dox concentration responsiveness of Ad vector-mediated RNAi (Fig. 4). A549 cells were coinfected with Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR at a molar ratio of 1:6, and were cultured with medium containing various concentrations of Dox. A Dox concentration of 10^{-1} μg/ml was enough to completely suppress the expression of p53 and c-Myc. At a Dox concentration of 10^{-2} μg/ml, intermediate levels of knockdown of p53 and c-Myc expression were obtained. Ad-H1tetO-p53 plus Ad-TR and Ad-H1tetO-Myc plus Ad-TR in the presence of Dox did not interfere with c-Myc and p53 expression, respectively, suggesting that the suppressive effect was target gene specific. These results suggest that the degree of knockdown of target gene expression can be modulated by Dox concentration.

We next performed a Northern blot analysis of shRNA (siRNA) expression in the presence and absence of Dox (Fig. 5). Levels of shRNA and siRNA expression for *p53* in both A549 and HepG2 cells transduced with Ad-H1tetO-p53 plus Ad-TR in the absence of Dox were significantly reduced compared with those in transduced cells in the presence of Dox (Fig. 5A). The signal of shRNA and siRNA in the absence of Dox was faint. These observations were marked in the case of c-*myc* (Fig. 5B). These results suggested that shRNA expression was tightly regulated in the Ad vector-mediated Dox-inducible RNAi system.

While this work was in progress, a plasmid vector-mediated inducible siRNA expression system using TetR was reported by van de Wetering *et al.* (2003). The mutant H1 promoter in their system contains the *tetO* sequence at a different position (by 1 bp) compared with the position of the *tetO* sequence in the present study. Both positions for insertion of the *tetO* sequence in the H1 promoter seem to be functional for regulated transcription. A similar system using the mutant U6 promoter containing the *tetO* sequence and TetR for inducible RNAi has also been reported (Matsukura *et al.*, 2003). Furthermore, a tetracycline repressor-based system was reported by two groups. In the study by Wiznerowicz and Trono, the *tetO* sequence was placed upstream of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003), whereas in the study by Chen *et al.*, the *tetO* sequence was placed in these three regions: (1) upstream of the U6 promoter, (2) between the distal promoter element and the core promoter (PSE) of the U6 promoter, and (3) between the PSE and TATA box of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003).

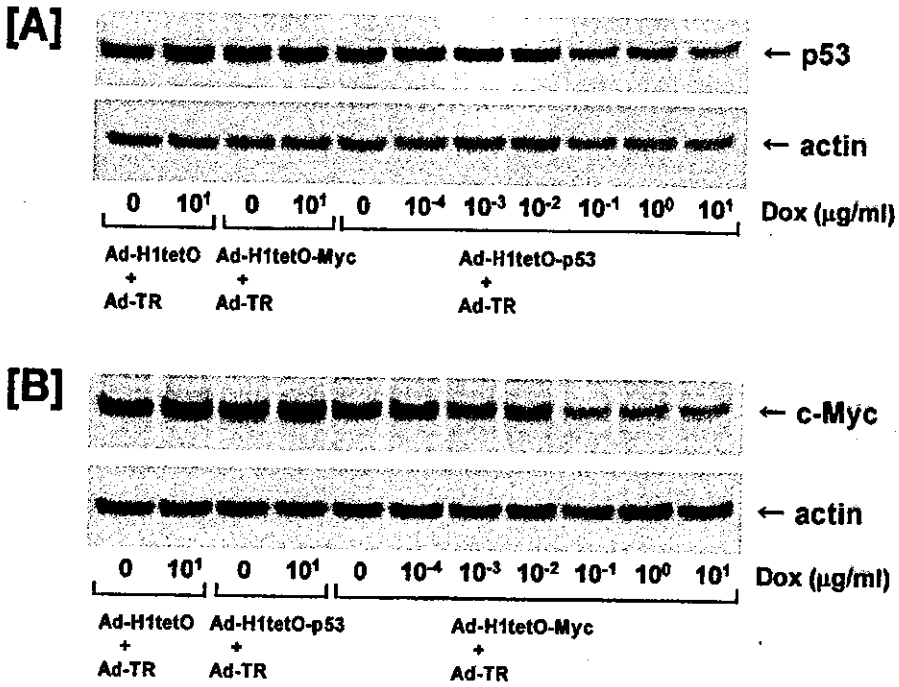

FIG. 4. Dox dose-dependent suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with various concentrations of Dox for 3 days. The cells were also infected with Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

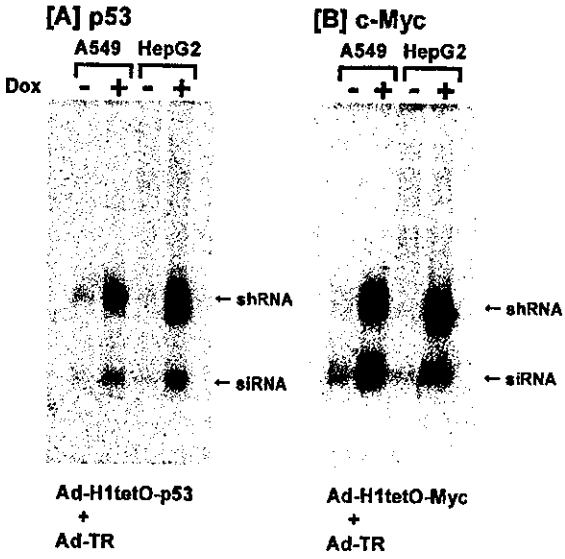

FIG. 5. Dox-inducible *p53* or c-*myc* siRNA expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 and HepG2 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with or without Dox (1 μg/ml) for 3 days. Total RNAs were then extracted from the cells, and levels of *p53* and c-*myc* siRNA expression were examined by Northern blotting.

Ad vector-mediated RNAi represents a new strategy for the study of gene function and therapeutic applications. Ad vector-mediated delivery of siRNA allows efficient transduction into a variety of cell types *in vitro* and *in vivo*. Several studies have reported Ad vector-mediated gene silencing using both the Pol II promoter, in which a mutant CMV promoter was used, and the Pol III (H1) promoter (Xia *et al.*, 2002; Zhao *et al.*, 2003). The combination of Ad vectors and an inducible siRNA expression system offers a superior strategy to researchers. To our knowledge, this study is the first to report on the development of Ad vector-mediated inducible RNAi. Various inducible siRNA expression systems, including a tetracycline repressor-based system, and a capsid-modified Ad vector to change viral tropism, can be easily combined. The system described here has great potential for therapeutic use as well as for a variety of applications, including the study of gene function.

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Efficient Gene Transfer into Human Trophoblast Cells with Adenovirus Vector Containing Chimeric Type 5 and 35 Fiber Protein

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Recombinant adenovirus (Ad) vectors based on Ad type 5 have been widely used for gene transfer experiments. Conventional Ad type 5 vectors have a narrow range of tropism and are limited by the size of the transgene that can be packaged. To overcome these limitations, we previously developed an Ad vector (Ad5/35 vector) containing a chimeric Ad type 5 and 35 fiber protein. In the current study, we evaluated the ability of the Ad5/35 vector to transfer genes into human trophoblast cell lines (JAR, JEG-3 and BeWo cells), which are used as in vitro models of human placenta. We compared the gene transfer efficiency of Ad5/35 to that of conventional Ad vector. We found that expression of CD46, which are receptors for Ad5/35 vector, are higher than that of coxsackievirus and adenovirus receptor in all 3 trophoblast cell lines, as determined by flow cytometry. Next, we compared the transducing activity of Ad5 vector and Ad5/35 vector that each expressed luciferase as a reporter gene. Ad5/35 vector had greater gene transfer activity than the conventional Ad vector in all 3 trophoblast cell lines (1.82-fold in JAR cells, 5.37-fold in BeWo cells, 6.11-fold in JEG-3 cells). Thus, Ad vector that contains chimeric type 5 and 35 fiber protein can be a powerful tool for gene transfer experiments in human trophoblast cell lines.

Key words adenovirus vector; chimeric fiber; gene therapy; trophoblast

The placenta, which is responsible for the development of the fetus, has broad-ranging functions that include transporting nutrients from maternal fluid into the fetus, secreting hormones, and preventing the transfer of toxic substances into the fetus.[1] The placenta contains a variety of cell types, including trophoblast cells, endothelial cells and epithelial cells. Trophoblast cells are believed to be important for fetal development because they transport nutrients from the mother to the fetus.[1] Human trophoblast cell lines functionally expressed transporters of monocarboxylic acids, folic acid and anti-cancer drugs.[2—5] Gene transfer into trophoblast cells can be a useful tool for clarifying the biology of placenta, but methods to transfer gene into trophoblast cells have never been fully investigated.

Recombinant adenovirus (Ad) vectors can introduce genes of interest into cells and tissues. There are more than 51 serotypes of Ad. Ad type 5 (Ad5) vector has been frequently used in basic research and clinical work.[6] Ad5, which belongs to subgroup C, has been used to prepare recombinant Ad vectors because its genetic and biological characteristics have been extensively studied. There are at least two steps to the infection of cells with Ad5. The first step is the attachment of the virus to coxsackievirus and adenovirus receptor (CAR) on the cell membrane *via* the knob domain of the fiber.[7,8] Then, Ad5 is internalized into the cell through the interaction of RGD (Arg-Gly-Asp) motifs on the penton base of the Ad5 surface with $\alpha v\beta 3$- and $\alpha v\beta 5$-integrins on the cell membrane.[9,10] Transgenes delivered by a conventional Ad5 vector are limited to a size of 8.1—8.2 kb,[11] and Ad5 has poor transduction efficiency in CAR-negative cells.[12] Thus, conventional first-generation Ad vectors have a limited transgene size as well as limited tropism.

To overcome these limitations, we previously developed Ad vectors containing chimeric type 5 and 35 fiber protein.[13,14] Ad type 35 (Ad35), which belongs to subgroup B, was initially isolated from the kidneys and lungs of a renal transplant patient.[15] CD46, which is a receptor for Ad35,[16] is ubiquitously expressed in human cells.[17] The Ad5/35 vector can package 8.8 kb of foreign DNA and can transduce CAR-negative cell lines and various human cell lines more effectively than Ad5 vector.[13,14] Thus, Ad5/35 vector is a promising candidate for mediating efficient gene transfer into human trophoblast cell lines.

In the present study, we examined the expression of CD46 in 3 human trophoblast cell lines (JAR, BeWo and JEG-3), which are used in human trophoblast research. We also evaluated the ability of Ad5/35 vector to transfer genes into the human trophoblast cell lines.

MATERIALS AND METHODS

Cell Culture The BeWo cells (clone b30) were obtained from Dr. Alan Schwarts (Washington University, MO, U.S.A.). BeWo cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% MEM non-essential amino acid solution (Gibco, MD, USA), 1.6 g/l sodium bicarbonate, 0.584 g/l L-glutamine and 3.5 g/l D-glucose. JAR and JEG-3 were obtained from American Type Culture Collection (Manassas, VA, U.S.A.) and were cultured with Minimum essential Eagle's medium (MEM) supplemented with 10% FBS and RPMI-1640 supplemented with 10% FBS, respectively.

Preparation of Ad Vectors Ad-L2, which is the conventional Ad vector derived from Ad type 5, and Ad-F35-L2, which contains chimeric type 5 and 35 fiber protein, were purified as previously described.[13,18,19] Both vectors expressed luciferase. The virus particle titer and infectious (plaque forming unit: PFU) titer were spectrophotometrically deter-

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mined by the methods of Maizel *et al.*[20] and by the method of Kanegae *et al.*,[21] respectively. The PFU to particle ratio was 1 : 14 for Ad-L2 and 1 : 15 for Ad-F35-L2.

Adenovirus-Mediated Gene Transduction into Human Trophoblast Cells Cells (1×10^4 cells) were seeded into a 96-well dish. On the following day, they were transduced with Ad-L2 or Ad-F35-L2 (3000 vector particles per cell) for 1.5 h. After culture for 48 h, luciferase production in the cells was measured using a luciferase assay system (PicaGene LT2.0, Toyo Inki Co., Ltd., Tokyo, Japan).

Flow Cytometry To detect the expression of human CAR on the membrane, cells were labeled with mouse monoclonal antibody RmcB (kindly provided by Dr. J. M. Bergelson, The Children's Hospital of Philadelphia, PA, U.S.A.). The cells were then incubated with fluorescein-labeled secondary antibody (Pharmingen, San Diego, CA, U.S.A.). To detect the expression of human CD46, the cells were labeled with fluorescein-conjugated anti-human CD46 (E4.3; Pharmingen). Labeled cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Tokyo, Japan).

Statistical Analysis The significant difference was calculated using one-way ANOVA followed by Dunnett's test.

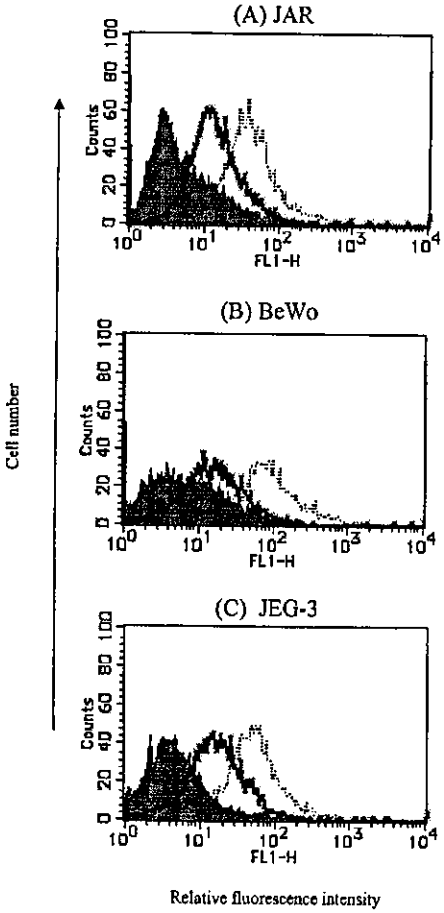

Fig. 1. Flow Cytometric Analysis of CAR and CD46 Expression in Human Trophoblast Cell Lines

Cells were incubated with: (1) anti-CAR antibodies followed by fluorescein-labeled secondary antibody, (2) fluorescein-labeled anti-CD46 antibody or (3) fluorescein-labeled goat IgG. Then, labeled cells were detected by flow cytometry. The dashed regions indicate cells labeled by anti-CD46, the shaded regions indicate cells labeled by goat IgG and the solid regions indicate cells labeled by anti-CAR antibody.

RESULTS AND DISCUSSION

To overcome the limited tropism of conventional Ad5 vectors, we previously developed Ad5/35 vectors that contain chimeric type 5 and type 35 fiber.[13,14] These chimeric Ad vectors can infect cells *via* CD46, a receptor of the type 35 fiber.[14] First, we investigated the expression of CD46 and CAR on the membranes of JAR, JEG-3 and BeWo cells. Flow cytometry analysis showed that CD46 and CAR were expressed on the membranes of all 3 trophoblast cell lines (Fig. 1), suggesting that Ad5/35 vector can infect with the trophoblast cell lines in a CD46-dependent fashion. To compare the transgene activities of the chimeric Ad5/35 vector (Ad-F35-L2) and the conventional Ad5 vector (Ad-L2), we used luciferase as a reporter gene. Ad5/35 mediated greater transgene activity than Ad5 in JAR (1.82-fold), JEG-3 (6.11-fold) and BeWo cells (5.37-fold) (Fig. 2). We previously reported that Ad5/35 vector mediated 100-fold greater expression of reporter gene than Ad5 vector in CAR-negative LN444 cells.[13] Taken together, Ad5/35 may infect with the trophoblast cells *via* different receptors from CAR. Although CD46 is known to be a receptor for Ad type 35,[16] the involvement of unidentified receptors for Ad type 35 in the infection of Ad5/35 vectors is not negligible. Indeed, Erikson *et al.* indicated Ad 35 infected with the cells *via* a trypsin-insensitive receptor.[22] The different transgene activity among JEG-3, JAR and BeWo cells may be caused by different expression profiles of receptors for Ad type 35 among them.

In summary, this is the first report of efficient gene delivery into JAR, JEG-3 and BeWo cells by Ad vector containing chimeric type 5 and type 35 fiber protein. The chimeric

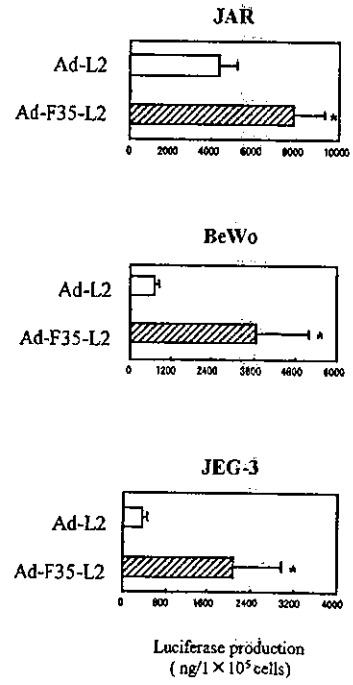

Fig. 2. Comparison of Luciferase Production in Human Trophoblast Cells Transduced by Ad-L2 or Ad-F35-L2

Cells were transduced with 3000 vector particles per cell of Ad-L2 or Ad-F35-L2 for 1.5 h. After culture for 48 h, luciferase production was measured by a luminescent assay. The data are expressed as mean±S.D. ($n=4$). * Significant difference from the Ad-L2-transduced group ($p<0.01$).

Ad5/35 vector can package a gene of up to 8.8 kb and has broad tropism.[13] Thus, Ad5/35 vectors may be powerful tools for gene transfer experiments in human trophoblast cells.

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A single intratumoral injection of a fiber-mutant adenoviral vector encoding interleukin 12 induces remarkable anti-tumor and anti-metastatic activity in mice with Meth-A fibrosarcoma☆

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Abstract

Cytokine-encoding viral vectors are considered to be promising in cancer gene immunotherapy. Interleukin 12 (IL-12) has been used widely for anti-tumor treatment, but the administration route and tumor characteristics strongly influence therapeutic efficiency. Meth-A fibrosarcoma has been demonstrated to be insensitive to IL-12 treatment via systemic administration. In the present study, we developed an IL-12-encoding fiber-mutant adenoviral vector (AdRGD-IL-12) that showed enhanced gene transfection efficiency in Meth-A tumor cells, and the production of IL-12 p70 in the culture supernatant from transfected cells was confirmed by ELISA. In therapeutic experiments, a single low-dose (2×10^7 plaque-forming units) intratumoral injection of AdRGD-IL-12 elicited pronounced anti-tumor activity and notably prolonged the survival of Meth-A fibrosarcoma-bearing mice. Immunohistochemical staining revealed that the IL-12 vector induced the accumulation of T cells in tumor tissue. Furthermore, intratumoral administration of the vector induced an anti-metastasis effect as well as long-term specific immunity against syngeneic tumor challenge.

Keywords: Interleukin 12; Meth-A fibrosarcoma; Recombinant adenoviral vector; Anti-tumor; Anti-metastasis; Intratumoral administration; IL-12 insensitive

The immunostimulating cytokine interleukin 12 (IL-12), a heterodimeric protein composed of two disulfide-linked subunits, is secreted by dendritic cells as well as macrophages and is a key mediator of immunity [1,2]. A variety of studies have focused on the use of IL-12 in cancer therapy and, in these experiments, IL-12 has exhibited potent anti-tumor activity in a number of tumor models [3–5]. IL-12 acts on T and natural killer (NK) cells by enhancing the generation and activity of cytotoxic T lymphocytes and inducing the proliferation and production of cytokines, especially interferon-γ [6]. In addition, IL-12 inhibits

☆ *Abbreviations:* Ad vector, adenoviral vector; AdRGD, RGD fiber-mutant Ad vector; FBS, fetal bovine serum; IL-12, interleukin 12; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PFU, plaque-forming units; $TCID_{50}$, tissue culture infectious $dose_{50}$.

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tumor angiogenesis mainly through IFN-γ-dependent production of the chemokine interferon-inducible protein-10 (IP-10) [7].

Several mechanisms of the anti-tumor activity of IL-12 have been identified, and each contributes differently to the overall therapeutic outcome in a given tumor model [8–10]. Further, some tumor models, such as Meth-A and MCH-1A1 cells, are resistant to treatment with systemically administered IL-12 [11,12]. For example, intraperitoneal administration of murine recombinant IL-12 failed to inhibit the growth of Meth-A fibrosarcoma, even at a dosage of 500 ng daily for 3 days [11]. Compared with so-called IL-12-sensitive tumor cells such as OV-HM ovarian carcinoma and CSA1M fibrosarcoma, which both exhibited notable tumor regression after IL-12-stimulated T-cell infiltration into tumor tissues, Meth-A and MCH-1-A1 tumors lacked similar accumulation of immune cells [12]. Furthermore, otherwise exciting tumor regression results from preclinical studies were moderated by the severe adverse effects that occurred after systemic administration of IL-12 in murine models [13]. The clinical development of IL-12 as a single recombinant protein for systemic therapy has been tempered by pronounced toxicity and disappointing anti-tumor effects [14].

Intratumoral administration of IL-12 may offer several potential advantages over systemic dosing, such as delivery of the gene directly to the tissue of interest and avoidance of the drawbacks of systemic delivery, including the induction of toxicity, acute allergic reactions, and other adverse effects due to the encoded gene [15]. The results of one clinical trial suggest that intratumoral injection of $\leqslant 3 \times 10^{12}$ viral particles of an IL-12-encoding adenoviral vector in patients with advanced gastrointestinal malignancies is feasible and well tolerated [16].

In the present study, we constructed a recombinant adenovirus (Ad) vector that encoded IL-12 (AdRGD-IL-12); the gene transfection efficiency of AdRGD-IL-12 was higher than that of a conventional Ad vector. We also investigated the feasibility of using a single intratumoral injection of AdRGD-IL-12 to provide effective cancer treatment for primary and metastatic Meth-A fibrosarcoma. Furthermore, immunostaining was used to measure the postinjection infiltration of immune cells into tumor tissue.

Materials and methods

Cell lines and animals. Meth-A fibrosarcoma cells (BALB/c origin) were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Osaka, Japan) and were maintained by intraperitoneal passage in syngeneic BALB/c mice. Human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% FBS. BALB/c female mice were obtained from SLC (Hamamatsu, Japan) and used at 6–8 weeks of age. All of the experimental procedures were performed in accordance with the Osaka University guidelines for the welfare of animals in studies of experimental neoplasia.

Vector construction. The replication-deficient AdRGD vector was based on the adenovirus serotype 5 backbone with deletions of E1/E3 region. The RGD sequence for αv-integrin targeting was inserted into the HI loop of the fiber knob by using a two-step method, as previously described [17]. AdRGD-Luc, which is identical to the AdRGD-IL-12 vectors but with the substitution of the luciferase gene expression cassette for the cytokine, was used as negative control vector in the present study. The replication-deficient AdRGD-IL-12, which carries the murine IL-12 gene derived from mIL-12 BIA/pBluescript II KS(−) [18] (kindly provided by Prof. Hiroshi Yamamoto, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan), was constructed by an improved in vitro ligation method using pAdHM15-RGD [19,20]. The expression cassette, which was designed to be transcribed in order from the IL-12 p35 cDNA through the internal ribosome entry site sequence to the IL-12 p40 cDNA under the control of the cytomegalovirus promoter, was inserted into the E1-deletion region of the E1/E3-deleted Ad vector (Fig. 1). All vectors were propagated in HEK293 cells, purified by two rounds of CsCl gradient centrifugation, dialyzed with phosphate-buffered saline (PBS) containing 10% glycerol, and stored at −80 °C. The number of viral particles in vector stock was determined spectrophotometrically by the method of Maizel et al. [21]. Titers (tissue culture infectious $dose_{50}$; $TCID_{50}$) of infective AdRGD particles were evaluated by the end-point dilution method using HEK293 cells and expressed as plaque-forming units (PFU).

Gene expression by AdRGD-Luc or conventional Ad-Luc in Meth-A cells. Meth-A cells were plated in 96-well plates at a density of 2×10^3 cells/well and incubated with Ad-Luc or AdRGD-Luc at concentrations of 1250, 2500, 5000, or 10,000 viral particles/cell for 1.5 h. Cells were then washed with PBS and cultured for an additional 48 h. Subsequently, the cells were washed, collected, and lysed with Luciferase Cell Culture Lysis buffer (Promega, USA), and their luciferase activity was measured by the Luciferase Assay System (Promega,

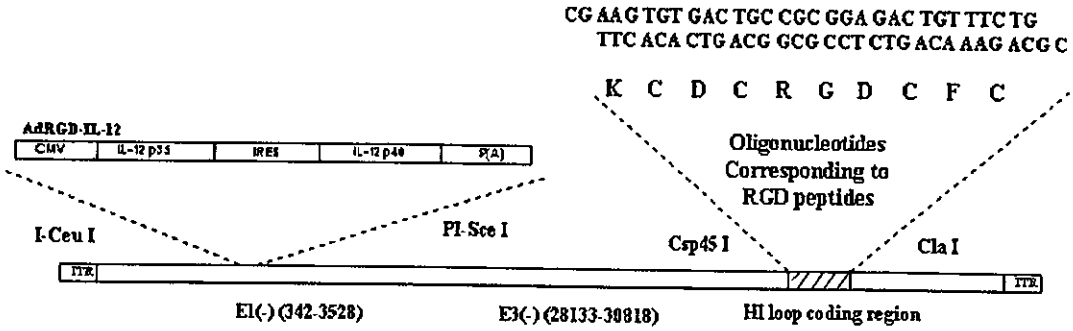

Fig. 1. Construction of IL-12 encoding fiber-mutant adenoviral vector.

USA) and Microlumat Plus LB96 (Perkin-Elmer) according to the manufacturer's instructions.

Analysis of gene transduction of AdRGD-IL-12 in vitro. Meth-A cells were plated in six-well plates at a density of 5×10^5 cells/well and transfected with AdRGD-IL-12 for 24 h at various multiplicities of infection (MOIs) in 2 ml RPMI 1640 medium containing 10% FBS. After three washes of the transfected cells with PBS, a 1.5-ml aliquot of culture medium was added to each well. The supernatants were collected after 24 h, and the amount of IL-12 p70 in each sample was measured with a murine IL-12 p70 ELISA kit (Biosource International, Camarillo, CA, USA) according to the manufacturer's instructions.

Tumor inoculation and intratumoral administration of vectors in animal experiments. Meth-A cells were inoculated intradermally into the flanks of BALB/c mice at 2×10^6 cells/mouse. After 7 days, established tumors (diameter, 9–10 mm) were injected with each vector at 2×10^7 plaque-forming units (PFU) in 50 μl PBS. Tumor size (length and width in mm) was measured twice weekly; animals were euthanized when either of the two parameters exceeded 20 mm. At 3 months after complete regression of the primary tumors, mice were challenged with freshly isolated Meth-A tumor cells or CT26 cells by intradermal injection of 1×10^6 cells into the flank.

Immunohistochemical staining. T-cell infiltration into the Meth-A tumors after intratumoral injection of AdRGD-IL-12 was determined by immunohistochemical analysis. Tumor-bearing mice were euthanized 6 days after administration of AdRGD-IL-12 or the control vector. The tumor nodules were harvested, embedded in OCT compound (Sakura, Torrance, CA, USA), and stored at −80 °C. Frozen thin (6-μm) sections of the nodules were fixed in 4% paraformaldehyde solution, washed with Tris-buffered saline (TBS), and incubated in methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase activity. The sections were incubated with the optimal dilution of the primary antibody—either rabbit anti-human CD3 antibody (DakoCytomation) or normal rabbit IgG (Santa Cruz Biotechnology)—for 60 min at room temperature. Bound primary antibody was detected after incubation with the secondary antibody from the EnVision+ System (DakoCytomation) for 30 min, followed by a 15-min wash in TBS. The sections were stained with DAB (DakoCytomation) and finally counterstained with hematoxylin (DakoCytomation). We randomly selected six fields from different tumor sections and counted the immunostained cells under a light microscope at 400× magnification.

Experiments on metastatic tumor. We intradermally inoculated mice with 2×10^6 Meth-A cells as described earlier and, 5 days later, injected 8×10^4 cells intravenously. Two days after the intravenous injection, intratumoral injection of AdRGD-IL-12 (2×10^7 PFU) was carried out. The size of the primary tumor was measured twice weekly, and the lungs were harvested 2 weeks after the intravenous injection. The lungs were weighed, sectioned for histology, and stained with hematoxylin and eosin. Metastases in the lungs were identified under a light microscope.

Statistical analysis. Student's t test was used for statistical comparison when applicable. Differences were considered statistically significant at $P < 0.05$.

Results

Meth-A tumor cells transfected with the fiber-mutant adenoviral vector induce higher luciferase gene expression than do those induced with the conventional vector

To evaluate the gene transfection efficiency of the fiber-mutant Ad vector developed for this study, Meth-A cells were transfected with either the conventional Ad-Luc vector or the fiber-mutant AdRGD-Luc vector at various MOIs and the luciferase activity was measured. The luciferase gene expression due to transfection of the fiber-mutant vector was much higher than that from the conventional vector (Fig. 2). For example, at 5000 and 10,000 viral particles/cell, 16.8-fold and 15.7-fold greater gene expression, respectively, was obtained in response to AdRGD-Luc than to Ad-Luc. These results show that insertion of the RGD peptide into the viral fiber enhanced the transfection efficiency of the Ad vector into Meth-A cells.

Expression of IL-12 p70 in Meth-A cells via transfection of AdRGD-IL-12

The IL-12-encoding fiber-mutant adenoviral vector AdRGD-IL-12 was developed as shown in Fig. 1. To confirm the biological activity of AdRGD-IL-12, we used an ELISA to measure the amount of IL-12 in the

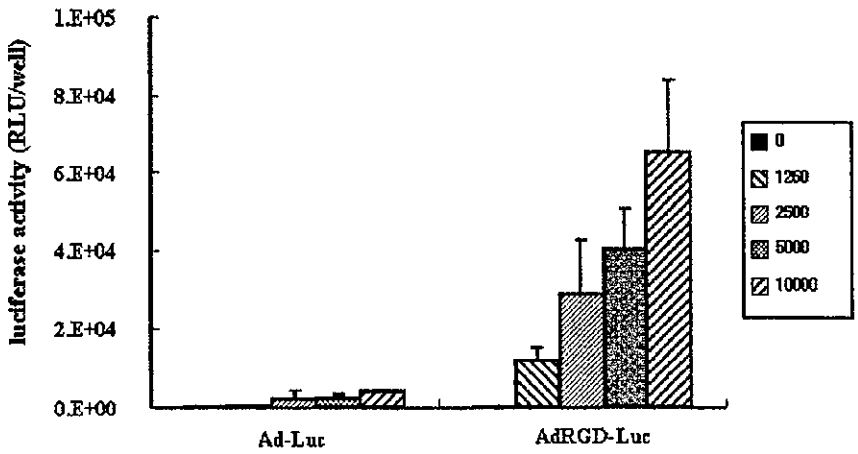

Fig. 2. Gene expression by AdRGD-Luc or conventional Ad-Luc in Meth-A cells. Meth-A cells (2×10^3/well) in 96-well plates were treated with Ad-Luc or AdRGD-Luc at the indicated numbers of viral particles/cell for 1.5 h. Cells were washed and cultured for an additional 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured. Data are presented as means ± SE of relative light units (RLUs)/well from three experiments.

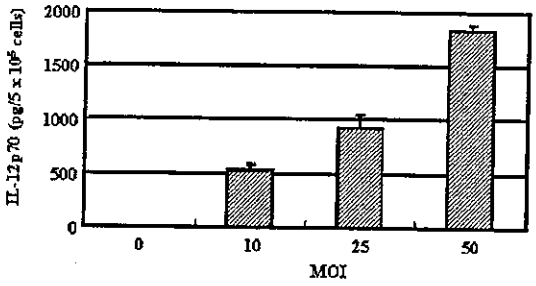

Fig. 3. Production of IL-12 p70 from Meth-A cells transfected with AdRGD-IL-12. We transfected 5×10^5 Meth-A cells with AdRGD-IL-12 for 24 h at the indicated multiplicities of infection (MOIs). Then the cells were cultured for a further 24 h with fresh medium. The supernatants were collected and the IL-12 p70 level was measured by ELISA.

supernatants of transfectants. Meth-A cells transfected with AdRGD-IL-12 showed dose-dependent concentrations of IL-12 p70 in the supernatants. In contrast, no detectable IL-12 p70 was present in the culture media of cells that had not been transfected (Fig. 3).

Anti-tumor activity and long-term specific immune response are induced by intratumoral injection of AdRGD-IL-12

The growth of Meth-A tumors was suppressed dramatically, and complete regression occurred in about 70% of the tumor-bearing mice after a single intratumoral injection of 2×10^7 PFU of AdRGD-IL-12. In contrast, the AdRGD-Luc group showed no apparent anti-tumor effect (Fig. 4A). In addition, the relative survival rates further demonstrated prolonged survival after treatment with IL-12 (Fig. 4B). In the rechallenge

Table 1
Specific long-term anti-tumor immune response to IL-12 treatment

Groups	Challenging cell	Tumor rejected mice/challenged mice
Intact mice	Meth-A[a]	0/5
Meth-A rejected[c]	Meth-A[a]	5/5
Meth-A rejected[d]	CT26[b]	0/3

[a] Challenged with 1×10^6 cells.
[b] Challenged with 3×10^5 cells.
[c] Meth-A cured; Meth-A rechallenged.
[d] Meth-A cured; CT26 rechallenged.

experiment, mice showing complete regression were reinoculated intradermally with Meth-A or CT26 cells 90 days after the initial injection of tumor cells. All of the mice challenged with Meth-A cells remained tumor-free for at least 2 months (Table 1). In contrast, 100% of the mice challenged with CT26 developed palpable tumors within 2 weeks. These results indicate the generation of specific immunity against Meth-A tumor cells in those mice that rejected Meth-A upon treatment with IL-12.

Intratumoral administration of AdRGD-IL-12 induces the infiltration of T cells into Meth-A tumors

To investigate the anti-tumor mechanism of AdRGD-IL-12, tumor tissues were subjected to immunohistochemical staining for CD3 six days after treatment with AdRGD-IL-12 or AdRGD-Luc. Tissues from mice that received AdRGD-IL-12 demonstrated significantly increased accumulation of $CD3^+$ T cells compared with animals injected with either AdRGD-Luc or PBS (Fig. 5).

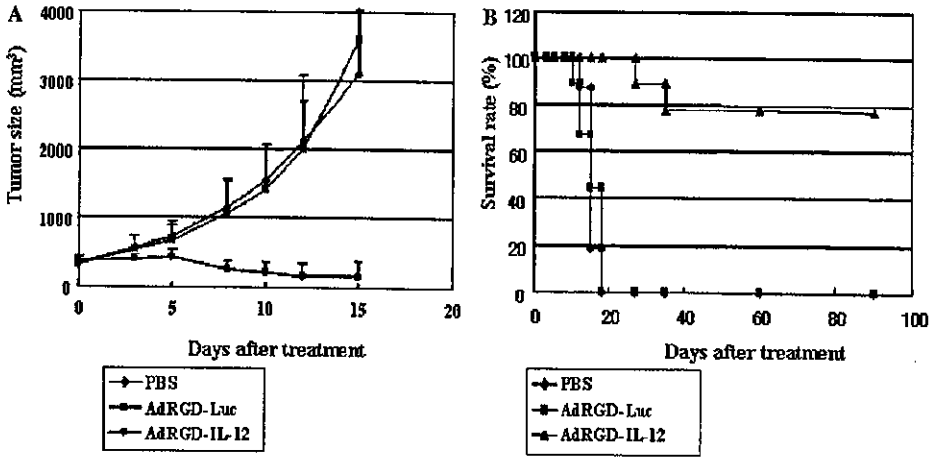

Fig. 4. Growth in BALB/c mice of established Meth-A tumor cells injected intratumorally with IL-12-encoding adenoviral vector. Mice were inoculated intradermally in the flank with 2×10^6 Meth-A cells (100 μl in RPMI 1640). They were then intratumorally injected with 2×10^7 PFU AdRGD-IL-12, AdRGD-Luc, or PBS. Tumor volume was calculated after measuring the length and width of tumors at the indicated time points, and data are expressed as means ± SE of results obtained from at least eight mice. Animals were euthanized when either the length or width of the tumor exceeded 20 mm. (A) Average tumor size. (B) Survival rate (%) of mice.

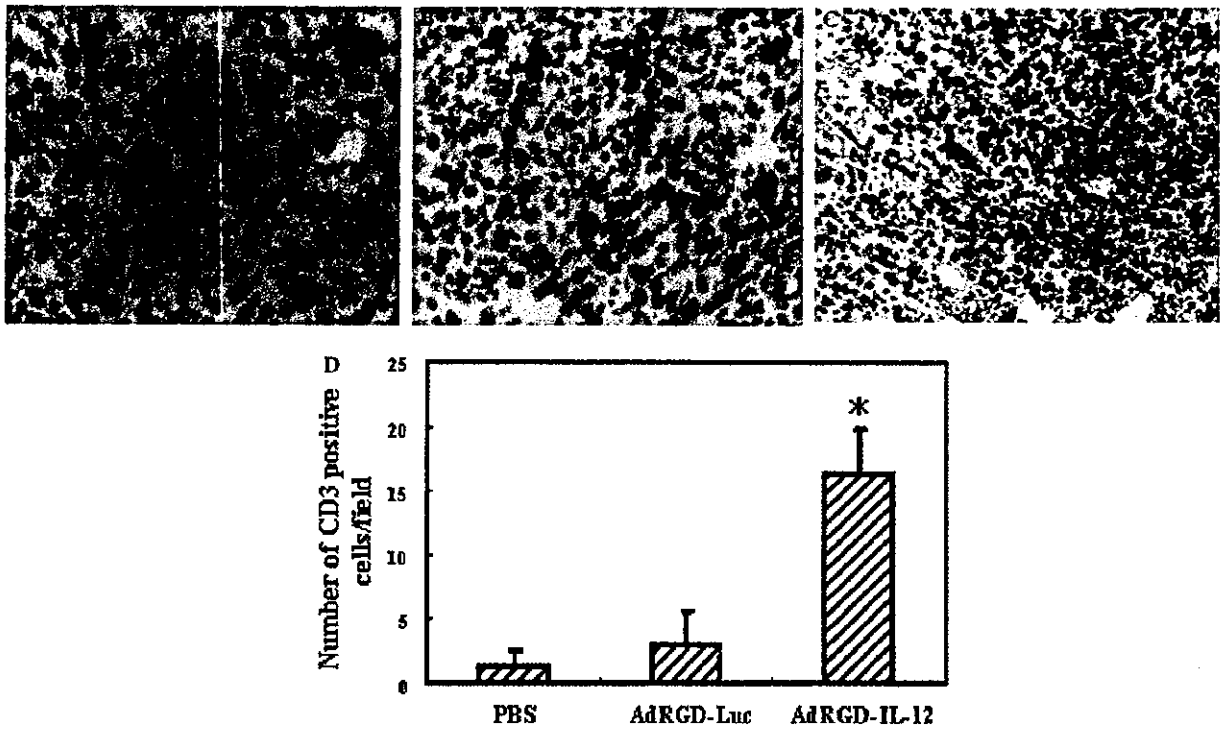

Fig. 5. Intratumoral injection of AdRGD-IL-12 induced the infiltration of $CD3^+$ T cells into Meth-A tumors. Representative views of tumor nodules from mice, harvested 6 days after intratumoral injection of the indicated vectors and controls, and stained for CD3. (A) PBS, (B) AdRGD-Luc, (C) AdRGD-IL-12. The photographs were obtained under light microscopy at 400× magnification. (D) Six fields from different tumor sections were randomly selected and positive cell number infiltrated into tumor tissue was counted. $^*P < 0.05$ with Student's t test in groups between treated with AdRGD-IL-12 and AdRGD-Luc or PBS.

Anti-metastatic activity is induced by intratumoral injection of AdRGD-IL-12

We then sought to evaluate whether intratumoral injection of AdRGD-IL-12 would induce anti-tumor effects against both the primary and metastatic tumors. Our results showed that single intratumoral injection of AdRGD-IL-12 induced pronounced anti-metastasis activity (Figs. 6A and B) while maintaining tumor-suppressive activity toward the primary tumor, similar to

A

Group	Metastasis-free mice/mice in group	Occurrence of Metastasis (%)
PBS	0/8	100%
AdRGD-Luc	1/9	89%
AdRGD-IL-12	8/9	11%

B

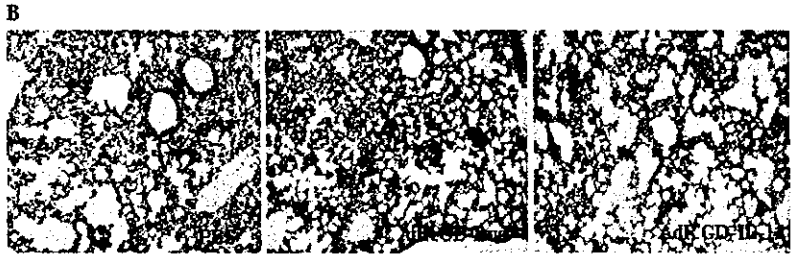

Fig. 6. Anti-metastatic activity due to intratumoral injection of AdRGD-IL-12 into Meth-A fibrosarcoma. (A) Incidence of metastasis in each group. (B) Photomicrographs of lung tissue harvested 2 weeks after treatment and stained with hematoxylin and eosin. The photographs were obtained under light microscopy at 10× magnification. The arrows indicate micrometastasizing tumor.