Original Paper

Engineering the Rapid Adenovirus Production and Amplification (RAPA) Cell Line to Expedite the Generation of Recombinant Adenoviruses

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Key Words

Adenoviral vectors • Adenovirus production • Packaging cell line • Gene transfer • Gene delivery

Abstract

Background/Aims: While recombinant adenoviruses are among the most widely-used gene delivery vectors and usually propagated in HEK-293 cells, generating recombinant adenoviruses remains time-consuming and labor-intensive. We sought to develop a rapid adenovirus production and amplification (RAPA) line by assessing human Ad5 genes (E1A, E1B19K/55K, pTP, DBP, and DNA Pol) and OCT1 for their contributions to adenovirus production.

Methods: Stable transgene expression in 293T cells was accomplished by using *piggyBac* system. Transgene expression was determined by qPCR. Adenoviral production was assessed with titrating, fluorescent markers and/or luciferase activity. Osteogenic activity was assessed by measuring alkaline phosphatase activity. **Results:** Overexpression of both E1A and pTP led to a significant increase in adenovirus amplification, whereas other transgene combinations

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did not significantly affect adenovirus amplification. When E1A and pTP were stably expressed in 293T cells, the resultant RAPA line showed high efficiency in adenovirus amplification and production. The produced AdBMP9 infected mesenchymal stem cells with highest efficiency and induced most effective osteogenic differentiation. Furthermore, adenovirus production efficiency in RAPA cells was dependent on the amount of transfected DNA. Under optimal transfection conditions high-titer adenoviruses were obtained within 5 days of transfection. **Conclusion:** The RAPA cells are highly efficient for adenovirus production and amplification.

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Introduction

Human adenovirus serotype 5 (Ad5) contains a linear 36 kb double-stranded genome [1, 2]. The adenovirus virion is a nonenveloped icosahedral particle about 70–90 nm in size with an outer protein shell surrounding an inner nucleoprotein core [2]. Recombinant replication-deficient adenoviruses are usually missing critical viral functions leading to 1) the inhibition of viral spreading that renders such viruses safe and 2) the insertion of foreign transgenes, secondary to the packaging space created by the deletion of viral genes [3, 4]. Recombinant adenoviruses are among the most widely used viral vectors for *in vitro* and *in vivo* gene delivery [1–5]. Recombinant adenoviruses offer several attractive features for gene transfer, including a large capacity for gene delivery, an efficient production of high titer viral stocks, high levels of transgene expression and the ability to transduce a wide range of mammalian tissues and dividing/non-dividing cells [4, 5]. Additionally, adenovirus-mediated gene delivery does not involve DNA integration into host genome so the risk of insertional mutagenesis is low [2, 4]. The utility of recombinant adenoviruses has further been benefited from the fact that the mechanisms underlying adenovirus DNA replication and adenovirus life cycle have been well studied [1, 2, 4].

For the past decades, recombinant adenovirus technologies have gone through rather extensive evolutions that led to the development of several generations of adenoviral vectors, including the most drastic form of gutless high-capacity adenoviral vector [2, 4]. The first-generation recombinant adenoviral vectors, which are most commonly used adenoviruses, usually have both the E1 and E3 regions deleted [1, 4]. The first generation adenoviral vectors can be propagated to high yields in permissive E1 complementation or packaging cell lines, such as HEK-293 cells that provide the E1 functions *in trans* [6]. HEK-293 cells were generated by transformation of human embryonic kidney cells with sheared adenoviral genomic DNA containing the Ad5 sequences between 1–4137nt integrated into chromosomal DNA [6, 7]. While the early methods to generate recombinant adenoviruses relied on less efficient homologous recombination between a shuttle vector and adenoviral backbone vector in HEK-293 cells [8], several technical advances, including direct ligation, efficient homologous recombination in bacteria, and the use of Cre recombinase-mediated integration, have significantly facilitated the generation of adenoviral vectors for the past decade [9–14]. Nonetheless, the initial generation and production of recombinant adenoviruses usually requires two to three weeks, and thus remains as a time-consuming and labor-intensive process.

Adenovirus production is a highly dynamic and complex cellular process, which requires the coordination among temporal viral gene expression, efficient adenoviral DNA replication, and adequate synthesis of viral capsid proteins for adenovirus virion assembly [1, 15]. Transcription of the adenoviral genome occurs on both strands and is coordinated through a precisely temporally regulated splicing program of almost all the transcripts [1, 15]. The *E1A* gene is the first viral gene to be expressed and its product acts as the major viral transcriptional transactivator to activate viral early transcription. As the E2 gene products (including E2A; DNA binding protein, DBP; E2B; preterminal protein, pTP; and Ad5 DNA polymerase, Ad5 DNA Pol) accumulate, viral DNA replication can commence. Viral DNA

synthesis occurs through protein priming mediated by the preterminal protein (pTP) at the inverted terminal repeats (ITRs) of the viral genome [1, 15]. Several host factors such as OCT1 and NF1 also play important roles in adenoviral DNA replication [15]. We have recently demonstrated that overexpression of human Ad5 pTP in HEK-293 cells (i.e., 293pTP) can enhance adenovirus amplification and production [16].

In this study, we engineered a rapid adenovirus production and amplification (RAPA) cell line through a functional analysis of five human Ad5 viral genes and a host factor, which are known to play important roles in adenovirus DNA replication, in 293T cells. Our results demonstrate that the dual E1A-pTP-overexpressing RAPA cells can package and yield high titers of recombinant adenoviruses within 5 days under optimal transfection conditions. Therefore, the use of RAPA cells should significantly expedite the generation and production of recombinant adenoviruses, which should be beneficial for most applications involving adenovirus-mediated gene deliveries in basic and translational research.

Materials and Methods

Cell culture and chemicals

HEK-293 and 293T cells were obtained from ATCC (Manassas, VA, USA). The iMEF cells are mouse mesenchymal stem cells (MSCs) previously described [17, 18]. These cells lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 100 g/ml streptomycin at 37°C in 5% CO₂ as described [19, 20]. Unless indicated otherwise, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Construction of piggyBac transposon vectors expressing pTP, E1A, E1B19K, E1B55K, DBP, and DNA Pol of human Ad5 or human OCT1

A homemade *piggyBac* vector pBC2, which contains *piggyBac* terminal repeats (PB-TRs), core insulators (CIs) and the Blasticidin S selection marker, was used as the base vector [21, 22]. The coding regions of human Ad5 pTP, E1A, E1B19K, E1B55K, DBP, and DNA Pol were PCR amplified and/or assembled together from adenoviral genome-containing plasmid pJM17. Coding region for human OCT1 was amplified from pCGN-OCT1 that was generously provided by Dr. Winship Herr [23]. PCR cloning primers are listed in Table 1. The PCR amplified fragments were subcloned into pBC2. All PCR-amplified fragments were verified by DNA sequencing. Detailed information regarding vector constructions is available upon request.

Establishment of 293T stable cell lines expressing individual transgenes, the M series and the RAPA cell line

293T expressing single genes, the M series, and the RAPA line were accomplished by co-transfection of single or combined pBC2 expression vectors with a *piggyBac* transposase expression vector into 293T cells with Lipofectamine (Invitrogen, Grand Island, NY, USA), as described [18]. Stable cells were selected in the presence of Blasticidin S (at the final concentration of 5 µg/ml) for 5-7 days with repeated plating.

The established 293T stable lines were designated as following: 293T-pTP, 293T-E1A, 293T-E1B19K, 293T-E1B55K, 293T-DBP, 293T-Pol, and 293T-OCT1 express the respective transgene. For the M series lines, M1 line (aka. RAPA) expresses both pTP and E1A; M2 line expresses pTP, E1A, E1B19K and E1B55K; M3 line expresses pTP, E1A, E1B19K, E1B55K and DBP; M4 line expresses pTP, E1A, E1B19K, E1B55K, DBP, and DNA Pol; M5 line expresses pTP, E1A, E1B19K, E1B55K, DBP, DNA Pol, and OCT1; and M6 line expresses pTP, E1A, E1B19K, E1B55K and OCT1. All stable lines were scaled up and stocked in LN2 tanks.

RNA isolation, semi-quantitative and quantitative PCR (TqPCR)

Total RNA was isolated using the TRIZOL Reagent (Invitrogen, Carlsbad, CA) and subjected to reverse transcription with hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The cDNA products were diluted 10- to 100-fold and used as PCR templates. PCR primers specific for human Ad5 pTP, E1A, E1B19K, E1B55K, DBP, DNA Pol, human OCT1, and GAPDH were designed by using the Primer3 Plus program [24] (Table 1). For the semi-quantitative PCR analysis, a touchdown PCR program was carried

Table 1. List of Oligonucleotides for Cloning and qPCR

Genes	Sequences	Use
Ad5 E1A	gtaccagactgtgacccggcgccgttaccacatgggcatgagacatattatctgccacggagtg	Forward
	catatgtacgtatctagattcgaagttttatggcctggggcgtttacagc	Reverse
Ad5 E1B19K	ggaggatccaccacatggaggttgggagtggttggag	Forward
	gaagaattctcattcccgagggtccagccggctc	Reverse
Ad5 E1B55K	ggaggatccaccacatggagcgaagaacccatctgagc	Forward
	gaagaattctcaatctgtatcttcctgctagag	Reverse
Ad5 DBP	ggaggatccaccacatggcctgggaagaggagcagc	Forward
	gaagaattcttaaaatcaagggttctgccgcgc	Reverse
Ad5 DNA Pol	ggaggatccaccacatggccttgcctcaagctcaccgggcccgtc	Forward
	gaagaattctcctacggcatctcgtacccagcatatc	Reverse
Ad5 pTP	GCGTCAACGATTGCGCGCGCCTG	Forward
	CTAAAAGCGGTGACGCGGCGAGCCCCCGGAG	Reverse
human OCT1	agtagtactaccacatggcgagggagcagcagtc	Forward
	ggcggcgccctcactgtgcttggaggcgggtggtg	Reverse
Ad5 E1A	cgaagatcccaacgagagg	Forward
	ggcatagaacccgacccaa	Reverse
Ad5 E1B19K	aggtttctgtgggctc	Forward
	cccgtcagatgggttctt	Reverse
Ad5 E1B55K	ggcttgtaggctacagagg	Forward
	aatctggcctaagtgccacc	Reverse
Ad5 DBP	agcagtctagcaagccaag	Forward
	cggctggcggttaggatac	Reverse
Ad5 DNA Pol	aaaatggccataggtcgcca	Forward
	aaagccgttgatgtgtggc	Reverse
Ad5 pTP	AGGCCCTTATGGAAGAGGAG	Forward
	TGTGTTCTGCCACGAAGAAG	Reverse
human OCT1	ctcaacaacacagcaaccg	Forward
	ggacaaaggagtgagggtgg	Reverse
GAPDH	gtcaaggctgagaacgggaa	Forward
	aaatgagccccagccttc	Reverse

out as described [25-27]. Briefly, the cycling program was as follows: 94°C x 2 min for one cycle, 4 cycles at 92°C x 20 sec, 68°C x 30 sec, and 72°C x 20 sec with a decrease of three degrees per cycle, and 25~30 cycles at 92°C x 20 sec, 57°C x 30 sec, and 72°C x 20 sec. The PCR products were resolved and visualized on 1.5% agarose gels.

The quantitative real-time-PCR analysis also was carried out using our recently optimized TqPCR protocol [28]. Briefly, the SYBR Green qPCR reactions (Bio-Rad Laboratories) were set up according to manufacturer's instructions. TqPCR reactions were carried out in triplicate using the following conditions: 95°C x 3' for one cycle; 95°C x 20", 66°C x 10", for 4 cycles by decreasing 3°C per cycle; 95°C x 20", 55°C x 10", 70°C x 1", followed by plate read, for 40 cycles. GAPDH was used as a reference gene.

Construction of recombinant adenoviral vectors AdR-GLuc and AdBMP9

Recombinant adenoviruses were generated using the AdEasy technology as described [10, 11, 29]. Briefly, the coding regions of monomeric RFP and Gaussia luciferase were PCR amplified and cloned into an adenoviral shuttle vector, and subsequently used to generate recombinant adenoviral vector pAdR-GLuc, which was used for recombinant adenovirus packaging assays in the engineered stable lines. The recombinant adenovirus plasmid that co-expresses human BMP9 and eGFP was previously described [30-32]. All transgenes are driven by the CMV promoter as independent expression cassettes. Detailed information about vector constructs is available upon request.

Determination of adenovirus titers

Adenoviral lysate samples were prepared in five-fold serial dilution and used to infect subconfluent HEK-293 cells seeded in 6-well cell culture plates as described [11, 16]. The numbers of GFP or RFP-positive cells were counted at 36-48h post infection. Virus titers (expressed as infectious units per ml, or iu/ml) were calculated by averaging the numbers of GFP or RFP-positive cells over multiple dilutions for each sample.

Gaussia luciferase assay

Gaussia luciferase assay was carried out as previously described [19, 33-35]. Briefly, 50µl culture medium of the adenovirus-infected cells was taken and mixed with 20µl of the BioLux Gaussia Luciferase

Flex Assay Kit (New England Biolabs) for bioluminescence measurement. The relative GLuc activities were expressed in arbitrary units. Each assay condition was done in triplicate.

Fluorescence-activated cell sorting (FACS) analysis

Subconfluent iMEF cells were infected with the adenoviral lysate. At 36h post infection, cells were trypsinized, washed with PBS, and subjected to flow cytometric analysis of GFP-expressing cells using the BD LSR II Flow Cytometer. Uninfected iMEF cells were used as blank controls. Each assay condition was done in triplicate. The data were analyzed with FlowJo software.

ALP activity assay

ALP activity was quantitatively assessed by using a modified Great Escape SEAP Chemiluminescence assay (BD Clontech, Mountain View, CA, USA) and/or histochemical staining assay (using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt) as described [20, 36]. Each assay condition was performed in triplicate.

Statistical analysis

All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean $\pm$ S.D. Statistical significances between groups were determined by one-way analysis of variance and the Student's t-test. A value of $p < 0.05$ was considered statistically significant.

Results

Overexpression of human Ad5 pTP or E1A in 293T cells facilitates the amplification and production of recombinant adenovirus

We sought to engineer a cell line that speeds up the production and amplification of recombinant adenoviruses by stably overexpressing the adenoviral genes and host factors that are known essential to adenovirus replication and production [1, 3, 15]. We previously showed that overexpression of human Ad5 pTP in HEK-293 cells can enhance the amplification and packaging of adenovirus [16]. However, the possible contributions of adenoviral genes, which are known to participate in viral gene expression and DNA replication, have not been comprehensively evaluated. Moreover, in our pilot experiments we found that HEK-293 derivative 293T cells exhibit much higher transfection efficiency than parental HEK-293 cells. Considering that high transfection efficiency may be a prerequisite for effective adenovirus production, we chose to establish 293T stable lines that express high levels of E1A, E1B19K, E1B55K, pTP, DBP, DNA Pol, or host factor OCT1, and tested their effects on enhancing the efficiency of adenovirus packaging and amplification.

We have recently optimized the *piggyBac* transposon system, which has been shown to have significant advantages over retroviral vectors in making stable lines with high levels of transgene expression [18, 21, 22]. Using the PBC2 as a base vector we subcloned six genes of human adenovirus type 5 (i.e., E1A, E1B19K, E1B55K, pTP, DBP, and DNA Pol) and the host factor OCT1 into this vector (Fig. 1A). Using the constructed *piggyBac* vectors expressing transgenes as depicted in Fig. 1A, we established the stable lines in 293T cells, designated as 293T-E1A, 293T-pTP, 293T-E1B19K, 293T-E1B55K, 293T-DBP, 293T-Pol and 293T-OCT1. We analyzed the transgene expression in these stable lines by comparing with that in the parental 293T cells. Using quantitative PCR (Fig. 1B), we found a much higher level of each transgene expression in engineered stable lines, compared with that in the parental 293T cells. Thus, these results demonstrate that these stable lines overexpress the desired transgenes at readily detectable levels.

To determine which transgene(s) would yield the most efficient production and amplification 293T lines, we used the pre-titrated AdR-GLuc, which expresses both RFP and Gaussia luciferase (GLuc) to infect the 293T stable lines with three different titers. Consistent with our earlier report on overexpressing pTP in HEK-293 cells [16], we found that 293T-pTP line exhibited the highest numbers of RFP⁺ cells. 293T-E1A line also had higher numbers of

Fig. 1. Overexpression of the genes that may facilitate the amplification and production of recombinant adenoviruses in 293T cells. (A) Schematic representation of *piggyBac* transposon-based vectors expressing different transgenes (a). PB-TR, *piggyBac* terminal repeats; CIs, core insulators; BSD, Blasticidin S selection marker; Pa, SV40 poly A signal; CMV, Cytomegalovirus promoter. Stable 293T lines were established by co-transfecting combinations of transgene vectors and selecting the transfected cells in BSD (5 µg/ml). (B) Quantitative qPCR analysis of transgene expression in the 293T stable lines. The cDNA samples prepared from each stable lines were subjected to TqPCR analysis using gene-specific primers. GAPDH was used as the reference gene. “***” $p < 0.001$ vs. that of 293T’s. (C) Comparison of adenovirus amplification efficiency in the 293T stable lines. The stable lines were seeded at the same cell density in 24-well cell culture plates and infected with AdR-GLuc virus at 2×10^5 iu/ml (multiplicity of infection or MOI = 1), 4×10^5 iu/ml (MOI = 2) and 8×10^5 iu/ml (MOI = 4). At 48h post infection, the RFP signal was recorded, and the culture medium from each line was taken for Gaussia luciferase assay for all three titers. Luciferase assays were done in triplicate. The relative GLuc activities were expressed in arbitrary units. “*” $p < 0.05$ and “***” $p < 0.001$ vs. luciferase activity of 293T cells’. (D) Comparison of adenovirus production efficiency in the 293T stable lines. The exponentially growing 293T lines were seeded in 24-well cell culture plates (in duplicate) and transfected with Pac I-linearized pAdR-GLuc plasmid DNA (0.5 µg/well). At 5 days after transfection, cells were collected and lysed in 0.2ml PBS by 4 cycles of freezing-thawing, followed by adenovirus titrating in HEK-293 cells. RFP-positive cells were counted at 48h post infection.

Fig. 2. Comparison of recombinant adenovirus amplification efficiency in the 293T M series lines expressing multiple Ad5 genes and/or OCT1. (A) Establishment and characterization of the M series stable lines. (a) List of the M lines and their combinations of different viral genes and/or OCT1 used for making 293T stable lines. The M1 line is also known as the RAPA line. (b) Semi-quantitative PCR determination of the transgene expression in the M series lines. Total RNA was isolated from the indicated cell lines and subjected to touchdown semi-quantitative PCR analysis. GAPDH was used as the reference gene. Representative results are shown. (c) Quantitative qPCR analysis of transgene expression in the M series lines. The cDNA samples prepared in (A-b) were subjected to TqPCR analysis using gene-specific primers. GAPDH was used as the reference gene. “*” $p < 0.05$, “***” $p < 0.001$ vs. that of 293T’s. (B) Comparison of adenovirus amplification efficiency in M series lines. The M series lines were seeded at the same confluence in 24-well cell culture plates and infected with AdR-GLuc at 2×10^5 iu/ml (multiplicity of infection or MOI = 1) and 4×10^5 iu/ml (MOI = 2). At 48h post infection, the RFP signal was recorded. Only the cells infected with 4×10^5 iu/ml are shown. (C) Quantitative GLuc activity assessment of the AdR-GLuc infected M series lines. The infection conditions were set up as described above (B). The culture medium from each M line was taken for Gaussia luciferase assay for both titers (a & b). Luciferase assays were done in triplicate. The relative GLuc activities were expressed in arbitrary units. “*” $p < 0.05$ and “***” $p < 0.001$ vs. luciferase activity of 293T cells’.

RFP+ cells, compared with that of 293T cells at 48h after infection. However, 293T-E1B19K, 293T-E1B55K, 293T-DBP, 293T-Pol and 293T-OCT1 lines did not exhibit any significant differences in terms of numbers of RFP+ cells when compared with parental 293T cells (data not shown).

We determined the GLuc activities of the infected lines and found that only 293T-pTP cells had significantly higher GLuc activities at the three tested titers (Fig. 1C), while 293T-E1A cells had significantly increased GLuc activities at lower titers (Fig. 1C-ab), compared with

that of 293T cells. Specifically, the GLuc activities in 293T-pTP cells increased 191%, 120% and 15% over that of 293T cells infected at 2×10^5 iu/ml, 4×10^5 iu/ml, and 8×10^5 iu/ml, respectively (Fig. 1C). 293T-E1A cells also yielded significant increases in GLuc activities at low titers (67% and 48% increases over 293T cells at 2×10^5 iu/ml and 4×10^5 iu/ml), while other 293T stable lines did not exhibit any significant differences compared with 293T cells (Fig. 1C).

We further tested the infectious adenoviral vector production efficiency in the stable 293T lines, and found that, similar to the results obtained for adenovirus amplification, 293T-pTP yielded the highest AdR-GLuc adenovirus titer at day 5 among the engineered 293T lines, and approximately 3.5-fold and 1.5-fold higher than that for 293T's and 293T-E1A's, respectively (Fig. 1D). AdR-GLuc samples packaged in 293T-E1A cells were titrated up to 2.3-fold higher than that packaged in parental 293T cells, while other stable 293T lines did not show any significant differential packaging efficiency compared with 293T cells. Collectively, these results suggest that expression of E1A or pTP may be sufficient to facilitate adenovirus packaging and amplification whereas overexpression of adenoviral genes E1B, DBP and DNA Pol, as well as host factor OCT1, may not facilitate the adenovirus packaging and amplification process.

Co-expression of human Ad5 pTP and E1A in 293T cells markedly facilitates the amplification and production efficiency of recombinant adenoviruses

We next determined whether co-expression multiple human Ad5 genes that are involved in viral replication in 293T cells may exhibit stronger effects on the amplification efficiency of recombinant adenoviruses. Using the same *piggyBac* vectors depicted in Fig. 1A, we established a panel of 293T stable lines, namely M1 through M6, which express combinations of different viral and/or host factor OCT1 (Fig. 2A-a). We analyzed the transgene expression in these stable lines, compared with that in 293T-E1A, 293T-pTP and parental 293T cells. Using both semi-quantitative PCR (Fig. 2A-b) and quantitative PCR (Fig. 2A-c), we found that as expected both E1A and pTP were highly expressed in the 293T M lines, while E1B19K and E1B55K were highly expressed in M2 to M6 lines, compared with that in 293T-E1A, 293T-pTP and parental 293T ($p < 0.001$) (Fig. 2A-c). Elevated expression of DBP and DNA Pol was only observed in M3, M4, and/or M5 lines whereas OCT1 was overexpressed mostly in M5 and M6 lines, compared with that in 293T cells' ($p < 0.001$) (Fig. 2A-c). Taken together, these results demonstrate that the stable lines overexpress the desired transgenes at readily detectable levels.

We also tested which combination(s) of the above transgenes would yield the most efficient production and amplification lines. To determine the amplification efficiency of these engineered cell lines, we infected six 293M lines, 293T-E1A, 293T-pTP and parental 293T cells with different titers of AdR-GLuc. Under the same infection conditions, we found that while 293T-pTP and 293T-E1A groups both had higher numbers of RFP+ cells compared with that of parental 293T at 48h after infection, M1 cells exhibited the highest numbers of RFP+ cells (Fig. 2B), while M2, M3, M4, M5 and M6 lines did not exhibit any significant differences in terms of numbers of RFP+ cells (Fig. 2B).

Quantitatively, we determined the GLuc activities of the infected lines and found that 293T-pTP and 293T-E1A cells had significantly higher GLuc activities than 293T cells, while M1 cells exhibited the highest GLuc activities at both titers (Fig. 2 C-ab). The GLuc activities in M1 cells increased 259% and 161% compared with that of the 293T cells infected at 2×10^5 iu/ml and 4×10^5 iu/ml, respectively. Other 293T M lines did not exhibit any significant differences when compared with 293T cells (Fig. 2C-ab). These results indicate that under the same infection conditions adenovirus amplification was the most efficient in M1 line among all tested lines. M1 line was referred to as RAPA thereafter.

We next compared adenovirus production efficiency among parental 293T, 293T-E1A, 293T-pTP and RAPA lines. By transfecting the Pac I-linearized pAdR-GLuc plasmid DNA into these lines under the same conditions (e.g., cell density and amount of DNA), we found that RFP signal did not differ significantly among 293T-E1A, 293T -pTP, RAPA, and 293T

Fig. 3. Efficient production of recombinant adenovirus in the RAPA cells. (A) Comparison of adenovirus production efficiency in the four cell lines. The three stable lines and 293T control cells were seeded in 35mm culture dishes at subconfluence and transfected with the *Pac I*-linearized pAdR-GLuc plasmid DNA (2.5 μ g/dish). RFP-positive cells were monitored at the indicated time points and the cells were collected and lysed in 0.5ml PBS followed by 4 cycles of freezing-thawing. The cleared viral lysate samples were used for titrating in HEK-293. The average virus titers are indicated. Representative results are shown. (B) Quantitative analysis of the produced AdR-GLuc virus from the four cell lines. Subconfluent HEK-293 cells were infected with 10% of the AdR-GLuc viral lysate (VL) packaged in 293T, 293T-E1A, 293T-pTP and RAPA lines for 3 or 5 days. RFP-positive cells were monitored at the different time points. Representative images are shown at 48h post infection (a). The cell medium collected from 48h infection was used for Gaussia luciferase assay (b). The relative GLuc activities were expressed in arbitrary units. Each assay condition was done in triplicate. “*” $p<0.05$, “***” $p<0.001$ vs. that of 293T control groups’.

parental control cells at 3 days after transfection cells (Fig. 3A), although the titers of the produced adenoviruses from these lines showed significant differences. The viral titer from the RAPA line was 8.6×10^3 iu/ml, about 1.5-fold higher than 293T-pTP’s (5.7×10^3 iu/ml) and 2-fold higher than 293T-E1A (4.0×10^3 iu/ml) and 6-fold higher than 293T’s (1.5×10^3 iu/ml) ($p<0.05$) (Fig. 3A, upper panel). At 5 days after transfection, the RAPA cells yielded significant numbers of comet-like foci (titered at 1.7×10^5 iu/ml, $p<0.001$), indicating active local productions of adenovirus, whereas no such foci were observed in the transfected 293T and 293T-E1A cells (titered at 3.4×10^4 iu/ml and 5.5×10^4 iu/ml, respectively), although the RFP signal was noticeably intensified in the 293T-pTP cells (titered at 7.6×10^4 iu/ml) (Fig. 3A, bottom panel). Therefore, at day 5 of transfection/packaging, the average adenovirus titer obtained from the RAPA cells was 2.2-fold higher than 293T-pTP cell line’s, 3.1-fold higher than 293T-E1A cell line’s and 5.0-fold higher than 293T parental cells’ (Fig. 3A, bottom panel).

Fig. 4. Flow cytometric analysis of the infectious vector production efficiency of AdBMP9 adenovirus in RAPA cells. Subconfluent 293T, 293T-E1A, 293T-pTP and RAPA cells were transfected with *Pac* I-linearized pAdBMP9 plasmid DNA, which co-expresses human BMP9 and eGFP. At 5 days after transfection, cells were collected and subjected to freeze-thaw cycles to prepare the adenoviral lysate samples. The average virus titers were 3.1×10^4 , 5.0×10^4 , 8.9×10^4 , and 7.5×10^5 iu/ml for AdBMP9 viral lysate samples prepared from 293T, 293T-E1A, 293T-pTP and RAPA lines, respectively. 5% or 10% of the above titrated AdBMP9 viral lysate (VL) prepared from each line were used to infect subconfluent MSC iMEF cells seeded in 60mm cell culture dishes. At 36h after infection, cells were collected and subjected to fluorescence-activated cell sorting (FACS) analysis to detect GFP-positive cells. Representative FACS analysis data from the 10% AdBMP9 viral lysate-infected cells are shown (A), and the % of GFP-positive cells was graphed for two infection titers (B). FACS assays were done in triplicate. “*” $p < 0.05$, “**” $p < 0.001$ vs. that of 293T control’s.

To further quantify the produced adenovirus titers, we used 10% of the above titrated AdR-GLuc virus lysate samples collected at 3 or 5 days after transfection to infect subconfluent HEK-293 cells. At 48h after infection, we found that the viral lysate (VL) samples prepared from RAPA cells exhibited the highest RFP signal intensity and RFP+ cells among all four lines at both time points (Fig. 3 B-a). We further assessed the GLuc activities and found that the GLuc activities of the cells infected with AdR-GLuc viral lysate (VL) collected at 3 days after transfection were 2.4, 3.0 and 3.5-fold higher than that of 293T's for 293T-E1A, 293T-pTP and RAPA cells, respectively (Fig. 3B-b, upper panel). Similarly, the GLuc activities for the cells infected with the VL collected at 5 days after transfection were 1.8, 2.5 and 3.9-fold higher than that of 293T's for 293T-E1A, 293T-pTP and RAPA cells, respectively (Fig. 3B-b, bottom panel). These results suggest that RAPA cells may drastically facilitate adenovirus packaging and production, even more efficiently than the 293T-pTP line, which should be equivalent to our previously characterized 293pTP cells [16].

AdBMP9 produced in RAPA cells exhibits the highest activity in inducing osteogenic differentiation of mesenchymal stem cells (MSCs)

We further tested the functionality of the packaged adenoviruses using the engineered stable lines. By using recombinant adenoviruses expressing 14 types of human bone morphogenetic proteins (BMPs), we previously demonstrated the BMP9 is among the most potent BMPs to promote osteogenic differentiation of mesenchymal stem cells (MSCs) [30-32, 37-39]. Here, we transfected the previously constructed, *Pac* I-linearized pAdBMP9 plasmid, which co-expresses BMP9 and eGFP, into 293T-E1A, 293T-pTP, RAPA, and 293T lines. At 5 days after transfection, we collected the AdBMP9 viral lysate samples and found the average virus titers were 3.1×10^4 , 5.0×10^4 , 8.9×10^4 , and 7.5×10^5 iu/ml for AdBMP9 viral lysates generated from 293T, 293T-E1A, 293T-pTP and RAPA lines, respectively. We used

Fig. 5. The initial AdBMP9 viral lysate produced in RAPA cells induces the highest activity of osteogenic marker ALP in iMEF mesenchymal stem cells. The viral lysate samples from different stable lines were prepared in the same fashion as described in Figure 4. The AdBMP9 titers were 3.1×10^4 , 5.0×10^4 , 8.9×10^4 , and 7.5×10^5 iu/ml for the viral lysate samples prepared from 293T, 293T-E1A, 293T-pTP and RAPA lines, respectively. Briefly, 5% or 10% of the AdBMP9 viral lysate (VL) from each line were used to infect subconfluent iMEF cells seeded in 60mm cell culture dishes. At 7 days after infection, cells were either fixed and subjected to histochemical staining of alkaline phosphatase (ALP) activity (A), or lysed for quantitative analysis of ALP activity (B). Each assay condition was done in triplicate. Fold of ALP activity changes was calculated by dividing the average ALP activity of the stable lines' with that of 293T line's. Representative images are shown. "*" $p < 0.05$, "**" $p < 0.001$ vs. that of the 293T control's.

5% and 10% of the above titered viral preparations to infect MSC line iMEF cells for 36h. Quantitative FACS analysis revealed that the GFP+ iMEF cells were 19.4%, 30.1%, 43.8% and 53.9% for the AdBMP9 viral lysate prepared from 293T, 293T-E1A, 293T-pTP and RAPA cell lines, respectively (Fig. 4A). In fact, the % of GFP+ cells was always the highest for RAPA cell-derived AdBMP9 viral lysate when the iMEFs were infected with different titers of the AdBMP9 viral lysate samples. Specifically, when iMEFs were infected with 5% of the AdBMP9 viral lysate, the average % of GFP+ cells was 11.2%, 17.4%, 24.5% and 34.1% for 293T, 293T-E1A, 293T-pTP and RAPA lines (Fig. 4B). Similarly, when iMEFs were infected with 10% of the AdBMP9 viral lysate, the average % of GFP+ cells was 18.2%, 33%, 41.6% and 54.2% for 293T, 293T-E1A, 293T-pTP and RAPA lines (Fig. 4B). These results indicate that the RAPA viral lysate samples possessed the highest titers among the viral lysate samples prepared under the same conditions.

We further analyzed the differential capability of the produced AdBMP9 adenovirus from the four lines in inducing early osteogenic marker alkaline phosphatase (ALP) in MSCs. We found that the AdBMP9 viral lysate from RAPA cells exhibited the highest and strongest ALP positive staining among the viral lysates prepared from all four cell lines (Fig. 5A). Quantitative ALP activity assay further indicates that, using 5% of the above titered AdBMP9

Fig. 6. The adenovirus production efficiency is dose-dependent on the recombinant adenovirus plasmid DNA in RAPA cells. Subconfluent RAPA cells were seeded in 24-well culture plates and transfected with the indicated amounts of *Pac* I-linearized pAdR-GLuc plasmid in triplicate. RFP signal was detected at days 2, 3, 4, and 5. Representative results are shown. Replicates were collected for viral lysate preparations and titrating assays as indicated in each condition.

lysates, the ALP activities of the AdBMP9 lysate prepared from 293T-E1A, 293T-pTP and RAPA were 1.68, 3.57 and 3.97-fold higher than that of the AdBMP9 lysate prepared from the 293T cells (Fig. 5B). Similarly, when using 10% of the titered AdBMP9 lysates, the ALP activities were shown to elevate 2.01, 3.28 and 4.05-fold over that of 293T controls for 293T-E1A, 293T-pTP and RAPA lines, respectively (Fig. 5B). Thus, these functional assays further demonstrate that the AdBMP9 packaged in the RAPA cells exhibited the highest titer and thus induced the most effective osteogenic differentiation of MSCs.

Efficient and rapid production of recombinant adenoviruses in RAPA cells is also dependent on the efficient transfection of a sufficient amount of adenovirus plasmid DNA

Based on our extensive experience on generating recombinant adenoviruses, the initial production and packaging efficiency are largely dependent on transfection efficiency and amount of DNA used for transfection. To accomplish efficient and rapid adenovirus production in RAPA cells, we further tested the effect of varied amounts of transfected plasmid DNA on adenovirus production efficiency. Using the *Pac* I-linearized pAdR-GLuc plasmid, we transfected RAPA cells with different amounts (0.5µg, 1.0µg and 2.0µg per well) of plasmid DNA and monitored the appearance and intensity of RFP+ cells. We found that, while there was a time-dependent increase in RFP+ cells in the wells transfected with 0.5µg DNA, no apparent cytopathic effect was observed at 5 days after transfection (Fig. 6a). When the amount of DNA increased to 1.0µg DNA per well, the presence of RFP+ cells was time-dependent and became apparent at day 4 while some cytopathic effects were visible at day 5 (Fig. 6b). Furthermore, when using 2.0µg DNA per well, there were higher numbers of the RFP+ cells than that of other two groups at day 3, and the cytopathic effect was observed at day 4, while a profound cytopathic effect was observed at 5 days after transfection (Fig. 6c), indicating a significant and rapid production of recombinant AdR-GLuc virus. Titer analysis of the packaged adenoviruses indicated that under the same transfection conditions virus production increased with the amounts of the adenoviral plasmid DNA. For example, at five days after transfection the adenovirus titer for the 2.0µg/well group (2.9×10^7 iu/ml) is approximately 6.9-fold of the 1.0µg/well group's (4.2×10^6 iu/ml) and 51.8-fold of the 0.5µg/well group's (5.6×10^5 iu/ml) (Fig. 6, bottom row). Thus, these results strongly suggest that

high titer of adenoviruses may be produced in the RAPA cells as rapidly as within 5 days if an efficient transfection of sufficient amount of recombinant adenoviral plasmid DNA is accomplished.

Discussion

Although recombinant adenoviral vectors have been widely used in basic and translational research, the efficient generation and production of recombinant adenoviruses remains a challenging and time-consuming task for many investigators [1, 4]. In order to expedite recombinant adenovirus production and amplification processes, we conducted a functional analysis of five human Ad5 viral genes, including E1A, E1B19K/55K, pTP, DBP and DNA Pol, and host factor OCT1, and through different combinations delineated their contributions to adenovirus production and amplification. Although HEK-293 cells harbor adenoviral E1A and E1B genomic DNA, their expression levels can be further enhanced by using our *piggyBac* transposon system. Consistent with our earlier studies [16], overexpression of pTP is beneficial for adenovirus production. Interestingly, the overexpression of E1B19K/55K, DBP, Ad Pol and/or OCT1 on top of the E1A and pTP expression did not improve the production and amplification efficiency of recombinant adenoviruses, suggesting that overexpression of E1B19K/55K, DBP, Ad Pol and/or OCT1 may interfere with adenovirus production facilitated by E1A and/or pTP. Such results were reproducible in HEK-293, 293T, and 293 Phoenix lines (data not shown). The exact cause(s) are not clearly understood. Given the complex functions of most adenoviral genes, it's conceivable that constitutive overexpression of E1B19K/55K, DBP, Ad Pol and/or OCT1 may negatively affect adenovirus genome DNA replication and hence virus production. Nonetheless, our results demonstrate that co-overexpression of E1A and pTP in 293T cells is sufficient to drive a rapid production of recombinant adenovirus.

Transcription regulation and viral DNA replication of the adenovirus life cycle offer tremendous insights into our efforts to develop a rapid adenovirus producer line [1, 2, 4, 15]. It was shown that adenovirus genome can be replicated *in vitro* by the combined action of three viral proteins (pTP, Ad Pol and DBP), two cellular transcription factors (NFI/CTFI and NFIII/OCT1), and topoisomerase I [40]. Our studies highlight the essential roles of E1A and pTP in adenoviral production in 293T cells. E1A is necessary for viral replication, as it is the most important viral transcriptional transactivator for the temporal transcription of the viral genome. It also reprograms the host cell transcription to induce S phase [1], through interactions with host cell transcription factors (e.g., ATF-2, YY1), co-activators (CBP/p300), co-repressors (CtBP), nucleosomal remodeling factors (SWI/SNF), and the general transcription machinery (TBP, TAFs) [15]. The Ad5 pTP also plays an essential role in viral DNA replication as the initiation of DNA replication starts by covalent coupling of the dCMP residue of the 5' termini inverted terminal repeats (ITRs) to pTP and Ad5 DNA Pol complex in 3'-OH group, which serves as a primer for further elongation by a strand displacement mechanism [15]. We have demonstrated that exogenous pTP expression in HEK-293 cells can accelerate the packaging and amplification of recombinant adenoviruses [16]. In the current study, we further show that overexpression of both E1A and pTP in 293T cell line leads rapid adenovirus production and amplification.

Most first-generation adenoviral vectors can be propagated to high yields in permissive E1 complementation or packaging cell line HEK-293 cells, which provide the E1 functions in *trans* [6]. HEK-293 cells were human embryonic kidney cells stably transduced with sheared adenoviral genomic DNA containing the first 4 kb of the Ad5 genome sequence [6, 7]. In addition to adenovirus packaging and amplification, the HEK-293 line is one of the most commonly used lines for molecular and cell biology studies, largely owing to its easy maintenance, cell line stability and high transfection efficiency. In fact, various HEK-293 derivative lines have been established for exogenous gene expression or transgene productions [41-43]. One such line is 293T. Among several HEK-293 derivative lines, we found that 293T cells exhibit high transfection efficiency, which should be beneficial for the effective transfer of large recombinant adenovirus plasmid molecules into packaging

cells. We demonstrated that RAPA adenovirus producer line allows us to obtain high titer adenoviruses as early as at 5 days after transfection.

In order to achieve a high level and stable transgene expression in 293 cells, we choose to use our optimized *piggyBac* transposon-based gene expression system [18, 21, 22], which can effectively integrate multiple copies of the transgene into relatively AT-rich regions of host chromosomes. We successfully established six types of genetically modified 293T M lines with different combinations of viral genes and/or OCT1 and found that the stable 293T M lines express high levels of transgenes. The engineered RAPA producer line overexpressing both E1A and pTP driven by constitutive CMV promoters as we previously showed that the constitutive CMV promoter drives the highest stable transgene expression using the *piggyBac* transposon system [44].

In summary, we engineered a rapid adenovirus production and amplification (RAPA) cell line through a functional analysis of five human Ad5 viral genes and the host factor OCT1. Our results demonstrate that RAPA cells can package and yield high titers of recombinant adenoviruses within 5 days under optimal transfection conditions. Thus, the use of RAPA cells should expedite the generation and production of recombinant adenoviruses and would be beneficial for most applications of adenovirus-mediated gene deliveries *in vitro* and *in vivo*.

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Disclosure Statement

The authors declare no conflicts of interest.

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