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# Establishment of a novel cell line for producing replication-competent adenovirus-free adenoviruses

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## Abstract

Adenoviruses are commonly utilized as viral vectors for gene therapy, genetic vaccines, and recombinant protein expression. To generate replication-defective adenoviruses, E1-complementing cell lines such as HEK293A are utilized; however, limitations remain. Repeated passage of E1-deleted virus in HEK293A cells increases the occurrence of replication-competent adenoviruses (RCAs). In the present study, we developed a novel cell line originating from human primary cells. L132 cells were transduced two times with E1-encoded retrovirus and three times with E1A-encoded retrovirus. Finally, we selected the most productive L132 cell line for generation of RCA-free adenovirus, GT541. GT541 can serve as an alternative cell line to HEK293A and other adenovirus-producing cells.

**Keywords** Adenoviruses, Replication-competent adenovirus, Cell line, E1, L132

## Introduction

Adenoviruses can be used as vectors for gene therapy, genetic vaccines, and recombinant protein expression [1, 2]. Compared with other viral vectors, including retro- or lentiviruses, adenoviruses possess a large gene delivery capacity, can infect both dividing and nondividing cells, and can efficiently deliver target genes in vivo and remains mainly episomal. Based on these advantages, adenoviruses are utilized as a vector for gene therapy and a delivery system for genetic vaccines in clinical settings [3–5].

The commonly used adenoviruses have been engineered as replication-defective adenoviruses in which early region 1 (E1) has been deleted [6]. This adenovirus can be replicated in high yields only in packaging cell lines that provide the E1 function. Human embryonic kidney 293 (HEK293) [7] and PER.C6 cells [8] are widely utilized E1-complementing cell lines for producing adenoviruses. However, these existing cell lines have limitations, necessitating the development of alternative cell lines for more efficient adenovirus production.

HEK293 cells contain the sequence from 1 to 4344 base pairs (bp) of the adenovirus nucleotide, facilitating E1 complementation. E1 is an essential gene for viral replication [9]. The adenoviral sequences in HEK293 cells contain regions homologous to the adenovirus vector, generating replication-competent adenoviruses (RCAs) through double crossover recombination. Repeated passaging during large-scale production and clinical applications increases RCA occurrence. In contrast, PER.C6 cells, originating from human embryonic retinal cell lines, have minimum adenovirus sequences (459–3510 bp of adenovirus sequences), which prevents RCA

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production but are still capable of producing “atypical” RCA [8, 10]. PER.C6 cells can be easily transfected, exhibit high expression of transferred genes, and are well characterized and fully documented [4]. PER.C6™ is licensed by various companies, including Merck & Co., GlaxoSmithKline, and Novartis (<https://www.bioprocessonline.com/doc/perc6-manufacturing-platform-0001>). However, owing to licensing costs, the general application of this cell line is strictly limited.

We generated E1-complementary cells which have a part of adenoviral E1 (560 bp–3509 bp of adenovirus sequences) to minimize the homologous recombination with viral genome. The present study introduces two distinctive technical aspects compared to existing technologies. First, stable insertion of the E1 gene into the host cellular genome is achieved by retroviral vectors, instead of plasmids. Second, to increase the production yield of recombinant adenovirus, total E1 gene was delivered into the host cells, and subsequently, additional E1A genes were transduced repeatedly. The E1 gene products are essential for virus replication [9]. E1A induces cell proliferation, which support viral replication. E1A inhibits negative regulators of cell cycle, which cause apoptosis rather than growth arrest [11]. On the other hand, E1B 55-kDa protein (E1B-55 K) of Ad5 interacts with p53, thus blocking p53-dependent apoptosis [12]. E1B 19-kDa protein (E1B-19 K) is also anti-apoptotic protein thorough interacting with Bax protein [13]. Therefore, both E1A and E1B is important to establish the E1-complementary cells for virus production. Finally, we selected

the most productive RCA-free adenovirus producing cell line, named as GT541 (Fig. 1).

The established GT541 is suitable for adenovirus production. Since GT541 is human origin cell, it can produce recombinant proteins which have the matched glycosylation patterns with endogenous proteins. Therefore, this cell-line may be an alternative to xenogenic cell lines such as Chinese hamster ovary cells and Madin-Darby canine kidney cells, as recombinant protein producing cell-lines.

## Materials and methods

### Cell-lines and reagents

BJ, ARPE-19 and HT1080 were purchased from the American Tissue Culture Collection (VA, USA). BJ cells are normal fibroblast cells derived from the foreskin of male neonates. They were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1% nonessential amino acids, and 1% penicillin. ARPE-19 cells are retinal pigment epithelial cells derived from the normal eyes of a man. These cells were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin–streptomycin. HT1080 cells are epithelial cells derived from the connective tissue of a patient with fibrosarcoma. L132 cells (Korean Cell Line Bank) were derived from the embryonic lung tissue. HT1080 and L132 cells were cultured in Eagle's minimum essential medium supplemented with 10% FBS and 1% penicillin–streptomycin. HEK293A cells (ThermoFisher, MA, USA) were cultured in DMEM supplemented with 10% FBS and

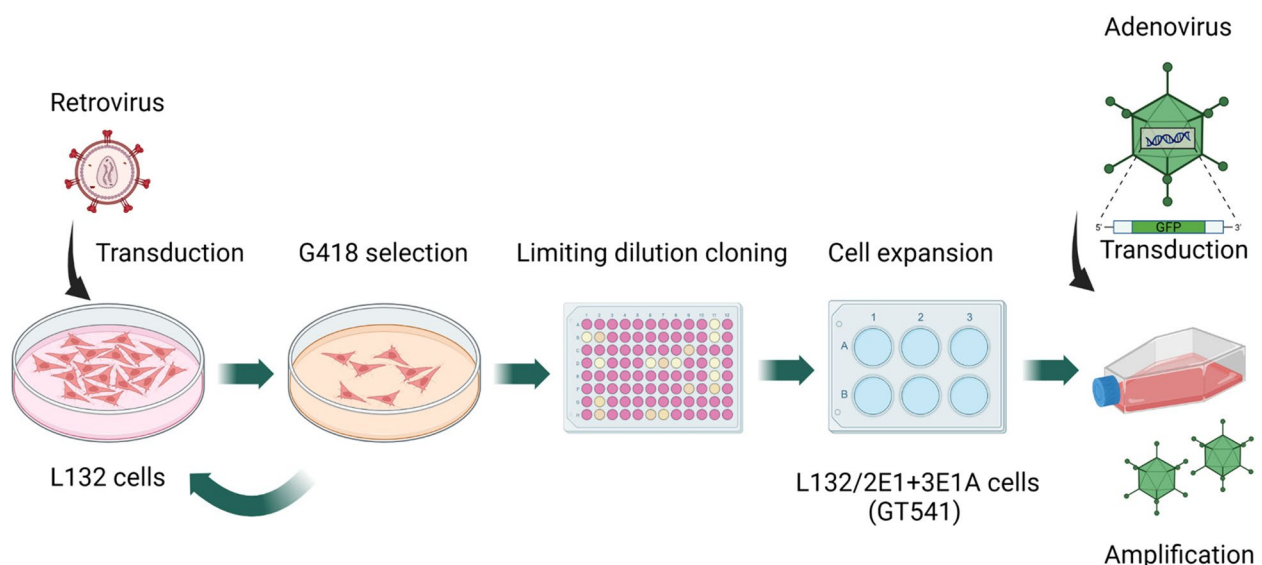


**Fig. 1** The overall workflow for the establishment of the adenovirus-producing cell line. L132 cells were transduced two times with E1-encoded retrovirus and three times with E1A-encoded retrovirus. G418-resistant cells were cloned via the limiting dilution method. The most productive L132 cell lines for generating RCA-free adenovirus were selected and named GT541. The image was created with BioRender.com

1% penicillin–streptomycin (all from Gibco, MA, USA). After transduction with retroviral vectors, each cell line was cultured in culture media supplemented with 1 mg/ml of G418 (Sigma, MA, USA).

### PCR

The total E1 gene (560–3509 bp) was obtained from the adenovirus type 5 genome via polymerase chain reaction (PCR) using a PCR Amplification Kit (TaKaRa, Japan). Next, using the total E1 gene as a template and E1A (560–1545 bp)-specific primers, PCR was performed to clone E1A. The primers contained BamHI and EcoRI restriction sites. The following primers (Cosmogenetech, Korea) were used: E1, Forward 5'-GGA TCC ATG AGA CAT ATT ATC TGC CAC GGA GGT-3', Reverse 5'-GAA TTC TCA ATC TGT ATC TTC ATC GCT AGA GCC-3'; E1A, Forward 5'-CAT GGA TCC ATG AGA CAT ATT ATC TGC CAC-3', Reverse 5'-CAT GAA TTC TTA TGG CCT GGG GCG TTT ACA-3'. Both genes were ligated into the pGEM-T easy vector (Promega, USA), generating the pGEM-T-E1 and pGEM-T-E1A plasmids, respectively.

To elucidate the insertion of E1 or E1A into the host cellular genome, Qiagen Tissue Kit (USA) was used to extract genomic DNA from retrovirus-transduced cells, followed by PCR amplification. The following primers (Cosmogenetech, Korea) and E1 primers were utilized: E1A (PCR), Forward 5'-TCC ACC TCC TAG CCA TTT TG-3', Reverse 5'-CCG TAT TCC TCC GGT GAT AA-3'.

To detect RCA production, HEK293A and GT541 cells were infected with the 10 multiplicity of infection (MOI) of adenovirus. After 3 days, the crude supernatants were subjected to CsCl gradient ultracentrifugation using an ultracentrifuge from Beckman Coulter (CA, USA). Then, we obtained adenovirus and performed PCR to amplify the adenoviral DNA. The E1(RCA) primer set (Cosmogenetech) was used: E1(RCA), Forward 5'-ATG AGA CAT ATT ATC TGC CAC-3', Reverse 5'-GTA AGT CAA TCC CTT CCT GCA C-3'.

### Production and titration of retrovirus

E1 and E1A DNA fragments were cloned into the pWZL-Neo vector (Cell Biolabs, Inc., CA, USA) to obtain retroviral expression clones. The retroviral expression and packaging vectors were co-transfected into 293-GP retroviral packaging cells using Jet-PEI (Polyplus Transfection, France). After 3 days, the virus released in the supernatant was harvested for concentration analysis. To obtain purified viruses, the virus-enriched supernatants were concentrated at 25,000 rpm for 2 h using an ultracentrifuge and washed with Opti-MEM (Gibco). The MinibEST Viral RNA/DNA Extraction Kit (Takara) was used

to extract retroviral nucleic acids. Real-time PCR titration was used to quantify the retroviruses.

### Reverse transcription (RT)-PCR

To measure E1 expression, HT1080 cells were transfected with the pWZL-Neo-E1 vector in the presence of JetPEI (Polyplus). Cells were harvested, followed by RNA extraction using an RNA Extraction Kit (Takara). With the gDNA Eraser Spin Column, pure RNA were obtained, and used in cDNA synthesis using a cDNA synthesis mix (Takara). To confirm E1 expression in retrovirus-transduced cells, total RNA was purified and used to synthesize cDNA. PCR was performed using the E1A, E1B-55 K, and GAPDH primer sets. The following primers (Cosmogenetech) were used: E1A (RT), Forward 5'-CGA AGA GGT ACT GGC TGA TAA TCT-3', Reverse 5'-CCG TAT TCC TCC GGT GAT AA-3'; E1B55K (RT), Forward 5'-TTA AGA AAT GCC TCT TTG AAA GGT-3', Reverse 5'-ATC ACA GGC TGG TTC CTA ATA TGT-3'; GAPDH, Forward 5'-GAG TCA ACG GAT TTG GTC GT-3', Reverse 5'-GTA AGT CAA TCC CTT CCT GCA C-3'.

### Establishment of E1-complementing cell lines

BJ, ARPE-19, and L132 cells were transduced with the recombinant retroviral particles containing E1 and E1A. Cells were pre-seeded in 60 mm dish at a density of  $2.5 \times 10^5$  cells/dish. On the following day, recombinant retroviruses were introduced to the cells at a 10 MOI in the presence of 8 µg/mL of polybrene (Sigma-Aldrich) to enhance transduction efficiency. After 2–3 days incubation, the transduced cells were then selected in a medium containing 1 mg/mL of G418 (Sigma-Aldrich). The selected cells were expanded and screened for E1 expression and adenovirus production yield. The same procedure was used for subsequent retro-E1 transduction. After performing retro-E1 transduction two times, the cells were transduced with retro-E1A three times. A monoclonal cell population was established using the limiting dilution approach. From this population, 3 to 6 clones of G418-resistant transduced cells were selected, and the adenovirus produced by each clone was titrated.

### Adenovirus production and titration

GT541 or HEK293A cells were pre-seeded in T75 flasks at a density of  $2.1 \times 10^6$  cells/flask. On the following day, the cells were transduced with 10 MOI of Ad-GFP (Invitrogen, USA), an adenovirus vector encoding green fluorescent protein (GFP), in 2 ml of DMEM supplemented with 5% FBS. After 1 h, 12 ml of DMEM supplemented with 5% FBS was added and incubated. After 3 days, cytopathic effect (CPE)-positive cells were harvested and

sonicated. The crude supernatants were collected after centrifugation.

**Real-time PCR**

To measure E1 and E1A expression in retrovirus-transduced cells, total RNA was extracted, purified, and reverse transcribed into cDNA. Quantitative PCR (qPCR) was performed using the above E1 (RCA) and E1A (RT) primer sets.

For titration, adenovirus samples were purified and amplified via qPCR. The purified retroviral genomic RNA was used for retroviral titration. A cDNA synthesis mix (Takara) and QuantiSpeed SYGreen Hi-ROX kit (Phile-Korea, Korea) were used to perform RT-qPCR. The following primers (Cosmogenetech) were utilized: Ad5, Forward 5'-CCG GGA ACT TAA TGT CGT TT-3', Reverse 5'-TTG CTT GAT CCA AAT CCA AA-3'.

**Fluorescent microscopy**

HEK293A and GT541 cells were transduced with Ad-GFP at 10 MOI and incubated for 72 h. GFP expression in the virus-infected cells was observed under a fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany).

**Viral titration**

Produced virus were titrated using 50% Tissue culture infectious dose (TCID<sub>50</sub>) method [14]. Serially diluted viral solution (50 μL/well) was added in collagen-coated 96-well plate. Then, we added HEK293A cell suspension to each well. Cells were incubated at 37 °C, with 5% CO<sub>2</sub>. We added 50 μl of culture media to each well at day 5 and day 10. We analyzed complete CPE using microscopy at day 14. We obtained TCID<sub>50</sub> statistically using Kåber's formula [15]. TCID<sub>50</sub> = (dilution rate giving highest CPE) × (dilution rate)<sup>Σ-0.5</sup> in

which Σ = total sum of (number of wells showing CPE) / (number of wells) at each step of dilution.

**Data visualization**

The data were presented using SigmaPlot software (version 14.5; Systat Inc. USA).

**Results**

**Cloning of the E1 of adenovirus type 5**

We obtained a part of adenoviral E1 (560 bp–3509 bp of adenovirus sequences) to avoid the homologous recombination with viral genome. The adenovirus E1 gene comprises two components: E1A and E1B (Fig. 2). The E1B gene produces two major products: E1B-19 K and E1B-55 K. The genomic DNA of adenovirus type 5 was used as a PCR template to amplify the E1 gene. A DNA band of approximately 3 kb was detected (Fig. 3a). PCR was performed using E1A-targeted primers and the adenovirus E1 gene as the template. A band of approximately 1 kb was detected (Fig. 3b). The obtained genes were cloned into the pGEM-T easy vector, followed by the confirmation of the nucleotide sequences.

**E1 gene-expressing retroviruses**

The pWZL-E1 plasmid was obtained by inserting the E1 gene into the retroviral vector pWZL-Neo. To confirm E1 expression, HT1080 cells were transfected with pWZL-E1. Then, RT-PCR was performed to measure E1 RNA expression (Fig. 4). HT1080 cells were used as a negative control. As expected, we observed the E1-specific band in HEK293A cells. The same cDNA band size of E1 was observed in pWZL-E1-transfected HT1080 cells. To exclude plasmid contamination, the DNA from pWZL-E1 was included in the gel electrophoresis analysis. Owing to the size difference between cDNA and genomic DNA

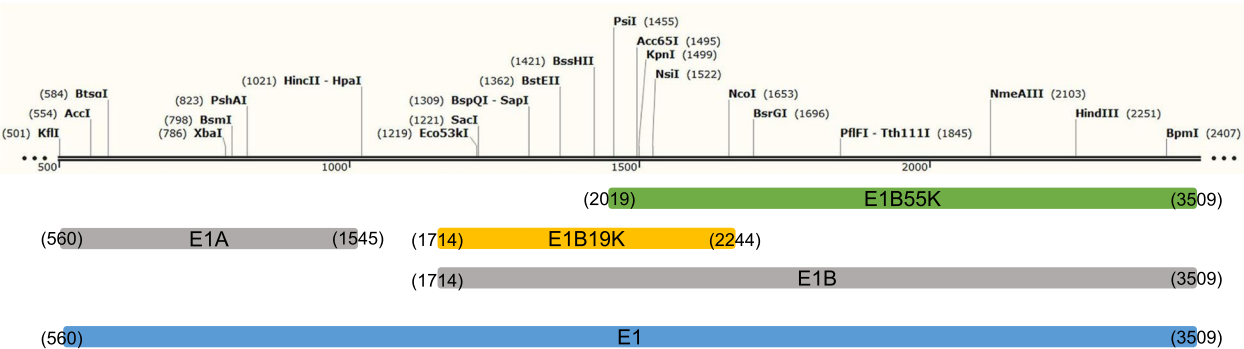


**Fig. 2** Schematic illustration of the adenovirus type 5 E1 gene structure. E1 is depicted in the blue-filled box, and E1A and E1B are depicted in the gray-filled box. The open reading frames of E1B-19 K and E1B-55 K are indicated. The restriction enzymes and cleavage sites are shown. The numbers in the parentheses indicate the locus of the adenovirus genome

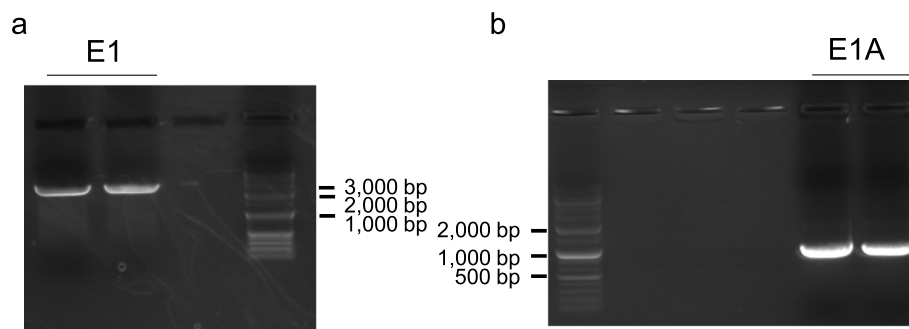


**Fig. 3** Cloning of E1 and E1A genes. **a** Adenovirus E1 gene (~3 kb size) was obtained from the genomic DNA of adenovirus type 5 via PCR. **b** E1A gene (~1 kb size) was cloned from the adenovirus E1 gene using an E1A-specific primer set. Uncropped gels are presented in supplementary Fig. 1

[16], we concluded that the E1 of pWZL-E1 was transcribed into the RNA in the transfected cells.

#### Adenovirus-producing cell line generation

The retroviral expression and packaging vectors were co-transfected into the 293-GP retroviral packaging cells. After 3 days, retroviruses were harvested and concentrated. E1-encoded retroviruses (retro-E1) and E1A-encoded retroviruses (retro-E1A) were obtained and used to deliver E1 and E1A genes for stable transduction.

First, L132 cells were transduced with retro-E1 and selected in G418-supplemented medium. Then, the cells were subjected to RT-PCR to measure E1A and E1B-55 K expression. Both E1A and E1B-55 K were detected in HEK293A cells. In the initial retro-E1-transduced L132 cells (L132/E1), the E1A band was barely visible, whereas the E1B-55 K band was marginally visible (Fig. 5a). A single transduction with retro-E1 was not sufficient for expressing E1A; therefore, an additional retro-E1 transduction was performed. This increased E1A and E1B-55 K expression in L132/2E1 cells compared with that in L132/E1 cells (Fig. 5b). However, E1A expression was lower in L132/2E1 cells than in HEK293A cells.

L132 cells, which are of normal cell origin, may have lower productivity than cells of cancerous origin. Therefore, E1A expression, which is closely associated with cell proliferation, is essential. As a result, we performed an additional transduction with retro-E1A. To enhance E1A expression, L132/2E1 cells were transduced three times with retro-E1A, generating L132/2E1 + 3E1A cells. After G418 selection, single cells were cloned from the transduced L132 cells using the limiting dilution approach. The generated clones were transfected with Ad-GFP to compare adenovirus production yields. First, L132/E1, L132/2E1, and L132/2E1 + 3E1A cells were transfected with Ad-GFP;

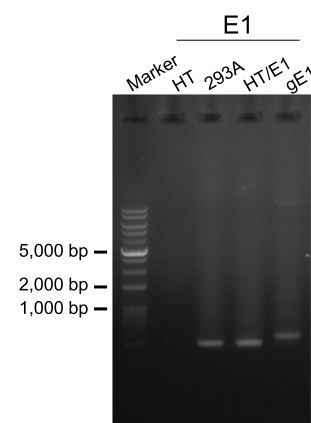


**Fig. 4** E1 mRNA expression in transfected cells with retroviral vector. Total RNA from HT1080 (HT), HEK293A (293A), or pWZL-E1-transfected HT1080 (HT/E1) cells was reverse-transcribed into cDNA. The DNA of the genomic E1-encoded vector, pWZL-gE1 (gE1), was included as a control. Genes were amplified via PCR. Uncropped gel is presented supplementary Fig. 2

then, the Ad-GFP production titer in each cell was analyzed (Fig. 5c). The Ad-GFP titer in L132/2E1 was approximately 3.8-fold higher than that in L132/E1. Furthermore, Ad-GFP production was significantly increased in L132/2E1 + 3E1A cells. In the comparative analysis of adenovirus production yield, we confirmed the effect of additional E1A transduction on adenovirus production. However, additional retro-E1A transduction on GT541 cells did not in a significant increase in E1A expression (data not shown).

#### Selection of the adenovirus-producing cell line

The other cell lines were established using the same developmental strategy. BJ and ARPE-19 cells were transduced two times with retro-E1 and three times with retro-E1A, followed by selection in G418-containing media. Real-time-PCR was performed to quantify Ad-GFP produced in each cell type (Fig. 5d). A remarkably higher degree of

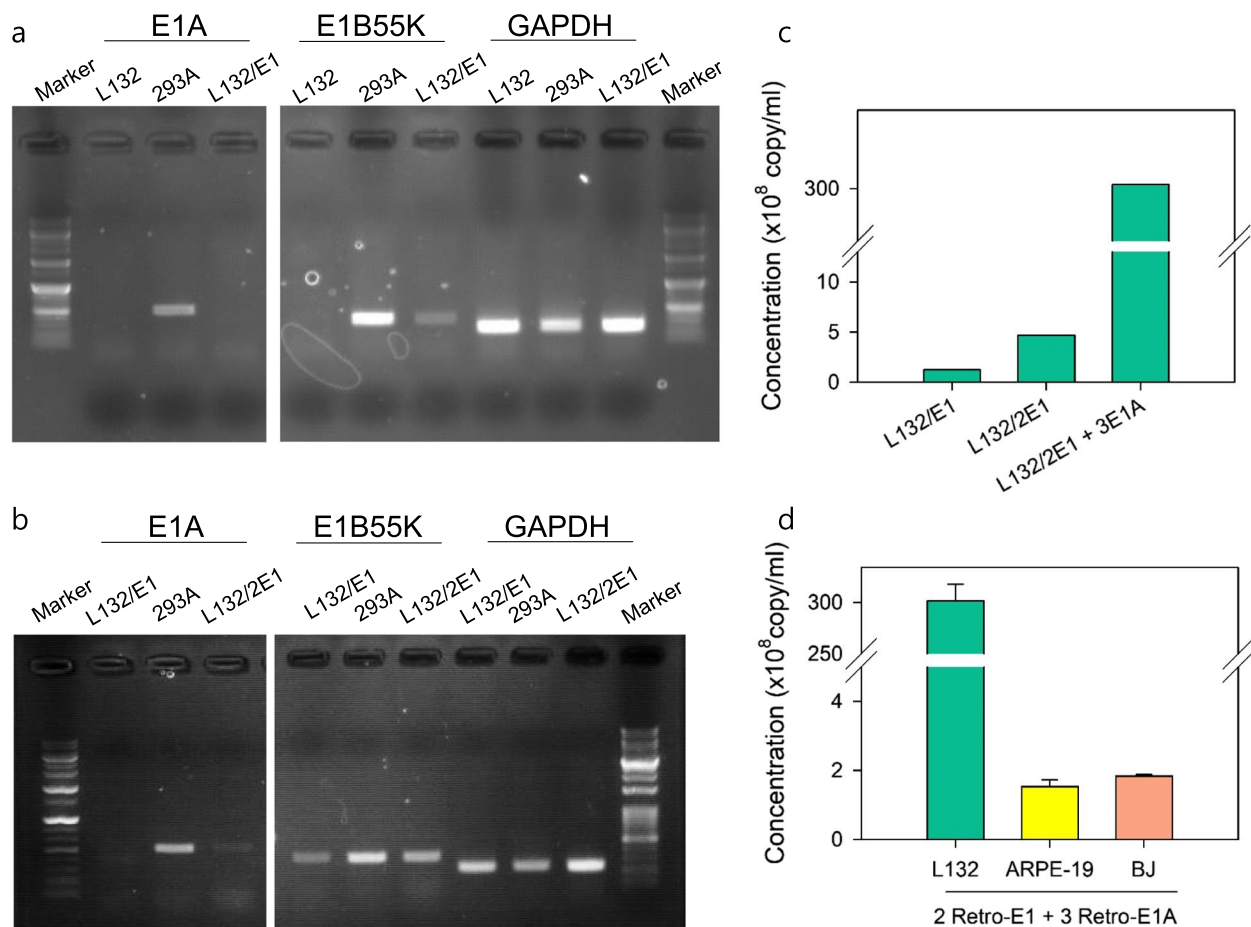


**Fig. 5** Expression of E1A and E1B in stably transduced L132 cells. **a** RNA purified from retro-E1-transduced L132 cells (L132/E1) were reverse-transcribed into cDNA. E1A, E1B55K, and GAPDH gene products were amplified via PCR. HEK293A cells were used as a positive control, whereas untransduced L132 cells were used as a negative control. **b** L132/E1 were additionally transduced with retro-E1 (L132/2E1). Expression of E1A, E1B55K were analyzed by RT-PCR. **c** Ad-GFP were generated using L132/E1, L132/2E1, or L132/2E1 + 3E1A. Each adenovirus was titrated via real-time PCR. **d** ARPE-19/2E + 3E1A and BJ/2E + 3E1A cells were generated via sequentially transducing retro-E1 two times and retro-E1A three times. The transduced cells were cultured in G418-supplemented media. The established cells were used to produce Ad-GFP. The produced adenoviruses were quantified using real-time PCR. Uncropped gels are presented in supplementary Fig. 3 and 4

adenovirus production was observed in L132 cells than in the other cell types. Therefore, L132/2E1 + 3E1A cells were selected as the best adenovirus-producing cell line and named GT541.

#### Characterization of GT541

When a gene is delivered using a retroviral vector, it is inserted into the genomic DNA of the host cell. To confirm the stable transduction of E1 and E1A genes, we performed PCR to detect the inserted E1 and E1A genes in the GT541 genome (Fig. 6a). Although the intensity was weaker than that observed in HEK293A cells, distinct bands of E1 (2962 bp) and E1A (421 bp) were detected in GT541 cells. Repeated transduction with retro-E1 and retro-E1A enhanced E1 expression. We performed

qPCR analysis to quantify E1 and E1A (Fig. 6b) expression. HEK293A and GT541 cells exhibited a difference in E1 transcription levels, with HEK293A exhibiting approximately 12 times higher expression than GT541 cells. Similarly, E1A transcription levels were 202 folds higher in HEK293A cells than in GT541 cells. Despite the lower expression of E1 and E1A in GT541 cells than in HEK293A, significant transcription was detected.

To compare the production yield of Ad-GFP between HEK293A and GT541 cells, the cells were transduced with Ad-GFP and incubated for 72 h. GFP expression was analyzed using fluorescence microscopy, revealing that GFP was efficiently expressed, with expression comparable with that in HEK293A (Fig. 6c) and GT541 cells (Fig. 6d). Produced Ad-GFP were quantified by real-time

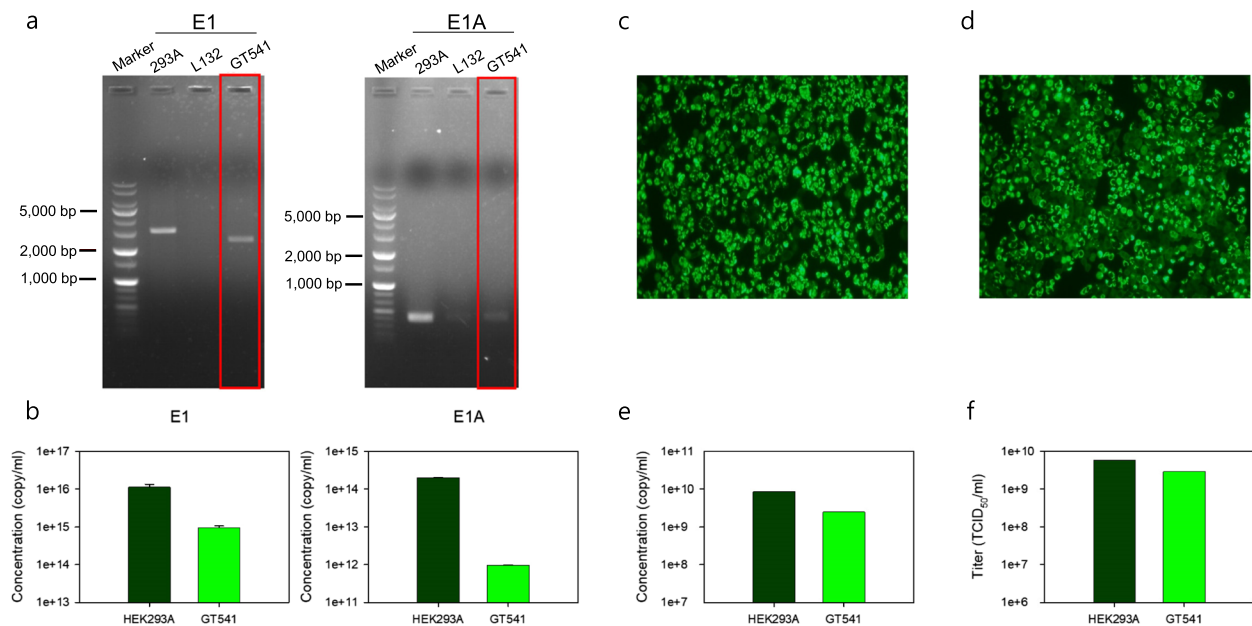


**Fig. 6** Characterization of adenovirus-producing cell lines. **a** E1 and E1A in each cellular genome were analyzed via PCR. HEK293A (293A) cells were used as a positive control, whereas untransduced L132 cells were used as a negative control. Uncropped gels are presented in supplementary Fig. 5. **b** qPCR analysis of E1 and E1A expression in HEK293A and GT541 cells. **c** and **d** GFP expression in Ad-GFP-producing cell lines. HEK293A (**c**), GT541 (**d**) cells were transduced with Ad-GFP at 10 MOI and incubated for 72 h. GFP expression in the virus-infected cells was observed under a fluorescence microscope. **e** HEK293A and GT541 were used to produce Ad-GFP. The produced adenoviruses were quantified using real-time PCR. **f** Virus in crude supernatant were quantified using TCID<sub>50</sub> method. Diluted viral solution was added to HEK293A cells. After 14 days, the number of cells showing CPE was determined. The values of TCID<sub>50</sub> were calculated using Käber's formula

PCR, and comparable levels of Ad-GFP were produced in HEK293A and GT541 (Fig. 6e). TCID<sub>50</sub> assay was used for quantification of viral infectious particles (Fig. 6f). The titer of Ad-GFP in crude supernatant of HEK293A was  $5.9 \times 10^9$  TCID<sub>50</sub>/ml, and the viral titer produced in GT541 was  $2.9 \times 10^9$  TCID<sub>50</sub>/ml. Although productivity was not higher than HEK293A cells, GT541 showed a comparable level of adenovirus productivity.

For the RCA assay, HEK293A and GT541 cells were passaged until passage 18. Adenoviruses produced from each cell line were analyzed for the presence of the E1 gene via PCR using specific primers (Fig. 7). None of the adenoviral samples produced in GT541 passaged 16–18 times carried the E1 gene, whereas adenovirus produced in HEK293A passaged 18 times contained the E1 gene. In summary, GT541 exhibits a high production capacity, which is comparable with that of HEK293A cells. Furthermore, the adenoviruses generated from GT541 are not contaminated with RCA.

## Discussion

At present, HEK293 and PER.C6 are representative virus-producing cell lines that are commonly utilized to produce agents for clinical trials or approved drugs. PER.C6 is actively utilized to produce recombinant adenoviral

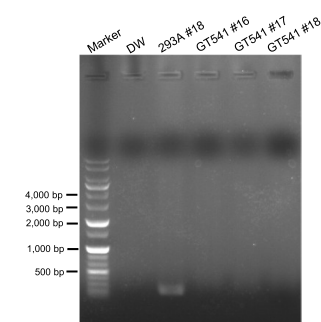


**Fig. 7** Assay for replication-competent virus in passaged GT541 cells. HEK293A and GT541 cells were passaged until passage 18. Ad-GFP were produced in each cell lines. DNA of Ad-GFP were subjected to PCR. E1 (RCA)-specific primers were used. Uncropped gel is presented in supplementary Fig. 6

vectors, influenza virus vaccines, and proteins, including monoclonal antibodies [17]. On the other hand, HEK293 cells are primarily utilized to produce adenoviruses and adeno-associated viruses [18].

Various attempts have been undertaken to develop alternative adenovirus-producing cell lines using cancer cells, including HeLa [19, 20] and A549 [21–23], that are free from RCA and licensing issues. However,

the high tumorigenicity of HeLa cells limits its commercial application [9]. Unmodified A549 cells have been tested under the guidance of the United States Food and Drug Administration (FDA) and may be utilized to produce replicating adenoviruses that do not require E1 complementation. However, the conversion of A549 cells to E1-complementing cells is warranted to produce E1-defective adenoviruses [24, 25].

Although several cell lines have been developed, they are currently used in preclinical studies and academic research. Cell line 911 is a human retinoblast cell line with a plasmid containing 79–5789 bp of the adenovirus type 5 genome [26]. The adenoviral vector production yield was higher using this cell line than using HEK293 cells; however, it exhibited similar frequencies of homologous recombination. Therefore, this cell line remains uncommercialized.

H2C16 (International publication number: WO2013077 645A1), GH329 [19], and HeLa-E1 [20] cells have been developed using HeLa cells. Various strategies have been developed to minimize RCA generation in viral products. H2C16 contains a modified E1 sequence (560–3509 bp of the adenovirus type 5 genome) that minimizes homologous recombination. In GH329 cells, overlapped sequences with an adenoviral vector have been removed at the 5′-end and minimized at the 3′-end. HeLa-E1, which contains the minimum E1 region (542–3526 bp of adenovirus type 5), did not generate RCA. However, the adenovirus production yield of HeLa-E1 was lower than that of 293 cells. In particular, HeLa cell-based cell lines have safety issues associated with their human papillomavirus oncogenes [27].

In a previous study, A549 cells were transformed with E1 expression vectors; however, this complementation cell line does not contain the left end of the adenoviral genome [22]. Furthermore, A549-originated cells, designated SL0003 cells, have mutant genes to constitutively express E1A and E1B-55 K, but not the E1B-19 K [25]. Adenoviral E1A interacts with p300 and p105 retinoblastoma (Rb) proteins, which are cellular proteins, and regulates various cellular pathways [28]. In SL0003 cells, mutant E1A proteins are defective in inducing cell apoptosis and may help overcome toxicity in host cells. E1B complementation increased production yield, in particular, in the condition of E1B-55 K expression without E1B-19 K [25]. The production yields were similar to those obtained using HEK293 cells without RCA generation.

The presence of RCA is strictly regulated for clinical applications based on FDA standards (1 RCA in  $3 \times 10^{10}$  viral particles). Therefore, the safety of RCA-free adenoviruses is crucial for their clinical application. In this study, we developed a novel cell line that expresses adenovirus E1 but shares minimal sequences with the adenovirus vector for producing RCA-free recombinant adenoviruses.

Traditional RCA assays are based on CPE analysis of the detector cells [29]. CPE results may be confirmed using more sensitive and specific assays, including PCR or immunofluorescence [30]. In the present study, we detected RCA using PCR; however, performing the RCA assay using additional methods may be warranted [31].

In this study, to increase the production yield of the recombinant adenovirus, we introduced the total E1 gene and additional E1A genes into L132 cells. E1A expression is essential for viral DNA replication [24] and for activating viral gene expressions [32]. E1B-55 K plays a role in promoting adenovirus infection [33] and contributes to growth promotion and delay of cellular apoptosis [12, 34]. Therefore, adenovirus-producing cell lines could be further optimized through additional transduction with E1B-encoded retroviruses. Notably, in the present study, although the two retro-E1 transductions successfully induced E1B-55 K expression, they did not induce sufficient E1A expression (Fig. 5b). However, our comparative analysis of adenovirus production yield confirmed that additional E1A transduction on significantly enhanced adenovirus production (Fig. 5c). These data suggest that high E1A expression contributes to enhanced adenovirus production.

At present, among the developed production cell lines, PER.C6 is the only commercialized recombinant adenovirus-producing cell line without issues of RCA generation. Our newly established cell line, GT541, may serve as an alternative to PER.C6 for producing recombinant adenoviruses because it does not generate RCAs and has adenovirus productivity comparable with that of HEK293 cells. The established GT541 cell line should be further characterized in subsequent studies, focusing on the genomic analysis of the E1 and E1A coding regions, assessment of batch variability, evaluation of long-term cultivation, testing for RCA at various passages, and large-scale scalability.

#### Abbreviations

|                 |                                                      |
|-----------------|------------------------------------------------------|
| bp              | Base pairs                                           |
| DMEM            | Dulbecco's modified Eagle medium                     |
| E1              | Early region 1                                       |
| E1B-19 K        | E1B 19-kDa protein                                   |
| E1B-55 K        | E1B 55-kDa protein                                   |
| FBS             | Fetal bovine serum                                   |
| FDA             | Food and Drug Administration                         |
| GFP             | Green fluorescent protein                            |
| HEK293          | Human embryonic kidney 293                           |
| L132/E1         | Retro-E1-transduced L132 cells                       |
| L132/2E1        | L132 cells transduced twice with retro-E1            |
| L132/2E1 + 3E1A | L132/2E1 cells transduced three times with retro-E1A |
| MOI             | Multiplicity of infection                            |
| PCR             | Polymerase chain reaction                            |
| qPCR            | Quantitative polymerase chain reaction               |
| retro-E1        | E1-encoded retroviruses                              |
| retro-E1A       | E1A-encoded retroviruses                             |
| Rb              | Retinoblastoma                                       |
| RCA             | Replication-competent adenovirus                     |

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12896-024-00894-x>.

Supplementary Material 1.

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Not applicable.

### Authors' contributions

E Y Han and Y-J Kim conceived and designed the analysis; E Y Han performed the research and acquired the data; E Y Han and Y-J Kim analyzed and interpreted the data. Both authors were involved in drafting and revising the manuscript.

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### Availability of data and materials

Data is provided within the manuscript or supplementary information files.

### Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

Eun Yeong Han has a patent on adenovirus producing cell lines. Yeon-Jeong Kim declare no conflict of interest.

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