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Development of a Novel Oncolytic Adenovirus Expressing a Short-hairpin RNA Against Cullin 4A

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Abstract. *Background: Arming of an oncolytic adenovirus (OAd) by inserting expression cassettes of therapeutic transgenes into the OAd genome is a promising approach to enhance the therapeutic effects of an OAd. Ideally, this approach would simultaneously promote the replication of an OAd in tumor cells and transgene product-mediated antitumor effects by expressing therapeutic transgenes. We previously demonstrated that knockdown of cullin 4A (CUL4A), which is an E3 ubiquitin ligase, significantly promoted adenovirus replication by increasing the c-JUN protein level. In addition, previous studies reported that CUL4A was highly expressed in various types of tumor, and was involved in tumor growth and metastasis. Materials and Methods: In this study, we developed a novel OAd expressing a short-hairpin RNA (shRNA) against CUL4A (OAd-shCUL4A). Results: OAd-shCUL4 mediated higher levels of cytotoxic effects on various types of human tumor cell than a conventional OAd. Higher levels of OAd genome copy numbers were found in the tumor cells for OAd-shCUL4A, compared with a conventional OAd. Conclusion: OAd-shCUL4A showed efficient antitumor effects by both enhancing OAd replication and inhibiting tumor cell growth.*

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Oncolytic viruses, which selectively propagate in and kill tumor cells without apparent damages to normal cells, offer an attractive therapeutic approach for treatment of multiple types of cancers. More than 10 species of oncolytic viruses, including herpesvirus, reovirus, and adenovirus, have been developed and tested in preclinical and clinical studies (1, 2). In 2015, the U.S. Food and Drug Administration approved an oncolytic herpes simplex virus (T-VEC) as the first oncolytic virus therapy.

Several approaches have been taken to enhance the antitumor effects of oncolytic viruses. Combination therapies with radio treatment (3), chemotherapy (4), photodynamic therapy (5), and immunotherapy (6) have exhibited promising therapeutic effects in preclinical and clinical studies. Other methods have been developed to enhance the antitumor activities of oncolytic viruses themselves. One highly promising approach is to insert therapeutic transgene expression cassettes into the oncolytic virus genome using genetic engineering. As the oncolytic virus proliferates in tumor cells, the therapeutic transgenes inserted into the oncolytic virus genome are efficiently expressed in a tumor cell-specific manner. Tumor cell-specific expression of the transgenes induces efficient antitumor effects with limited systemic toxicity due to transgene expression. Various types of therapeutic genes, including cytokine genes that can activate antitumor immunity [*e.g.* interferon genes, interleukin (IL)-12 gene, and granulocyte macrophage

Table I. *The oligonucleotides and primers used in this study.*

No.	Name	Sequence (5'-3')
1	Ad-F	GGGATCGTCTACCTCCTTTTGA
2	Ad-R	GGGCAGCAGCGGATGAT
3	Ad-probe	FAM-ACAGAAACCCGCGCTACCATACTGGAG-TAMRA
4	GAPDH-F	GGTGGTCTCCTCTGACTTCAACA
5	GAPDH-R	GTGGTCGTTGAGGGCAATG
6	GAPDH-probe	FAM-CACTCCTCCACCTTTGACGCTGGG-TAMRA
7	CUL4A-F	ACCTCGCACAGATGTACCAG
8	CUL4A-R	AGGTTGACGAACCGCTCATTC
9	shCUL4A-S	GATCCCAAGCATGAGTGCGGTGCAGCCTTCAAGAGAGGCTGCACCGCACTCATGCTTTTTTTGGAAAT
10	shCUL4A-AS	CTAGATTTCCAAAAAAAGCATGAGTGCGGTGCAGCCTCTCTTGAAGGCTGCACCGCACTCATGCTTGG

colony-stimulating factor (GM-CSF) gene] (7-9) and apoptotic genes (*e.g.* p53 gene, tumor necrosis factor-related apoptosis-inducing ligand gene, and Fas ligand gene) (10-12) have been inserted into oncolytic virus genomes. Insertion of these cytokine and apoptotic genes into oncolytic virus genomes provides significant indirect antitumor effects *via* mechanisms which are distinct from those of virus replication-mediated tumor cell lysis. Ideally, however, it is desirable not only to mediate indirect antitumor effects but also enhance virus replication-mediated tumor cell lysis by promoting the propagation of an oncolytic virus in tumor cells.

For this purpose, we focused on the cullin 4A (*CUL4A*) gene. CUL4A is an E3 ubiquitin ligase involved in ubiquitin-proteasome-mediated degradation of various proteins involved in cell-cycle progression, apoptosis, development, and DNA repair (13, 14). We demonstrated that dicer-mediated processing of virus-associated RNA (VA-RNA) II, which is an adenovirus-expressing noncoding RNA, produced VA-RNAII-derived microRNA (mivaRNAII), which knocked down the CUL4A gene *via* post-transcriptional gene silencing following infection with a wild-type adenovirus (15). Knockdown of *CUL4A* led to an elevation in c-JUN expression, resulting in up-regulation of the transcription of adenovirus genes and wild-type adenovirus infection. Moreover, previous studies demonstrated that *CUL4A* expression was significantly up-regulated in various types of tumor and that up-regulation of *CUL4A* was associated with growth and invasion of tumor cells (16-19). Knockdown of *CUL4A* significantly suppressed the tumor cell growth (20, 21). These findings led us to hypothesize that both promotion of adenovirus infection and suppression of tumor cell growth might be achieved by insertion of an expression cassette for a short-hairpin RNA (shRNA) against CUL4A (shCUL4A) into an oncolytic adenovirus (OAd) genome.

Materials and Methods

Cells and mice. HeLa (a human cervical adenocarcinoma cell line: RCB0007), Panc-1 (a human pancreatic cancer cell line), HuH-7 (a human hepatoma cell line: RCB1366), HepG2 (a human hepatocellular carcinoma cell line: RCB1648), HEK293 (a transformed embryonic kidney cell line), and MRC-5 (a normal embryonic lung fibroblast line: CCL-171) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). H1299 (a human non-small cell lung carcinoma cell line) cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum, streptomycin (100 mg/ml), and penicillin (100 U/ml). Human umbilical vein endothelial cells (HUVECs) were cultured in the medium recommended by the supplier (Lonza, Basel, Switzeland). HeLa, HuH-7, and HepG2 cells were obtained from the JCRB Cell Bank (Tokyo, Japan). The other cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

Plasmids. pAdHM19-hAIB-shCUL4A, a plasmid for a telomerase-specific replication-competent adenovirus expressing shCUL4A, was constructed as follows. Firstly, pENTR1A-H1T2 (22) was digested with *Bgl*II/*Xba*I and then ligated with oligonucleotides encoding shCUL4A, resulting in pENTR1A-H1T2-shCUL4A. Next, pENTR1A-H1T2-shCUL4A was digested with *Bam*HI/*Xba*I and then ligated with *Bam*HI/*Xba*I-digested pHM13 (23), resulting in pHM13-H1T2-shCUL4A. Finally, pHM13-H1T2-shCUL4A was digested with *Cla*I and then ligated with a *Csp45*I-digested fragment of pAdHM19-hAIB(22), resulting in pAdHM19-hAIB-shCUL4A. The oligonucleotide sequences are described in Table I.

Viruses. Recombinant OAds were prepared as follows. *Pac*I-digested pAdHM19-hAIB and pAdHM19-hAIB-shCUL4A were transfected into HEK293 cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), producing conventional OAd and OAd-shCUL4A, respectively. These recombinant OAds were amplified in H1299 cells, and purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80˚C (24). The virus particle (VP) titers were determined using a spectrophotometric method (25).

Determination of adenovirus genome copy numbers in cells. Cells were infected with an OAd, and then total DNA, including

adenovirus genomic DNA, was isolated from the cells using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). After isolation, adenovirus genome copy numbers were quantified using a StepOnePlus real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) as previously described (26).

Antibodies and western blotting analysis. Rabbit antibody to human CUL4A was purchased from Abcam (Cambridge, UK). Rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Trevigen (Gaithersburg, MD, USA). Horseradish peroxidase-labeled anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Western blotting analysis was performed as previously described (27). Briefly, whole-cell extracts were prepared and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions, followed by electrotransfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% skim milk or 5% bovine serum albumin prepared in Tris-buffered saline with Tween-20 (Tween-20, 0.1%), the membranes were incubated with primary antibodies, followed by incubation in the presence of secondary antibodies. The protein bands were visualized with a chemiluminescence kit (ECL Plus Western blotting detection system; Amersham Biosciences, Piscataway, NJ, USA).

Quantitative RT-PCR analysis. Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA Synthesis Kit (Life Technologies). Quantitative RT-PCR analysis was performed using Fast SYBR Green Master Mix (Life Technologies) and a StepOnePlus Real-Time PCR system (Life Technologies). The sequences of the primers used in this study are described in Table I.

siRNA knockdown. Cells were seeded on a 96-well plate at 1×10^4 cells/well. On the following day, cells were transfected with an siRNA against *CUL4A* (siCUL4A) (15) (Gene Design, Osaka, Japan) and control siRNA (AllStars Negative Control siRNA; Qiagen) at 50 nM using Lipofectamine RNAiMax (Life Technologies). Cell viabilities were determined as described below.

Cell viability assay. Cells were seeded on a 96-well plate at 1×10^4 cells/well. On the following day, cells were infected with OAds at 100 or 300 VP/cell. Cell viabilities were determined on days 2, 3, 4, and 5 following infection by staining with AlamarBlue (Life Technologies) according to the manufacturer's instructions.

Statistical analysis. Statistical significance of differences between groups was determined using Student's *t*-test. Data are presented as the means±S.D.

Results

CUL4A expression in human tumor cell lines. Firstly, we examined the CUL4A expression level in several types of human tumor cell lines and normal cells by western blotting analysis. The human tumor cell lines H1299, Panc-1 and HepG2 showed higher levels of CUL4A expression than did the normal human cells, MRC-5 and HUVECs (Figure 1A). CUL4A levels in Huh-7 and HeLa cells were similar or slightly lower than those in MRC-5 cells and HUVEC.

Next, in order to examine whether *CUL4A* knockdown inhibited tumor cell growth, siCUL4A was transfected into the tumor cells. We previously reported that 50 nM of the siCUL4A used in this study mediated approximately 80% knockdown at the mRNA level in HeLa cells 48 h after transfection (15). *CUL4A* knockdown significantly suppressed the growth of all tumor cell lines tested by approximately 20-50% on day 5 (Figure 1B). The CUL4A expression levels in the tumor cells did not appear to be correlated with the level of siCUL4A-mediated growth suppression. These data indicated that *CUL4A* knockdown suppressed tumor cell growth.

Tumor cell killing activity of an OAd expressing an shRNA against CUL4A. In order to suppress CUL4A expression in tumor cells following OAd infection, we inserted a human H1 promoter-driven expression cassette for an shRNA against *CUL4A* into the E3-deleted region of the OAd genome, producing OAd-shCUL4A (Figure 2A). OAd-shCUL4A possessed a human telomerase reverse transcriptase (*hTERT*) promoter-driven E1 gene expression cassette in the E1 gene-deleted region for tumor cell-specific replication of the OAd. OAd-shCUL4A significantly knocked down *CUL4A* expression at both the mRNA and protein levels in HeLa cells, while a conventional OAd appeared not to mediate any reduction in CUL4A expression at the titers used in this study (Figure 2B and C).

Next, in order to examine the tumor cell-killing activity of OAd-shCUL4A, human tumor cell lines were treated with OAd-shCUL4A. For all the human cell lines examined, OAd-shCUL4A lysed the tumor cells more efficiently than did the conventional OAd, and did so in a dose- and time-dependent manner (Figure 3A). We did not observe any correlation between the tumor cell viabilities following OAd infection and the CUL4A expression levels (Figure 1A) in the tumor cells. Higher levels of OAd genome copy numbers were found in HeLa cells treated with OAd-shCUL4A than in those treated with the conventional OAd (Figure 3B). On the other hand, neither OAd-shCUL4A nor the conventional OAd showed any apparent reduction in the viability of MRC-5 cells, which are normal human fetal lung fibroblasts (Figure 4A). The genome copy numbers of OAd-shCUL4A and a conventional OAd increased by 17- to 30-fold from 3 to 24 h after treatment (Figure 4B), but the OAd genome copy numbers in MRC-5 cells were much lower than those in HeLa cells 24 h after treatment. We did not observe any statistically significant increases in the OAd genome copy numbers in MRC-5 cells treated with either the conventional OAd or OAd-shCUL4A. These data indicated that OAd-shCUL4A showed higher levels of tumor cell-killing activity than the conventional OAd without apparent toxicity to human normal cells.

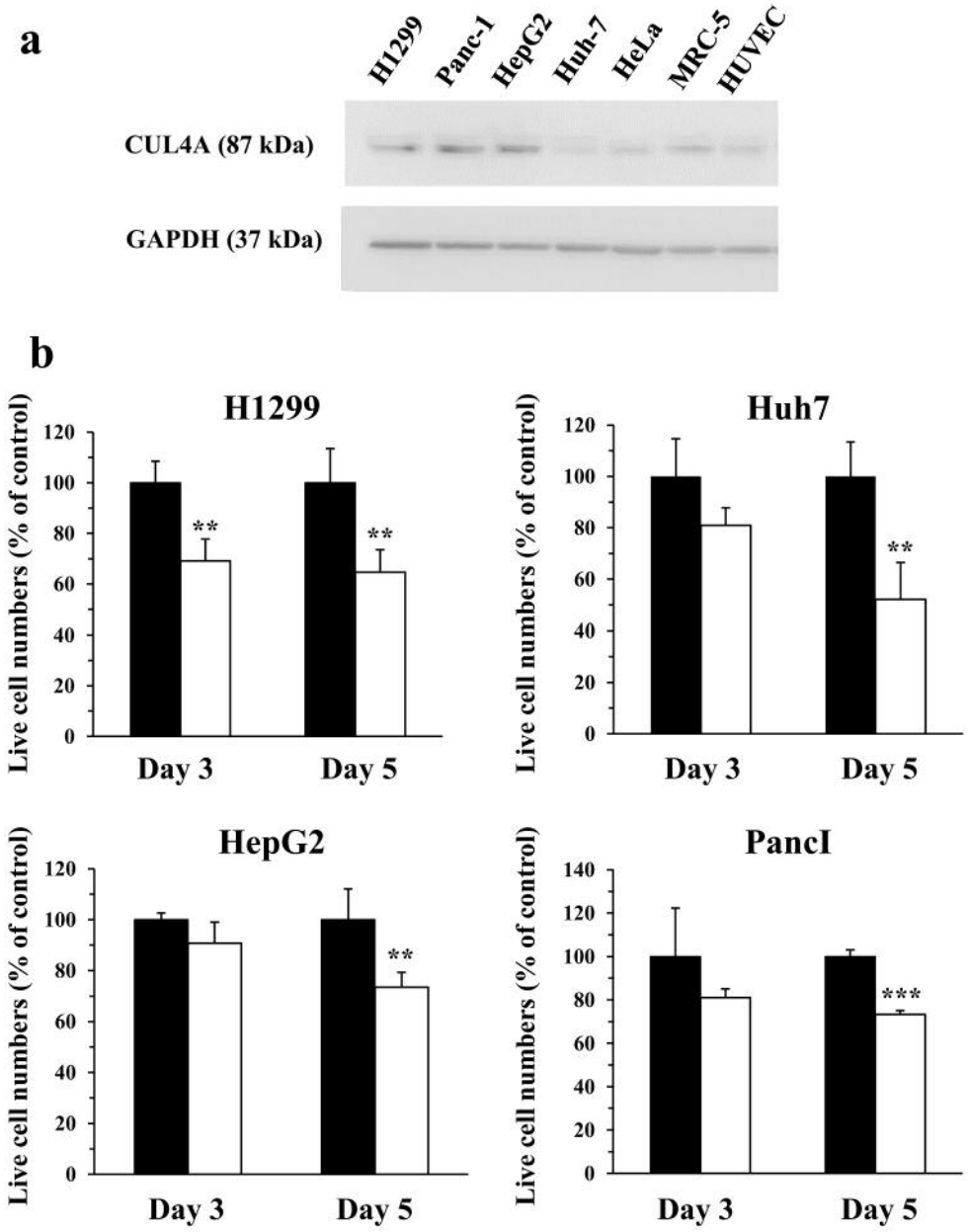

Figure 1. *A: Cullin 4A (CUL4A) expression levels in several human tumor cell lines and normal cells (MRC-5 fibroblasts; human umbilical vein endothelial cells, HUVECs). CUL4A protein levels were evaluated by western blotting analysis. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. B: Growth of the human tumor cells following siRNA-mediated knockdown of CUL4A. Live cell numbers were evaluated by an Alamar blue assay on days 3 and 5 following transfection with control siRNA (closed bar) and siCUL4A (open bar). The data were normalized by the data of the control siRNA-treated group. Significantly different at **p<0.01 and ***p<0.001.*

Discussion

In this study, an shCUL4A expression cassette was inserted into the OAd genome to mediate superior antitumor effects. CUL4A is considered to be an ideal target for OAd-mediated knockdown because knockdown of *CUL4A* not only enhances Ad propagation but also suppresses tumor cell growth. CUL4A has been shown to be overexpressed in clinical samples of numerous cancer types, including breast, colorectal, lung, and liver cancer (16-20). Moreover, CUL4A expression has been associated with poor prognosis of patients with cancer (16, 17). CUL4A is an E3 ubiquitin ligase, and is

involved in degradation of tumor-suppressor proteins, including p53 (28), p21 (29), and NF2 (30). *CUL4A* knockdown significantly suppressed tumor growth and invasion, and increased sensitivity to anticancer chemotherapy (20, 21). These findings indicated that OAd-mediated knockdown of *CUL4A* significantly enhanced the antitumor activities of OAd. This study also demonstrated that OAd-shCUL4A led to higher levels of tumor cell-killing activities in multiple types of tumor cells than did a conventional OAd.

CUL4A knockdown also enhanced OAd propagation, leading to efficient tumor cell-killing activities in this study (Figure 3). We previously reported that knockdown of *CUL4A* led to up-regulation of c-JUN, resulting in transcription of adenovirus genes and wild-type adenovirus infection (15). Much as in this previous study, our present finding that OAd-shCUL4A was more efficient replicated than a conventional OAd was likely attributable to up-regulation of c-JUN and c-JUN-mediated transcription of adenovirus genes by knockdown of *CUL4A*.

As described above, the knockdown of *CUL4A* both suppressed tumor cell growth and promoted propagation of OAd-shCUL4A, leading to lower viability of the tumor cells following treatment with OAd-shCUL4A, compared with a conventional OAd. However, the promotion of OAd-shCUL4A propagation appeared to make a larger contribution to the efficient tumor cell-killing of OAd-shCUL4A, because siRNA-mediated knockdown of *CUL4A* achieved a less than 20% reduction in the number of live tumor cells, although statistically significant differences were found between the control siRNA-treated and siCUL4A-treated groups. In contrast, OAd-shCUL4A achieved a more than two-fold increase in OAd genome copy numbers compared to the conventional OAd at 72 h after infection (Figure 3B).

We previously reported that *CUL4A* was significantly knocked down by mivaRNAII following infection with a wild-type adenovirus at 100 VP/cell in HeLa cells (15); however, the *CUL4A* knockdown efficiency following infection with a conventional OAd was slightly lower than those by the wild-type adenovirus shown in the previous study (15), even though the conventional OAd efficiently infected the tumor cells. In order to achieve more efficient knockdown of *CUL4A*, we inserted an expression cassette of an shRNA against *CUL4A* into the OAd genome. OAd-shCUL4 exhibited higher levels of *CUL4A* knockdown than a conventional OAd (Figure 2B and C). For more efficient knockdown of *CUL4A* following OAd-shCUL4A treatment, several modifications can be applied to an OAd. For example, the insertion of multiple shCUL4A expression cassettes in the OAd genome improves *CUL4A* knockdown efficiency. We previously demonstrated that insertion of multiple expression cassettes of an shRNA against luciferase into a replication-incompetent adenovirus vector genome significantly improved the knockdown efficiencies (31).

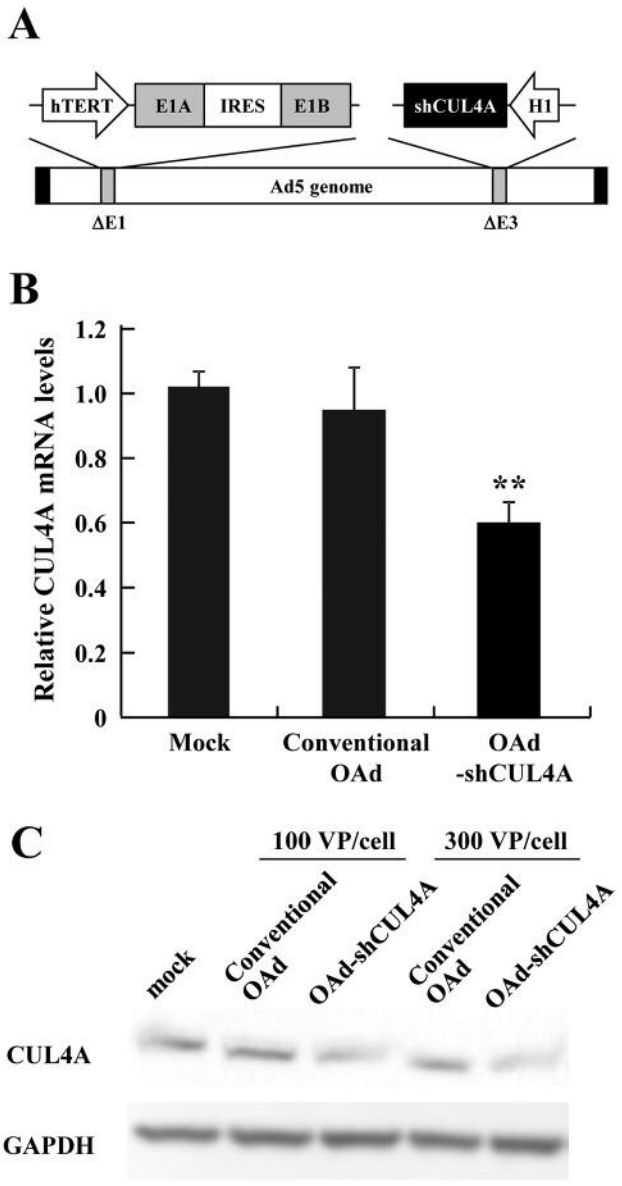

Figure 2. *A: Schematic diagram of the oncolytic adenovirus (OAd) expressing a short-hairpin RNA against CUL4A (shCUL4A) genome. hTERT: Tumor-specific human telomerase reverse transcriptase promoter; IRES: internal ribosome entry sites; H1: an H1 promoter. CUL4A mRNA (B) and protein (C) levels in HeLa cells following infection with an OAd. HeLa cells were infected with an OAd at 100 or 300 virus particles/cell. After 48 h of incubation, CUL4A mRNA and protein levels were determined by real-time reverse transcription polymerase chain reaction and western blotting analyses, respectively. **Significantly different at p<0.01.*

In summary, we demonstrated that OAd-shCUL4A mediated more efficient tumor cell-killing activities in several types of tumor cells *via* both suppression of tumor growth and promotion of virus propagation. These results

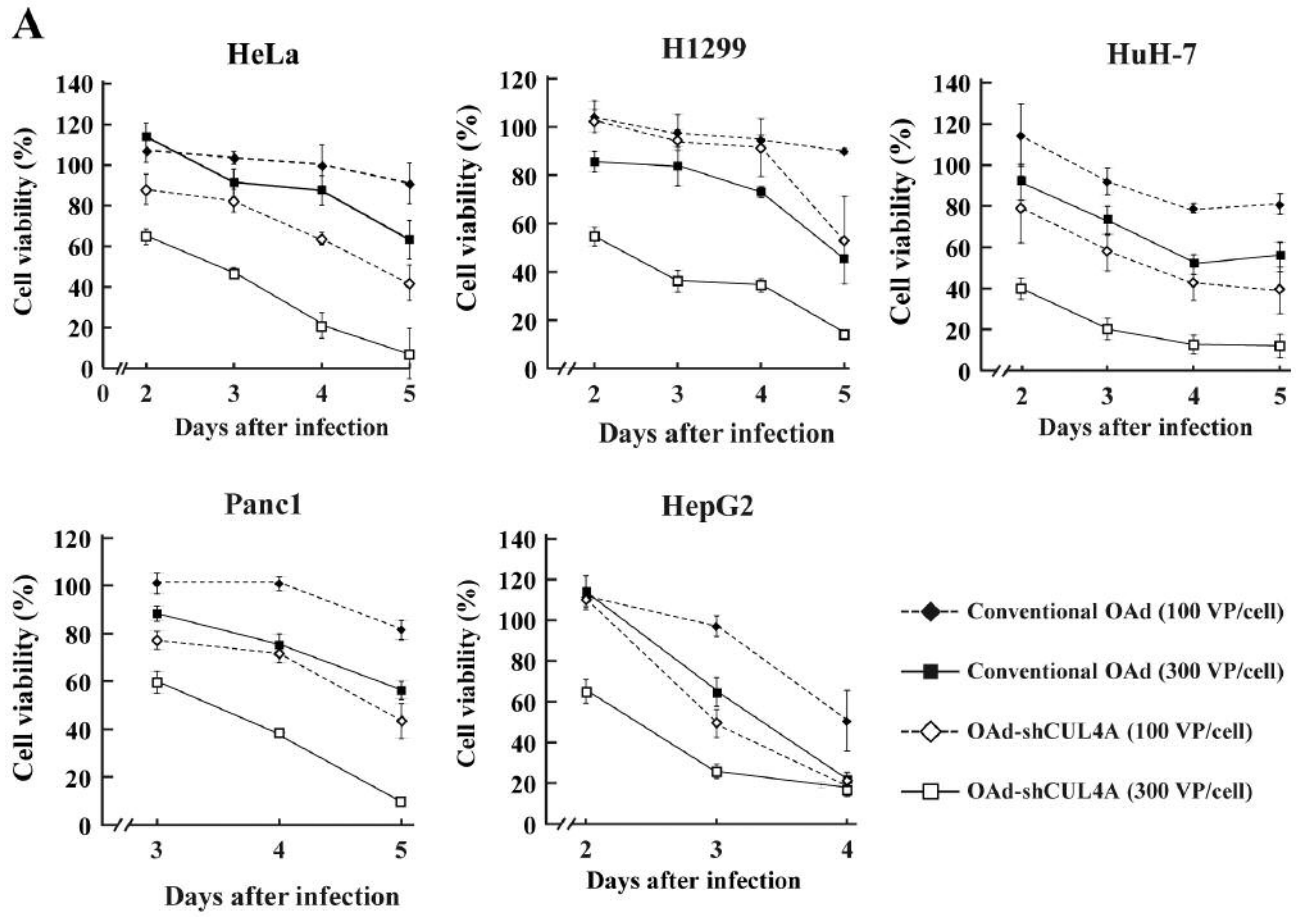

Figure 3. *A: Tumor cell-killing activities of oncolytic adenovirus (OAd) expressing a short-hairpin RNA against CUL4A (shCUL4A). Several human tumor cell lines were infected with an OAd at 100 and 300 VP/cell. At the indicated time points, cell viabilities were determined by an Alamar blue assay. The data were normalized by the data of the mock-infected group. B: OAd genome replication efficiencies in tumor cells. HeLa cells were infected with OAd at 100 virus particles (VP)/cell. At the indicated time points, the OAd genome copy numbers were determined by real-time polymerase chain reaction analysis and expressed as relative values (conventional OAd, 3 h=1).*

suggest that OAd-shCUL4A is a promising antitumor biotherapeutic for various types of tumor.

Conflicts of Interest

The Authors declare no competing financial interests in regard to this study.

Authors' Contributions

K.W. and F.S. designed and performed the experiments, analyzed data, and wrote the article. R.O. designed and performed the experiments, and analyzed data. T.F. provided the materials and supported the projects. H.M. wrote the article, and supervised the projects.

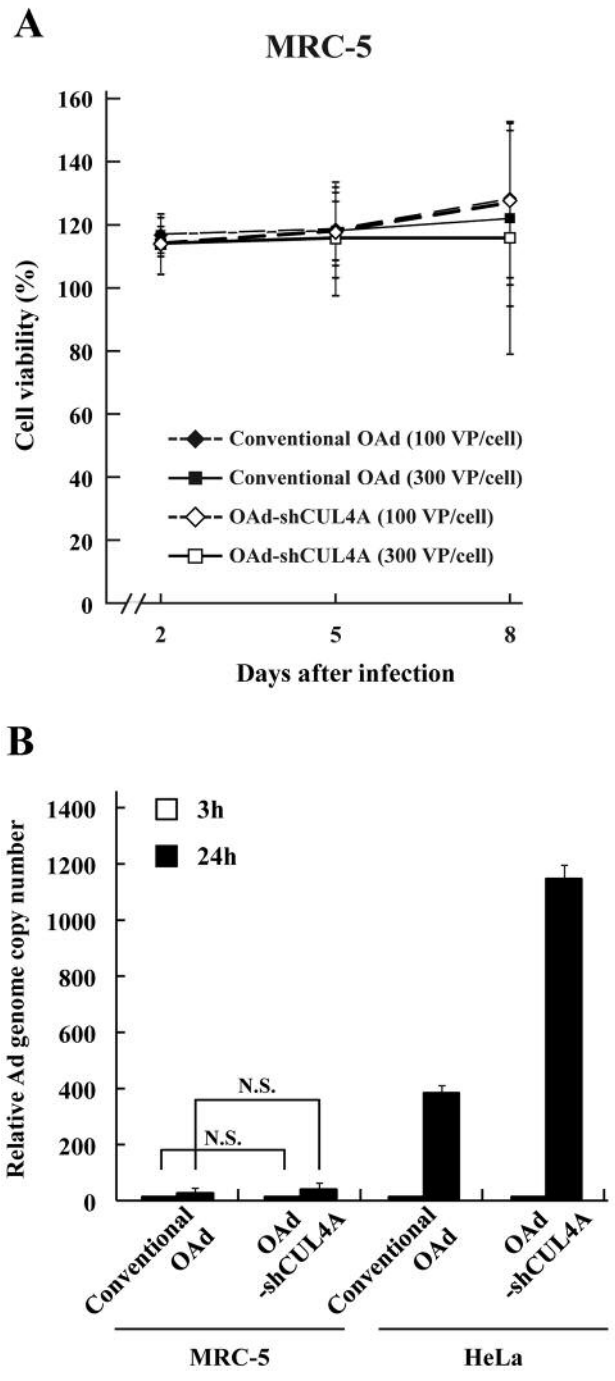

Figure 4. *A: Cytotoxicity levels of oncolytic adenovirus (OAd) expressing a short-hairpin RNA against CUL4A (shCUL4A) in human normal cells. MRC-5 cells were infected with an OAd at 100 and 300 virus particles (VP)/cell. At the indicated time points, the cell viability was determined by Alamar blue assay. The data were normalized by the data of the mock-infected group. B: OAd genome replication efficiencies in human normal cells. MRC-5 cells were infected with OAd at 100 VP/cell. After 3 and 24 h of incubation, the OAd genome copy numbers were determined by real-time polymerase chain reaction analysis and expressed as relative values (conventional OAd, 3 h=1). Note that the data for the HeLa cells are the same as those in Figure 3B. N.S., Not significantly different.*

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