CRISPR-Cas12a with an oAd Induces Precise and Cancer-Specific Genomic Reprogramming of *EGFR* and Efficient Tumor Regression

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CRISPR-Cas12a represents a class 2/type V CRISPR RNA-guided endonuclease, holding promise as a precise genome-editing tool *in vitro* and *in vivo*. For efficient delivery of the CRISPR-Cas system into cancer, oncolytic adenovirus (oAd) has been recognized as a promising alternative vehicle to conventional cancer therapy, owing to its cancer specificity; however, to our knowledge, it has not been used for genome editing. In this study, we show that CRISPR-Cas12a mediated by oAd disrupts the oncogenic signaling pathway with excellent cancer specificity. The intratumoral delivery of a single oAd co-expressing a Cas12a and a CRISPR RNA (crRNA) targeting the epidermal growth factor receptor (*EGFR*) gene (oAd/Cas12a/crEGFR) induces efficient and precise editing of the targeted *EGFR* gene in a cancer-specific manner, without detectable off-target nuclease activity. Importantly, oAd/Cas12a/crEGFR elicits a potent antitumor effect via robust induction of apoptosis and inhibition of tumor cell proliferation, ultimately leading to complete tumor regression in a subset of treated mice. Collectively, in this study we show precise genomic reprogramming via a single oAd vector-mediated CRISPR-Cas system and the feasibility of such system as an alternative cancer therapy.

INTRODUCTION

The type II clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated proteins (Cas) system has been widely used for genome editing and treatment of diverse genetic and non-genetic diseases.^{1–4} When used to target oncogenes, Cas9 derived from *Streptococcus pyogenes* (SpCas9) has led to potent antitumor effects.^{2,5,6} However, concerns about SpCas9-mediated off-target nuclease activity are well known.^{7–9} CRISPR-Cas12a, a CRISPR effector from *Prevotella* and *Francisella* 1 (Cpf1) derived from a type V CRISPR system, differs from SpCas9 in several key aspects.^{10,11} First, Cas12a exhibits ribonuclease activity, processing precursor CRISPR RNAs (crRNAs) into mature crRNAs, and is guided by a single crRNA, without the need for a *trans*-activating crRNA

(tracrRNA).^{10,11} Second, Cas12a recognizes a T-rich protospacer adjacent motif (PAM) (5'-TTTV-3') at the 5' end of a protospacer, generating staggered double strand breaks.¹² Third, Cas12a exhibits less off-target nuclease activity than does SpCas9, enabling more precise genome editing for therapeutic applications.^{7,8} Finally, the size of Cas12a is smaller than SpCas9. In particular, LbCas12a derived from *Lachnospiraceae* bacterium ND2006 has the smallest gene size among Cas12a orthologs (1,228 amino acids residues encoded by 3,684 bp genes). This difference provides an advantage for packaging of genes encoding LbCas12a and its crRNA into a single viral vector that is efficiently delivered to target cells,¹³ because optimal activity of viral vectors can only be achieved when the total size of the inserted transgenes does not exceed the packaging limitation.

Since the US Food and Drug Administration approved the first oncolytic virus therapy (Imlygic) in 2015,¹⁴ the numbers of oncolytic virus-related clinical trials and studies have grown rapidly.^{15,16} Recently, oncolytic adenovirus (oAd) has been highlighted as a promising alternative to conventional cancer therapy because of its ability to selectively propagate in and eradicate cancer cells through a domino-like cascading infection of tumor cells over multiple replication cycles; this is a unique feature of oncolytic viruses that other conventional therapeutics cannot mimic.^{17,18} In addition, oAd exerts anti-angiogenic effects,^{19,20} strong antitumor immunity,^{21–24} and synergistic anticancer effects when used in conjunction with standard cancer therapy (e.g., radiotherapy, chemotherapy, or

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immunotherapy).^{25–27} Despite many advantages of oAd, the virus lacking therapeutic transgenes has failed to demonstrate sufficient therapeutic benefit as a single agent in clinical trials.^{28,29} Thus, oAds armed with various therapeutic transgenes are under extensive investigation in the preclinical and clinical environment.³⁰ Such armed oAds induce a high level of transgene expression in targeted cancer cells but not in normal cells,³¹ improving both the therapeutic efficacy and safety profile in the complex human tumor environment.^{30,32}

Lung cancer causes more deaths than any other form of cancer.³³ About 75% of all lung cancers are non-small-cell lung cancers (NSCLCs), which exhibit high expression levels of receptor tyrosine kinases (RTKs).³⁴ Among several RTKs, the human epidermal growth factor receptor (EGFR) is the most commonly overexpressed in NSCLC; EGFR dysregulation leads to activation of oncogenic signaling pathways and exacerbates malignant phenotypic changes.^{35,36} Thus, tyrosine kinase inhibitor (TKI) therapies against EGFR have been actively developed by pharmaceutical companies to address this issue. Indeed, several clinically approved TKIs, which are a conventional treatment option for NSCLC, initially exert potent anticancer effects in NSCLC patients.^{37,38} However, the therapeutic benefit of TKIs drastically decreases during the treatment course due to acquisition of drug resistance by the tumors, thus severely restricting the benefit of these therapies in advanced stages of cancer and in recurrent tumors.^{39,40} These shortcomings necessitate the development of alternative and improved targeted therapies to address unmet patient needs.

Given these points, in this study, we hypothesized that an oAd expressing CRISPR-Cas12a that specifically targets *EGFR* in lung cancer would elicit potent antitumor effects through both virus-mediated oncolysis and CRISPR-Cas12a-mediated gene editing. To this end, we constructed an oAd co-expressing LbCas12a and a crRNA targeting *EGFR* (crEGFR) (oAd/Cas12a/crEGFR) and assessed its capability on genome editing and anticancer efficacy. We found that oAd/Cas12a/crEGFR disrupted the targeted oncogenic *EGFR* gene in a cancer-specific manner with high efficiency, while exerting minimal off-target nuclease activity in the whole genome, ultimately leading to potent anticancer effects both *in vitro* and *in vivo*. Collectively, these results indicate that the oAd/Cas12a/crEGFR-induced precise and selective genomic reprogramming and killing effects on cancer cells could be a promising strategy that ultimately enhances the therapeutic effects and safety profiles for treatment of cancer patients.

RESULTS

Genome Editing by CRISPR-Cas12a Targeting the *EGFR* Gene

To identify an effective crRNA for targeting *EGFR*, we constructed seven crRNAs that hybridize with different target sites in the *EGFR* gene (Table S1). Each crRNA includes an extra guanine (g) at the 5' end to allow for transcription under the control of the U6 promoter; the remaining 23 nt (X23) hybridize with a 23-nt target DNA sequence upstream of the PAM (5'-TTTV-3') sequence in the *EGFR* oncogene.

To evaluate Cas12a-mediated DNA cleavage in *EGFR*, plasmids encoding LbCas12a and each *EGFR*-specific crRNA were co-transfected into 293T cells, after which next-generation sequencing was used to determine the genome editing frequencies at the seven targeted *EGFR* sites. Cas12a-induced small insertions/deletions (indels) were detected in the *EGFR* gene with frequencies ranging from $48.2\% \pm 1.3\%$ to $22.0\% \pm 0.3\%$ at 48 h post-transfection (Figure S1). We determined that 54%–69% of the indels represented out-of-frame mutations (Figure S2). For use in further experiments, we selected crRNA-7 (crEGFR-TS7), which targets *EGFR* exon 3 (Figure 1A), because it resulted in a high indel frequency ($44.9\% \pm 0.8\%$) and a high ratio of out-of-frame mutations ($68.7\% \pm 0.3\%$) (Figures S1 and S2). In A549 human lung cancer cells, Cas12a with crEGFR-TS7 induced indels at the *EGFR* gene with a frequency of $22.7\% \pm 0.6\%$ at 48 h post-transfection (Figure S3).

Therapeutic Genome Editing by oAd Co-expressing Cas12a and crRNA Targeting the *EGFR* Gene *In Vitro* and *In Vivo*

With the aim of efficiently and specifically disrupting the *EGFR* gene and inducing a therapeutic effect in lung cancer cells, we applied an oAd-based CRISPR-Cas12a system. Given the small size of the genes encoding Cas12a and its crRNA, we successfully generated a single oAd vector co-expressing Cas12a and crEGFR-TS7 (oAd/Cas12a/crEGFR), as well as a control oAd expressing Cas12a alone (oAd/Cas12a), at high titers (Figure 1B; Figure S4). oAd/Cas12a/crEGFR-induced indel frequencies at the *EGFR* target site were $96.6\% \pm 0.4\%$, $92.4\% \pm 0.3\%$, and $78.7\% \pm 0.1\%$ in A549, H1299, and PC9 lung cancer cell lines, respectively, 24 and 48 h after Ad infection at an MOI of 200, whereas no indels were detectably observed in normal human dermal fibroblasts (HDFs) (Figure 1C). These results suggest that oAd/Cas12a/crEGFR can efficiently disrupt target loci in a cancer-specific manner.

To further evaluate the genome editing efficiencies of oAd/Cas12a/crEGFR *in vivo*, A549 xenograft tumors were intratumorally treated with 2×10^{10} viral particles (VP) of oAd/Cas12a, oAd/Cas12a/crEGFR, or phosphate-buffered saline (PBS) alone. The oAd/Cas12a/crEGFR treatment led to indels with a frequency of $35.4\% \pm 3.3\%$, $5.12\% \pm 2.1\%$, or $1.16\% \pm 0.5\%$ at 24, 48, or 72 h after virus injection into A549 tumors, respectively, whereas oAd/Cas12a- or PBS-treated tumors did not show any detectable indels (Figure 1D; Figure S5). Of note, no indels were detected in normal tissues in any of the three groups, demonstrating the cancer specificity of oAd/Cas12a-mediated genome editing. Of the indels induced by oAd/Cas12a/crEGFR, $71.0\% \pm 2.7\%$ were out-of-frame mutations (Figure 1E), suggesting that approximately 25.8% of *EGFR* sequences in lung tumor xenografts are efficiently knocked out via a single injection of oAd/Cas12a/crEGFR.

Genome-wide Analysis of oAd/Cas12a/crEGFR Specificity *In Vitro* and *In Vivo*

We next investigated whether Cas12a exhibits off-target nuclease activity in various lung cancer cells or in the A549 xenograft model. For genome-wide analysis of the specificity of the *EGFR*-targeting Cas12a

Figure 1. oAd/Cas12a/crEGFR-Mediated Genome Editing in A549 Lung Cancer Cells

(A) The protospacer of crRNA sequences (1–7) in *EGFR* gene. The PAM sequence and the crRNA7 target sequence are shown in red and blue, respectively. (B) Vector schematics of oAds expressing either Cas12a (oAd/Cas12a) or co-expressing Cas12a and crRNA targeting the *EGFR* gene in a single oAd vector. (C) *EGFR* mutation frequencies in A549, H1299, and PC9 lung cancer cell lines and normal HDFs infected with oAd/Cas12a or oAd/Cas12a/crEGFR. Error bars indicate Standard error of the mean (SEM) ($n = 3$). (D) *EGFR* mutation frequencies in A549 xenograft tumors following intratumoral injection of 2×10^{10} VP of oAd/Cas12a or oAd/Cas12a/crEGFR. Error bars indicate SEM ($n = 3$ –4). (E) The ratio of mutant sequences with in-frame versus out-of-frame indels at the *EGFR* TS7 target site in A549 tumors. Error bars indicate SEM ($n = 4$). The number of mutant sequence reads binned by the deletion or insertion size in base pairs and representative five top mutant *EGFR* sequences are presented. The red triangle indicates the cleavage position.

Figure 2. Specificity of oAd/Cas12a/crEGFR-Mediated Genome Editing

(A) Genomic DNA isolated from A549 cells was digested *in vitro* by a Cas12a ribonucleoprotein complex targeted to the *EGFR* gene and subjected to WGS. The Circos plot shows DNA cleavage scores across the human genome. Arrows indicate on-target (ON) or off-target (OT) sites. N indicates the number of *in vitro* cleavage sites identified by Digenome-seq. (B) Indel frequencies in oAd/Cas12a/crEGFR-infected A549 cells at the six OT sites and one ON site identified by Digenome-seq. At 24 h post-infection with oAd/Cas12a/crEGFR, genomic DNA was isolated from A549 cells and subjected to targeted deep sequencing. (C) Indel frequencies in oAd/Cas12a/crEGFR-treated A549 tumor xenografts at the six OT sites and one ON site identified by Digenome-seq. Mismatched nucleotides are shown in red, and PAM sequences are shown in blue. Error bars indicate SEM (n = 4).

2C; Table S3). Off-target indels were also not detectably induced in A549, H1299, and PC9 lung cancer cells at 48 h post-infection (Figure S6). These results demonstrate that the oAd/Cas12a/crEGFR is targetable to *EGFR* in a highly specific manner both *in vitro* and *in vivo*.

Efficient *EGFR* Downregulation and Potent Cancer Cell Killing Effect of oAd/Cas12a/crEGFR in Lung Cancer Cell Lines

To investigate whether CRISPR-Cas12a-mediated cleavage and disruption of the *EGFR* gene would translate into a reduction in the *EGFR* expression level, we performed quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and fluorescence-activated cell sorting (FACS) analysis. As shown in Figures 3A and 3B, *EGFR* mRNA and protein expression levels were significantly decreased in A549 cells by oAd/Cas12a/crEGFR treatment compared to oAd/Cas12a (**p < 0.001). Similarly, oAd/Cas12a/crEGFR-mediated attenuation of *EGFR* expression levels were observed in H1299 and PC9 lung cancer cells (Figures S7 and S8; ***p < 0.001, **p < 0.01). In sharp contrast, oAd/Cas12a/crEGFR did not alter *EGFR* expression levels in normal HDFs. These results indicate that efficient and cancer-specific genome editing mediated by oAd/Cas12a/crEGFR directly translates into inhibition of *EGFR* expression.

nuclease, we first carried out nuclease-digested whole-genome sequencing (Digenome-seq). A549 cell-free genomic DNA was digested *in vitro* using an *EGFR*-targeting Cas12a ribonucleoprotein (RNP) complex and then subjected to whole-genome sequencing (WGS). Seven cleavage sites, including the one on-target site and six off-target sites, were observed in the human genome (Figure 2A; Table S2).

Next, to evaluate the target specificity of the oAd-mediated CRISPR-Cas12a, targeted deep sequencing was performed at the six off-target sites captured by Digenome-seq using genomic DNA isolated from cells and tumor tissues 24 h after oAd/Cas12a/crEGFR treatment. No off-target indels were detectably induced in either A549, H1299, or PC9 lung cancer cells or A549 tumor xenografts (Figures 2B and

2C; Table S3). Off-target indels were also not detectably induced in A549, H1299, and PC9 lung cancer cells at 48 h post-infection (Figure S6). These results demonstrate that the oAd/Cas12a/crEGFR is targetable to *EGFR* in a highly specific manner both *in vitro* and *in vivo*.

Next, we assessed the cancer cell-specific killing effect of oAd/Cas12a/crEGFR via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in lung cancer and normal cells. Both oAd/Cas12a and oAd/Cas12a/crEGFR induced a cancer cell killing effect in a time-dependent manner (Figure 3C). Importantly, oAd/Cas12a/crEGFR elicited a 2.2-, 2.2-, or 1.6-fold more potent cancer

Figure 3. oAd/Cas12a/crEGFR Inhibits *EGFR* Expression and Induces Cancer Cell-Specific Toxicity

(A) Total RNA was isolated from A549 or HDF cells infected with oAd/Cas12a or oAd/Cas12a/crEGFR, and quantitative real-time PCR was performed using an *EGFR* primer set. Bars represent mean $\pm$ standard deviation (SD) (** $p < 0.001$). (B) A549 and HDF cells were infected with oAd/Cas12a or oAd/Cas12a/crEGFR (A549 cells, MOI of 50; HDFs, MOI of 200). Cell pellets were probed with EGFR-specific antibodies. Fluorescein isothiocyanate (FITC) intensity was assessed by flow cytometry. *** $p < 0.001$. (C) A549, H1299, PC9, and HDF cells were infected with oAd/Cas12a or oAd/Cas12a/crEGFR. At 24, 48, and 72 h post-infection, an MTT assay was performed to quantify the percentage of living cells. ** $p < 0.01$, *** $p < 0.001$, oAd/Cas12a versus oAd/Cas12a/crEGFR. (D) Total DNA was extracted from oAd/Cfp1/crEGFR-treated A549, H1299, PC9, and HDF cells. The Ad genome copy number was determined by quantitative real-time PCR.

cell killing effect than did control oAd/Cas12a in A549, H1299, or PC9 lung cancer cells, respectively, at 72 h post-infection (** $p < 0.001$). In marked contrast, both oAd/Cas12a and oAd/Cas12a/crEGFR induced negligible and comparable cytotoxicity in normal HDFs. In line with these results, viral replication of oAd/Cas12a/crEGFR occurred at 64.9- to 8,894.9-fold higher levels in lung cancer cells versus normal HDFs (Figure 3D; *** $p < 0.001$), suggesting that cancer-specific replication of oAd at a high level contributes to the potent anticancer effect of oAd/Cas12a/crEGFR and its minimal cytotoxicity in normal cells. To evaluate the effects of Cas12a and crEGFR on normal cells upon their release resulting from virus replication-induced cytolysis of cancer cells, we co-cultured oAd/Cas12a/crEGFR-infected A549 and HDF cells in a transwell system (Figure S9). The cell viability of A549 cells was significantly decreased by oAd/Cas12a/crEGFR at 72 h. In contrast, no observable cytotoxicity was observed in normal HDFs. Moreover, the indels were not detectably induced in normal HDFs. Collectively, these results indicate that oAd/Cas12a/crEGFR can effectively induce a potent anticancer effect through cancer-specific editing of *EGFR* and viral replication-mediated preferential cytolysis of cancer cells.

Potent Antitumor Effect of oAd/Cas12a/crEGFR in the Lung Cancer Xenograft Model via Efficient Genome Editing

To evaluate the antitumor effect of the oAd-based CRISPR-Cas12a system, A549 xenograft tumors were treated with PBS, oAd/Cas12a, or oAd/Cas12a/crEGFR via intratumoral injection (three injections of 2×10^{10} VP). As shown in Figure 4A, PBS-treated tumors grew rapidly and reached an average volume of $1,340.6 \pm 133.4 \text{ mm}^3$ by day 35 after the initial treatment. Both oAd/Cas12a- and oAd/Cas12a/crEGFR-treated tumors showed a significant reduction in tumor volume, by 42.2% and 88.6%, respectively, compared to the PBS control (** $p < 0.001$). Similar trends were observed by end-point tumor imaging and weighing (Figure 4B; *** $p < 0.001$, ** $p < 0.01$). Taken together, these results indicate that the oAd-mediated Cas12a system targeting *EGFR* induces a potent antitumor effect without observable toxicity.

To further assess the mechanism behind the potent antitumor effect of the oAd-based gene editing system, tumor tissues were assessed by histological and immunohistochemical analysis. Hematoxylin and eosin (H&E) staining revealed large areas and quantities of tumor

Figure 4. Antitumor Effect of oAd/Cas12a/crEGFR in the A549 Tumor Xenograft Model

(A) On day 1, A549 tumor-bearing mice were given an intratumoral injection of 2×10^{10} VP of oAd/Cas12a or oAd/Cas12a/crEGFR, along with PBS as a control. Tumor growth was monitored every other day until the end of the study. Values represent the mean $\pm$ SD ($n = 6$ per group). *** $p < 0.001$. (B) At 35 days after the initial treatment, the mice were sacrificed, and the tumors were excised. Tumor sizes and weights were then compared. ** $p < 0.01$, *** $p < 0.001$. (C–E) Tumors treated with PBS, oAd/Cas12a, or oAd/Cas12a/crEGFR were harvested on day 7 for histological and immunohistochemical analysis ($n = 3$ per group). H&E staining (C) and immunohistochemical staining (D and E) of PCNA (D) and EGFR (E), respectively.

cells in PBS-treated tumor tissues, whereas markedly reduced numbers of tumor cells, as well as moderate or extensive necrosis, were observed in oAd/Cpf1- or oAd/Cpf1/crEGFR-treated tumor tissues, respectively (Figure 4C). The frequencies of terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive spots in tumor tissues were in line with the results from H&E staining, as oAd/Cpf1/crEGFR-treated tumors showed more apoptotic cells than did any other tested group (Figure S10). In addition, oAd/Cas12a/crEGFR-treated tumor tissues showed a lower level (75.9% or 72.5%, respectively) of proliferating cell nuclear antigen (PCNA)-positive spots than did those treated with PBS or oAd/Cas12a (Figure 4D; $***p < 0.001$ and $*p < 0.05$, respectively). Importantly, the tumor tissues treated with oAd/Cas12a/crEGFR showed a significant reduction in EGFR expression (91.6% or 78.1%, respectively) compared with those treated with either PBS or oAd/Cas12a (Figure 4E; $*p < 0.05$ and $**p < 0.01$, respectively), implying that oAd-expressing CRISPR-Cas12a can efficiently target and attenuate EGFR expression. Taken together, these results show that oAd-mediated CRISPR-Cas12a can efficiently target and attenuate EGFR expression in lung tumors, ultimately leading to inhibition of tumor cell proliferation and an increase in apoptotic tumor cell death.

DISCUSSION

The field of genome editing has been growing at an exponential rate, since the adaptation of the CRISPR-Cas-based system as a genetic reprogramming tool within the last decade.^{9–12} Despite this rapid growth, there are only a few reports available to date exploring the therapeutic potential of genome editing technology in cancer therapy.^{2,4} In this regard, our pioneering work in 2017 utilizing replication-incompetent Ads (two vector system)-based genome editing of the oncogenic *EGFR* mutant allele in lung cancer clearly illustrated that the CRISPR-Cas system could translate into an anticancer effect.⁵ Based on these promising results, we sought to further improve the anticancer effect of the genome editing system by utilizing oAd as the vector of choice. To this end, in this study, we demonstrate the potent anticancer effect of genome editing of an oncogene via a single oAd vector system and its therapeutic potential. Our findings demonstrate that LbCas12a induces efficient knockout of an oncogene in a cancer-specific mode, ultimately leading to potent therapeutic efficacy for the treatment of lung cancer with minimal off-target effects.

In the last two decades, oAd has been extensively developed and actively tested in clinical trials, since the first clinical evaluation of ONYX-015 (now marketed in China as Oncorine). Current generation of oAds in ongoing clinical trials use more extensive and diverse genetic modifications than are present in ONYX-015 to achieve better antitumor efficacy and cancer selectivity. For example, DNX-2401⁴¹ is an oAd that is actively being investigated in several ongoing clinical trials (ClinicalTrials.gov: NCT03714334, NCT03178032, and NCT02798406). In line with these recent clinical developments, oAd/Cas12a/crEGFR utilized in the present study was constructed from an oAd backbone (RdB-RGD).⁴² RdB-RGD contains (1) an

Rb binding site mutation in the Ad E1A region and E1B55 kDa gene deletion that strengthen tumor specificity of Ad;^{42,43} (2) E1B19 deletion enhancing cancer cell killing efficacy of Ad;⁴³ and (3) insertion of the tumor targeting RGD motif in the fiber region of the viral capsid to enhance internalization of Ad into cancer cells.⁴⁴

The oAd-mediated CRISPR-Cas12a provides multiple advantages with respect to safety. First, CRISPR-Cas12a induces fewer off-target cleavages than does the most commonly and extensively studied system, CRISPR-SpCas9.^{7,8} In line with this trait, our current study clearly demonstrated that oAd/Cas12a/crEGFR induced high frequencies of indels at the targeted site (Figure 1C) with no observable off-target genome editing *in vitro* or *in vivo* (Figures 2B and 2C). Furthermore, cancer-specific replication of oAd should provide an additional layer of cancer specificity for genome editing because oAd-induced expression of therapeutic transgenes is selectively and exponentially amplified along with viral replication in a cancer cell-specific manner.⁴⁵ This phenomenon was confirmed by our results demonstrating excellent cancer-specific viral replication, cell killing effects, and genome editing by oAd/Cas12a/crEGFR (Figures 1C, 1D, and 3). Considering that many of the new cancer therapeutics currently being tested in phase I trials fail to overcome safety issues, the oAd-mediated CRISPR-Cas12a system could be an excellent candidate that demonstrates strong safety profiles as well as therapeutic efficacy.

In-frame mutations caused by the deletion of 3, 6, or 9 nt or other triple nucleotide combinations are more likely to be induced by Cas12a than by Cas9.⁷ In-frame deletions can potentially lead to the production of partially functional proteins; therefore, out-of-frame mutations are preferred in gene-nullifying applications. Thus, our system described in the present report utilized crEGFR-TS7, which induces a high overall indel frequency with the highest proportion of out-of-frame mutations (Figures S1 and S2) that eventually leads to efficient knockout of *EGFR* gene in cancer cells following oAd/Cas12a/crEGFR treatment (Figures 3A, 3B, and 4E).

Of interest, a time-dependent decrease in *EGFR* indel rate was observed following oAd/Cas12a/crEGFR infection of A549 tumors (24–72 h after the intratumoral injection; Figure 1D; Figure S5). One possible explanation for a time-dependent decrease is due to the cell death of infected tumor cells by potent oncolytic and gene editing effects of oAd/Cas12a/crEGFR. It is supported by diminished cancer cell viability to an extremely low level as early as 24 h post-infection (Figure 3A).

The CRISPR-Cas12a system can simultaneously edit multiple genes through the use of a single RNA polymerase III (RNA Pol III) promoter regulating the expression of a crRNA multiplex.⁴⁶ This attribute is particularly promising for cancer therapy where tumor heterogeneity and complex oncogenic alterations affecting multiple signaling axes pose numerous challenges for both conventional and targeted cancer therapies. For example, targeted therapeutics such as TKIs can be easily and completely nullified via a minute change

such as a single amino acid substitution or compensating activation of an alternative oncogenic signaling pathway.^{47,48} In this regard, we foresee that oAd-induced CRISPR-Cas12a multiplexing systems, which could potentially nullify multiple oncogenes, could be a powerful extension of our current approaches to address the complexity of heterogenic tumors where the concurrent activation of multiple oncogenic signaling pathways increases the overall malignancy of the disease.

Collectively, our findings clearly demonstrate for the first time that oAd-mediated gene editing can (1) precisely and selectively reprogram a targeted oncogene, (2) attenuate potential risks that may arise from off-target genome editing, (3) efficiently reduce the *EGFR* expression level, and (4) induce cancer cell killing through cancer-specific editing of *EGFR* and viral replication-mediated preferential cytolysis of cancer cells, thus leading to potent antitumor effects. Our findings indicate that CRISPR-Cas12a-mediated oncogene disruption, in conjunction with the strong oncolytic effect of oAd, will be an effective strategy for inducing potent antitumor effects clinically.

MATERIALS AND METHODS

Cell Lines and Cell Culture

HEK293 cells (a human embryonic kidney cell line expressing the Ad E1 region), human lung cancer cell lines (A549, H1299, and PC9), and normal HDFs were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), L-glutamine (2 mM), penicillin (100 IU/mL), and streptomycin (50 µg/mL).

Genomic DNA Extraction

293T cells (1×10^5) were seeded into 24-well plates 1 day prior to transfection and transfected with the crRNA plasmid (1,500 ng) and the Cas12a plasmid (500 ng) using 4 µL of Lipofectamine 2000 (catalog no. 11668019, Invitrogen, Carlsbad, CA, USA). Cells were maintained in DMEM supplemented with 10% FBS. At 48 h after transfection, genomic DNA was isolated using a DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany). To assess the genome editing efficiency of oAd/Cas12a/crEGFR, various lung cancer cells and normal cells were treated with oAd/Cas12a or oAd/Cas12a/crEGFR; genomic DNA was extracted from these cells at 24 h post-infection. Genomic DNA was isolated from tumors treated with either oAd/Cas12a/crEGFR or oAd/Cas12a (a total of 2×10^{10} VP in 30 µL of PBS) 24 h after injection.

Construction of Plasmids Encoding Cas12a and crEGFR

A human codon-optimized LbCas12a coding sequence, derived from *Lachnospiraceae* bacterium ND 2006 (Addgene, Watertown, MA, USA) was cloned into a pcDNA3.1 vector plasmid. The *EGFR*-specific crRNA sequence 1–7 (Table S1) was inserted to pRG vector plasmid (Addgene). crRNAs were transcribed under the control of the U6 promoter,

and LbCpf1 expression was controlled by the cytomegalovirus (CMV) promoter.

Mutation Analysis

On-target or predicted off-target loci were amplified from genomic DNA for targeted deep sequencing. Deep sequencing libraries were generated by PCR. TruSeq HT dual index primers were used to label each sample. Pooled libraries were subjected to paired-end sequencing using MiniSeq (Illumina, San Diego, CA). Indel frequencies are described in Tables S2 and S3.

Construction of oAd Encoding Cas12a and crEGFR

To generate an oAd construct that co-expresses Cas12a at the E1 region and an *EGFR*-targeting crRNA sequence at the E3 region, we first obtained a fragment containing a human codon-optimized Cas12a sequence (derived from *Lachnospiraceae* bacterium ND 2006) by digesting pcDNA3.1-hCas12a (Addgene) with *Sna*BI/*Xho*I. The Cas12a sequence was then subcloned into the modified Ad E1 shuttle vector, pXC1-RdB,⁴² which had been digested with *Sna*BI/*Sa*II, to create pXC1-RdB-hCas12a. The newly generated shuttle vector was linearized by *Nru*I digestion and transformed, together with *Bst*BI-digested Ad total vector harboring an RGD motif in the HI loop of Ad, into *Escherichia coli* BJ5183 for homologous recombination, generating RdB-RGD/hCas12a (oAd/Cas12a). Then, an Ad E3 shuttle vector harboring the U6 promoter and crEGFR (5'-GTAGTACATA TTTCTCTGATGA-3') was generated through ligation. The E3 shuttle vector was linearized with *Xmn*I and co-transformed into *Escherichia coli* BJ5183 along with *Spe*I-digested RdB-RGD/hCas12a for homologous recombination, generating RdB-RGD/Cas12a/crEGFR (oAd/Cas12a/crEGFR).

Ad Production

All viruses were propagated in HEK293 cells, and the purification, titration, and quality analysis of all Ads were performed as previously described.⁴⁹ The number of VP was calculated from measurements of optical density at 260 nm (OD₂₆₀), at which an absorbance value of 1 is equivalent to 1.1×10^{12} VP/mL. Infectious titers (plaque-forming units per milliliter) were determined by a limiting dilution assay on HEK293 cells.⁵

Dig genome-Seq

Genomic DNA was isolated from A549 cells using a DNeasy Blood & Tissue kit (QIAGEN) according to the manufacturer's instructions. Genomic DNA (8 µg) was mixed with Cas12a protein (300 nM) and crRNA (900 nM) in 400 µL of reaction buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, and 100 µg/mL BSA) and the mixture was incubated for 8 h at 37°C. Digested genomic DNA was then incubated with RNase A (50 µg/mL) for 30 min at 37°C to degrade crRNAs and purified again with a DNeasy Blood & Tissue kit (QIAGEN). Digested DNA was fragmented using the Covaris system and ligated with adapters for library formation. DNA libraries were subjected to WGS using an Illumina HiSeq X Ten sequencer (Macrogen, South Korea). We used the Isaac aligner to generate a Bam file using the following parameters: ver. 01.14.03.12; human genome reference, mm10 from

UCSC; base quality cutoff, 15; keep duplicate reads, yes; variable read length support, yes; realign gaps, no; and adaptor clipping, yes (adaptor: 5'-AGATCGGAAGAGC*-3', 5'-*GCTCTTCCGATCT-3'). A DNA cleavage score was assigned to each nucleotide position across the entire genome, using WGS data, according to the equation presented in Kim *et al.*⁷ These equations assume that Cas12a produces 1- to 5-nt 5' overhangs. *In vitro* cleavage sites with DNA cleavage scores above the cutoff value of 2.5 were computationally identified.

MTT Assay

To evaluate the cancer cell killing effect of oAd/Cas12a/crEGFR, 2×10^4 A549 or HDF cells were plated onto a 24-well plate and then infected with oAd/Cas12a or oAd/Cas12a/crEGFR at an MOI ranging from 100 to 200. At 24, 48, and 72 h after treatment, the MTT assay was carried out as previously described.⁴⁹ In brief, 200 μ L of MTT (Sigma, St. Louis, MO, USA) in PBS (2 mg/mL) was added to each well. After a 4-h incubation at 37°C, the supernatant was discarded and the precipitate was dissolved in 1 mL of dimethyl sulfoxide. Plates were then read on a microplate reader at 540 nm.

qRT-PCR

Total RNA was isolated from A549 and HDF cells treated with oAd/Cas12a or oAd/Cas12a/crEGFR, as well as untreated cells as a negative control, using TRIzol reagent (Invitrogen). cDNAs were synthesized using 5 μ g of total RNA, oligo(dT) (2.5 mM), and SuperScript II reverse transcriptase (200 U) in 20- μ L total volume using a reverse transcription kit (Invitrogen). qPCR was performed using SYBR Green master mix (Applied Biosystems, Carlsbad, CA, USA). The following qPCR oligonucleotide primers sets were used: EGFR forward (5'-CCAAGGGAGTTTGTGGAGAA-3'), EGFR reverse (5'-CTTCCAGACCAGGTGTTGT-3'), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward (5'-CCCCTTCATTGACCTCAACTAC-3'), and GAPDH reverse (5'-TCTCGCTCCTGGAAGATGG-3').

FACS Analysis

A549 and HDF cells treated with oAd/Cas12a or oAd/Cas12a/crEGFR, along with untreated cells as a negative control, were probed with anti-EGFR antibodies (Millipore, Burlington, MA, USA). Goat anti-mouse immunoglobulin (Ig)G antibody conjugated with FITC (SouthernBiotech, Birmingham, AL, USA) was utilized as secondary antibody. Quantitative analysis of the sample was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data from 10,000 events.

Animal Experiments

All aspects of animal care and treatment were performed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal studies were performed according to the institutionally approved protocols of Hanyang University. All mice were housed for 1 week for acclimatization, and *ad libitum* access to food and water was provided. Tumors were implanted in the abdomens of 5- to 6-week-old male nude mice by subcutaneous

injection of A549 cells (5×10^6 cells in 100 μ L of Hank's balanced salt solution [HBSS; Gibco-BRL]). When tumor volumes reached 70–100 mm³, animals were randomly assigned to one of three groups to receive PBS, oAd/Cas12a, or oAd/Cas12a/crEGFR (n = 6 per group). The first day of treatment was designated as day 1. All treatments were administered intratumorally (a total of 2×10^{10} VP in 30 μ L of PBS) on day 1. Tumor growth inhibition was assessed every other day by measuring the length (*L*) and width (*w*) of the tumor and determining the tumor volume, which was calculated using the following formula: volume = $0.523L(w)^2$. To evaluate the efficacy of gene editing in the tumor xenograft model, subcutaneously established A549 (5×10^6 cells) tumors were randomly assigned to one of three groups to receive 2×10^{10} VP of oAd/Cas12a or oAd/Cas12a/crEGFR, along with PBS as negative control. Indel frequencies were assessed by deep sequencing of genomic DNA isolated from tumors 24, 48, or 72 h after injection.

Histological and Immunohistochemical Analysis

Tumors treated with PBS, oAd/Cas12a, or oAd/Cas12a/crEGFR were harvested on day 7 for histological and immunohistochemical analysis (n = 3 per group). Representative sections were stained with H&E and then examined by light microscopy (Carl Zeiss, Oberkochen, Germany). For immunohistochemistry, slides were deparaffinized in xylene and then processed as previously described.^{20,31,50,51} A549 tumor tissue sections were incubated at 4°C overnight with mouse anti-proliferating cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark) or goat anti-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies, rinsed, and then incubated for 30 min with biotinylated anti-mouse (Cell Signaling Technology, Danvers, MA, USA) or anti-goat secondary antibodies (Dako), respectively. Sections were then rinsed in PBS and incubated with streptavidin-peroxidase. All slides were counterstained with Meyer's hematoxylin (Sigma). The immunohistochemical staining results were semiquantitatively analyzed using MetaMorph image analysis software (Universal Image, Buckinghamshire, UK).

Statistical Analysis

No statistical methods were used to predetermine sample sizes for *in vitro* or *in vivo* experiments. All results are expressed as the mean $\pm$ SEM unless indicated otherwise. Comparisons between groups were made using the two-tailed Student's *t* test or one-way ANOVA and Tukey's *post hoc* tests for multiple groups. Statistical significance is denoted as **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Statistical analysis was performed in GraphPad Prism 5 (GraphPad, San Diego, CA, USA) or using SPSS software (SPSS, Chicago, IL, USA).

Data Availability

The deep sequencing data from this study have been submitted to the NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under accession number PRJNA560758. We have included the read depth information of the deep sequencing data in Tables S2 and S3.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.ymthe.2020.07.003>.

AUTHOR CONTRIBUTIONS

Conception and design: A.-R.Y., T.K., and C.-O.Y. Performing the experiments: A.-R.Y., B.-K.J., E. Chung, E. Choi, and J.H. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.-R.Y. and T.K. Writing, review, and/or revision of the manuscript: A.-R.Y., T.K., J.H., and C.-O.Y. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.-R.Y., J.-S.K., T.K., and C.-O.Y. Study supervision: J.-S.K., T.K., and C.-O.Y.

CONFLICTS OF INTEREST

J.H. is a researcher at GeneMedicine. C.-O.Y. is the CEO at GeneMedicine. The remaining authors declare no competing interests.

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