Biotechnology and Bioprocess Engineering 27: 344-352 (2022)
DOI 10.1007/s12257-021-0222-y

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Bacterial Artificial Chromosome-based Protein Expression Platform Using the Tol2 Transposon System

Myeong Uk Kuk, Ji Yun Park, Eun Seon Song, Haneur Lee, Yun Haeng Lee, Junghyun Joo, Hyung Wook Kwon, and Joon Tae Park

Received: 6 August 2021 / Revised: 16 November 2021 / Accepted: 10 December 2021

Abstract Conventional vector systems, including plasmid-based vectors, are mainly used in mammalian cells for the production of biopharmaceuticals. Plasmid-based vectors express transgene by the random integration of recombinant transgenes into the genome. Transgene expression is greatly influenced by the surrounding chromatin, and in most cases, expression is weak and tends to be suppressed over time. Therefore, a novel strategy is required to create clones that maintain increased transgene expression. In this study, we used a bacterial artificial chromosome (BAC) containing the *Rosa26* locus, which allows constitutive and ubiquitous gene expression. Moreover, we improved the *Rosa26* BAC-mediated expression system by incorporating the Tol2 transposon system with helper vector, resulting in improved efficiency of protein production and maintained productivity even in single-cell clones. Furthermore, the recombinant *Rosa26* BAC was improved in terms of protein productivity by using helper mRNA instead of helper vector. Finally, establishment of an optimal molar ratio between the recombinant *Rosa26* BAC and helper mRNA helped to achieve maximal protein productivity. Taken together, our results provide an effective strategy for improving BAC-based expression systems for biopharmaceutical production.

Myeong Uk Kuk, Ji Yun Park, Eun Seon Song, Haneur Lee, Yun Haeng Lee, Junghyun Joo, Hyung Wook Kwon*, Joon Tae Park*
Division of Life Sciences, College of Life Sciences and Bioengineering, Incheon National University, Incheon 22012, Korea
Convergence Research Center for Insect Vectors, Incheon National University, Incheon 22012, Korea
Tel: +82-32-835-8090; Fax: +82-32-835-0754
E-mail: hwkwon@inu.ac.kr
Tel: +82-32-835-8841; Fax: +82-32-835-0754
E-mail: joontae.park@inu.ac.kr

Keywords: *Rosa26* BAC, Tol2 transposon system, helper mRNA, bacterial artificial chromosome

1. Introduction

Mammalian cell lines are used as hosts in the production of biopharmaceuticals, with Chinese hamster ovary (CHO) cells accounting for the largest proportion [1,2]. In mammalian cells, more than 100 therapeutic proteins are being produced, and this number is expected to rise substantially as new therapeutic proteins are developed [3]. Plasmid-based vectors are primary tools used for protein production [4]. Plasmid-based vectors persist as extra-chromosomal replication episomes in mammalian cells, allowing for peak gene expression between 24-96 h post-transfection [5]. However, episomal stability is limited, resulting in progressive loss of the transfected vector [6]. To circumvent the inconsistent expression issues, stably-transfected cells usually have to be selected and cultured in various ways. For example, the limited dilution method can enable selection of genetically homogeneous clonal populations [7]. During the selection process, a population in which the introduced plasmid is integrated into the host genome is selected, enabling stable expression of the introduced transgene as opposed to transient expression in which the introduced DNA persists for several days in cells [8]. However, in stable clones, transgene expression is strongly influenced by the chromatin surrounding the integration site as the recombinant constructs are randomly integrated into the genome. For example, when a vector integrates into a region of silent chromatin, its expression tends to be suppressed over time (*i.e.*, positional chromatin effects) [9]. Given these findings, the use of plasmid-based

vector systems in protein production has been questioned; more effective strategies are needed to avoid these shortcomings [10,11]. Bacterial artificial chromosomes (BAC) have been proposed as novel vector platforms for avoiding positional chromatin effects. BAC can cover a 150-350 kb genomic region that accommodates complete genes, including all *cis*-acting regulatory elements (enhancers, promoters, insulators, *etc*.) in their native configuration [12]. Furthermore, BAC can maintain the expected expression pattern with minimal interference from surrounding chromatin [9]. Thus, BAC-based expression systems are considered complete expression units and are unaffected by the surrounding chromatin [13].

Rosa26, located on chromosome 6 in mice, was originally identified by a gene-trap mutagenesis screen [14]. *Rosa26* supports constitutive gene expression and is not subjected to gene-silencing effects. More than 560 *Rosa26* knock-in lines have been generated and have demonstrated constitutively expressed transgenes *in vivo* [15]. Recently, BAC containing the *Rosa26* locus (*Rosa26* BAC) has emerged as a reliable resource for generating animal models to analyze biological processes [16]. Transgenes integrated into *Rosa26* BAC faithfully reproduced the expression pattern of the original *Rosa26* locus with minimal interference from chromatin regions next to the integration site. *Rosa26* BAC-based expression system has been used for recombinant protein production in mammalian cells [17]. The importance of *Rosa26* BAC as an expression vector is supported by the discovery that a *Rosa26* BAC-based expression system increased protein productivity 10-fold compared to conventional vector system [18]. However, since the BAC size is much larger than that of a normal plasmid, the efficiency of transgene integration is very low [19]. These drawbacks cause less BAC integration into the genome, resulting in lower protein productivity than plasmid-based vectors. Thus, an enormously time-consuming screening procedure is required to find the correct clones that produce the desired amount of protein, which in turn delays the cell line development period [19]. Given these findings, more effective strategies are needed to overcome these obstacles in the *Rosa26* BAC-based expression system.

Transposon systems like as Sleeping Beauty (SB), piggyBac (PB), and Tol2 have been investigated for efficient protein production by facilitating the integration of genes into the genome [20,21]. Transposon systems consist of genetic components that mobilize transgenes and incorporate them at a genomic locus [22]. They differ in size limitations with respect to the transgene's carrying capacity [23]. Specifically, since the BAC contains a 150-350 kb genomic region, a transposon system capable of mobilizing big transgenes is preferred [24]. It was known that when SB transposon carried a 6 kb transgene, its transposition efficiency was reduced by 50% [21]. PB transposons could carry up to 14 kb, but their efficiency of transposition was significantly reduced [25]. However, the Tol2 transposon can carry a 10 kb insert without reducing transposition efficiency significantly [24].

In this study, we aimed to evaluate the use of the *Rosa26* BAC-based expression system. We improved its possible shortcomings by integrating the Tol2 transposon system. Here, we report a novel BAC-based expression system that will open a new era of recombinant protein production with higher and more stable productivity.

2. Materials and Methods

2.1. Plasmid design and construction

Standard cloning techniques were used to construct recombinant plasmids. A BAC targeting vector was designed as described previously [26]. A chloramphenicol resistance (*CamR*) targeting vector (131590; Addgene, Watertown, MA, USA) was used to integrate Tol2 transposon system. A helper vector was constructed by inserting a cDNA encoding Tol2 transposase into the pcDNA 6 vector (V22020; Invitrogen, Carlsbad, CA, USA). A control vector was constructed for the cytomegalovirus (CMV) promoter-driven luciferase gene.

2.2. Helper mRNA (Tol2 transposase mRNA) synthesis

mRNA was synthesized using the MEGAscript™ T7 Transcription Kit (AM1333; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol.

2.3. Bacterial strain, BAC clones and BAC recombineering

SW105 bacteria were generously provided by the Copeland laboratory at the National Cancer Institute. The *Rosa26* BAC clone (RP24-85L15) was purchased from BACPAC Resources Center (BACPAC Genomics, Emeryville, CA, USA). BAC recombineering was performed according to the λ Red recombineering procedure [27].

2.4. Cell culture

CHO DG44 cells (A1100001; Thermo Fisher Scientific) were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose supplemented with 10% fetal bovine serum (SH30919.03; Hyclone, Waltham, MA, USA), 10 mM sodium hypoxanthine and 1.6 mM thymidine (2068642; Gibco, Waltham, MA, USA) along with 100 U/mL penicillin and 100 μg/mL streptomycin (SV30079.01; Hyclone). The cells were cultured in ambient air (20% O_2) supplemented with 5% CO_2. Confluent cells were split 1:4, and the culture medium was changed every 2 days. Cell density and viability were assessed using Cedex HiRes

Analyzer (05650216001; Roche, Basel, Switzerland).

2.5. Transfections and cell line development

CHO DG44 cells were transfected using Lipofectamine™ 2000 (11668030; Thermo Fisher Scientific) according to the manufacturer's instructions. Cells (2×10^6) were transfected with a 0.3 μg control vector (size: 7,123 bps) or 6 μg recombinant *Rosa26* BAC (size: 222,649 bps). The transfected copy ratio between the control vector and recombinant *Rosa26* BAC was set to 2:1. For the transfection of recombinant *Rosa26* BAC and helper vector, cells (2×10^6) were co-transfected with 6 μg recombinant *Rosa26* BAC and 0.667 μg helper vector. For the transfection of recombinant *Rosa26* BAC and helper mRNA, cells (2×10^6) were co-transfected with 6 μg recombinant *Rosa26* BAC and 0.667 μg helper mRNA. To optimize the amount of recombinant *Rosa26* BAC, cells (2×10^6) were transfected with varying amounts of recombinant *Rosa26* BAC (2, 4, 8, 16, 32, and 64 μg). To determine the optimal ratio between the recombinant *Rosa26* BAC and helper mRNA, cells (2×10^6) were co-transfected with 8 μg recombinant *Rosa26* BAC and varying amounts of helper mRNA (0, 0.444, 0.889, 2.667, 5.333, and 8 μg). Transfected cells were selected for two weeks using 500 μg/mL G418 (antgn-1; Invitrogen). During selection, the medium was changed every two days. After selection, single cells were seeded per well in 150 μL medium in 96-well plates (353072; Falcon, Franklin Lakes, NJ, USA) and incubated for 15 days.

2.6. Measurement of luciferase activity

The cell number was determined using the Cedex HiRes Analyzer, and luciferase protein concentration was analyzed using a luciferase assay system (E1500; Promega, Madison, WI, USA). Cells (2×10^6) were centrifuged at 200 × *g* for 2 min and washed twice with phosphate-buffered saline (PBS). Cells were vortexed in 100 μL of 1× cell culture lysis reagent and 100 μL of PBS. Lysed samples (100 μL) were transferred into each well of a white 96-well plate (30396; SPL Life Sciences, Pocheon, Korea), and 100 μL of luciferase assay reagent II was added to each well. Luciferase activity was evaluated by measuring the luminescence intensity on a VICTOR multi-label plate reader (2030-0050; PerkinElmer, Waltham, MA, USA).

2.7. Western blot analysis

Western blotting was performed as described previously [28]. Proteins were detected with the SuperSignal™ West Pico chemiluminescence solution (34577; Thermo Fisher Scientific) using a Chemidoc XRS+ system (1708265; Bio-Rad, Hercules, CA, USA). The primary antibodies used in this study were horseradish peroxidase (HRP)-conjugated anti-luciferase antibody (sc74548; 1:1,000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) and HRP-conjugated β-actin (sc47778; 1:1,000 dilution; Santa Cruz Biotechnology). The band densities of luciferase and β-actin were quantified by using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

2.8. Statistical analyses

Statistical analyses were performed using a standard statistical software package (SigmaPlot 12.5; Systat Software, San Jose, CA, USA). Student's *t*-test was used to determine statistical significance.

3. Results

3.1. Establishment of BAC-based expression system

Two strategies were used to construct robust BAC-based expression vectors suitable for protein production. First, we used the *Rosa26* BAC containing the *Rosa26* locus, which acts as a hotspot to enable constitutive and ubiquitous expression [29]. For BAC recombineering, a BAC targeting vector was designed wherein the 5′ homology region (HR) and 3′ HR flanked the CMV promoter-driven luciferase gene (Fig. 1A). Previously, *Rosa26* BAC-based expression was regulated by the CAG (CMV enhancer, chicken beta-actin promoter, and rabbit beta-globin splice acceptor site) hybrid promoter [18], but in this study, the CMV promoter was used because it enables transgene expression of the transgene over a longer period than the CAG hybrid promoter [30]. Second, to increase the genomic integration of *Rosa26* BAC, we used the Tol2 transposon system, which significantly increased the genomic integration rate in the germline [22,31]. For the recombineering, a *CamR* targeting vector was designed to contain 5′ *CamR* HR, 3′ *CamR* HR, a pair of inverted terminal repeats (ITRs), an ampicillin resistance (*AmpR*) gene, and a neomycin resistance (*NeoR*) gene (Fig. 1A). Following BAC recombineering, the recombinant region was verified. DNA sequencing revealed recombinant sequences, indicating successful homologous recombination in the *Rosa26* BAC (recombinant *Rosa26* BAC) (Fig. 1B). The helper vector, designed to bind to ITRs and facilitate transposon-mediated transgene integration, encodes the Tol2 transposase (Fig. 1C) [32]. The control vector contained only the CMV promoter-driven luciferase gene to compare expression levels with the recombinant *Rosa26* BAC-based expression system (Fig. 1D).

The cells were transiently transfected with the designated vectors, and luciferase activity was measured. The recombinant *Rosa26* BAC exhibited significantly increased luciferase activity compared with the control vector, supporting the

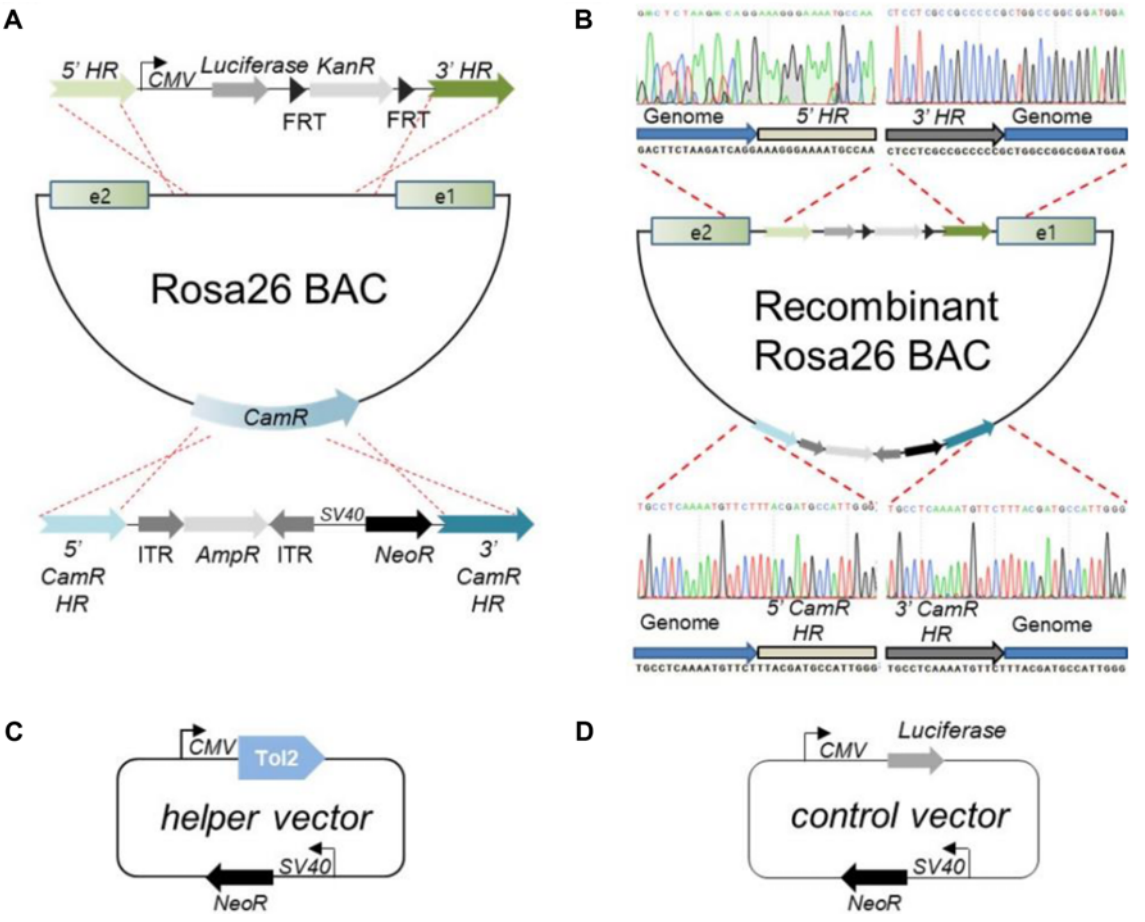

Fig. 1. Comparison of expression platform between recombinant *Rosa26* bacterial artificial chromosome (BAC) and conventional vector. (A) Schematic of the BAC recombination procedure for integrating the expression cassette and Tol2 transposon system. (B) Chromatogram results showing that the sequence of the recombinant *Rosa26* BAC was conserved compared to the reference sequence. (C) Helper vector containing a cytomegalovirus (CMV) promoter-driven Tol2 transposase gene. (D) Control vector containing a CMV promoter-driven luciferase gene. e1: exon1, e2: exon2, HR: homology region, *KanR*: kanamycin resistant gene, *CamR*: chloramphenicol resistant gene, *AmpR*: ampicillin resistant gene, *NeoR*: neomycin resistance gene, ITR: inverted terminal repeat, FRT: flippase recognition target.

previous finding that the *Rosa26* BAC-based expression system significantly improved protein productivity compared with the conventional vector system (Fig. 2A) [18]. Furthermore, when the helper vector was used together with the recombinant *Rosa26* BAC, the productivity improvement effect by the recombinant *Rosa26* BAC was increased. Specifically, the recombinant *Rosa26* BAC/helper vector facilitated the significant increase in luciferase activity compared to control vector and the marked increase compared with the recombinant *Rosa26* BAC alone (Fig. 2A). We then examined the efficiency of transgene integration to rule out the idea that the luciferase activity-inducing effect was not derived from our novel vector platform. Recombinant *Rosa26* BAC or recombinant *Rosa26* BAC/helper vector showed significantly lower transgene integration efficiency than control vector vectors, whereas these groups exhibited significantly higher luciferase activity (Fig. S1 and Fig. 2A). These data indicated that groups with lower transgene integration exhibited higher luciferase activity, thereby ruling out the possibility that differences in physical transfer efficiency could lead to differences in luciferase expression (Fig. S1 and Fig. 2A). Based on these data, we concluded that the luciferase activity-inducing effect was primarily attributed to the effect of our new vector platform.

Single clones should be established from cell pools to meet regulatory criteria, as single clones assure monoclonality and provide consistent results [33]. Single-cell isolation was performed using limiting dilutions to guarantee that the cellular characteristics of the cell pool remained the same for single clones, and 30 single clones were established for each group (Fig. 2B). Single-cell clones established using the recombinant *Rosa26* BAC/helper vector significantly increased luciferase activity compared with those established

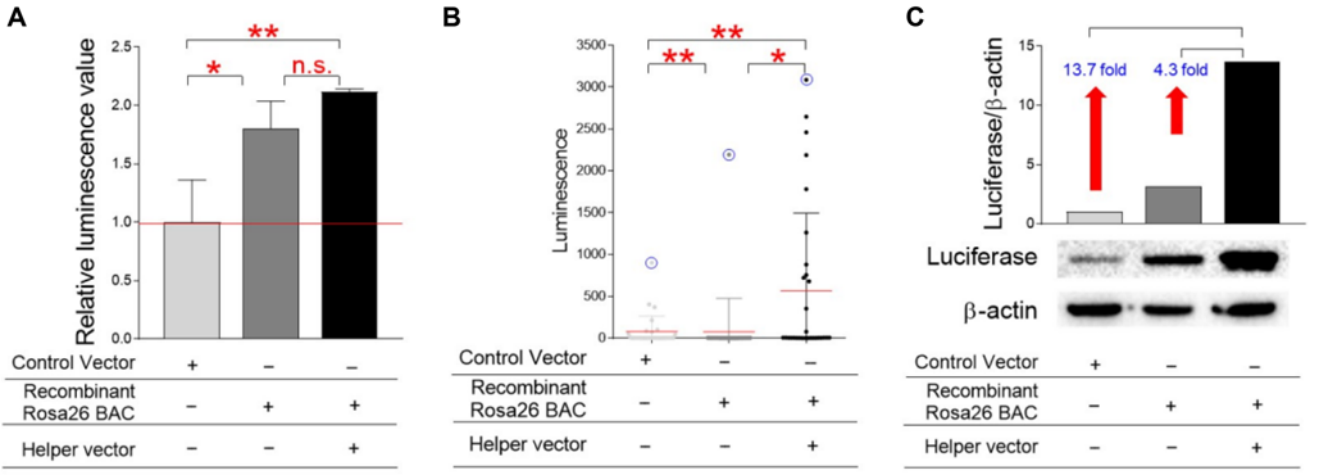

Fig. 2. Effect of Tol2 transposon system on the recombinant *Rosa26* BAC-mediated expression system. (A) Relative luciferase activity of cells transfected with control vector, recombinant *Rosa26* BAC, and recombinant *Rosa26* BAC/helper vector. *p < 0.05, **p < 0.01, Student's *t*-test. Means ± standard deviation (SD), N = 3. (B) Luciferase activity in single-cell derived stable clones established using control vector, recombinant *Rosa26* BAC, and recombinant *Rosa26* BAC/helper vector. Each dot represents the luciferase activity of single cell clones. The horizontal bar symbolizes the average of luciferase activity for each condition. Single-cell clones indicated as circles were used in western blot experiments. *p < 0.05, **p < 0.01, Student's *t*-test. Means ± SD, N = 30, for each condition. (C) Western blot analysis of protein productivity in single-cell derived stable clones established using control vector, recombinant *Rosa26* BAC, and recombinant *Rosa26* BAC/helper vector. The band density of luciferase was normalized to that of β-actin. BAC: bacterial artificial chromosome, n.s.: not significant.

using the control vector and the recombinant *Rosa26* BAC alone (Fig. 2B). These results indicate that the recombinant *Rosa26* BAC/helper vector-mediated productivity-inducing effect is maintained even in single-cell clones, justifying the use of recombinant *Rosa26* BAC/helper vector in cell line development. We then examined the protein production level by Western blotting. The recombinant *Rosa26* BAC/helper vectors exhibited in 13.7-fold and 4.3-fold increase in luciferase protein production compared with the control vector and the recombinant *Rosa26* BAC alone, respectively (Fig. 2C).

3.2. Effect of helper mRNA (Tol2 transposase mRNA) on recombinant *Rosa26* BAC-mediated expression system

Tol2 transposon system is driven by a cut-and-paste mechanism in which the Tol2 transposase recognizes a pair of ITRs in the transposon vector [34]. Tol2 transposase is introduced via a helper vector, but sustained expression of the helper vector may lead to remobilization and reintegration of the transposon vector, increasing potential damage to chromosomes [11]. Therefore, in this study, as an alternative, helper mRNA (Tol2 transposase mRNA) was used to address the shortcomings that may result from using a helper vector. The recombinant *Rosa26* BAC/helper vector exhibited a significant increase in luciferase activity compared with the control vector (Fig. 3). Furthermore, when the helper mRNA was used together with the recombinant *Rosa26* BAC, the productivity improvement

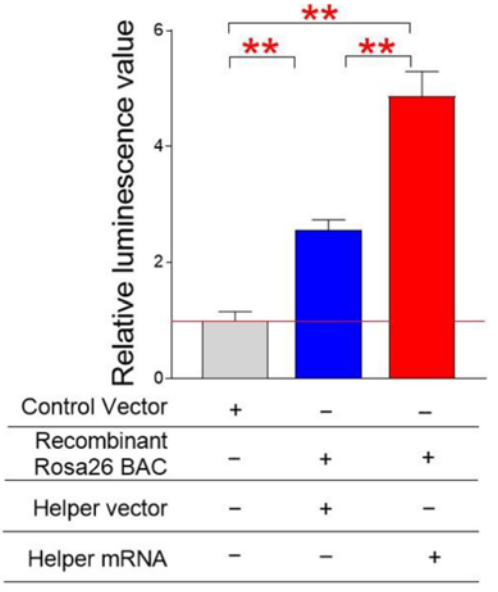

Fig. 3. Role of helper mRNA in the recombinant *Rosa26* BAC-mediated expression system. Relative luciferase activity of cells transfected with control vector, recombinant *Rosa26* BAC/helper vector, and recombinant *Rosa26* BAC/helper mRNA. **p < 0.01, Student's *t*-test. Means ± standard deviation, N = 3. BAC: bacterial artificial chromosome.

effect was enhanced. Specifically, the recombinant BAC/helper mRNA significantly increased luciferase activity compared with the control vector and the recombinant BAC/helper vector, indicating that providing helper mRNA

can be utilized as an effective approach to boost protein production (Fig. 3).

3.3. Optimization of the recombinant *Rosa26* BAC/helper mRNA-based expression system

The improvement in protein productivity due to the use of helper mRNA in the recombinant *Rosa26* BAC-based system suggested the possibility of achieving the highest protein productivity by optimizing the transfection conditions of recombinant *Rosa26* BAC and helper mRNA. We optimized the amount of recombinant *Rosa26* BAC by transfecting cells with varying amounts of BAC. Cells transfected with either 8 μg or 64 μg of recombinant *Rosa26* BAC showed a significant increase in luciferase activity compared to the control vector (Fig. 4A). However, cells transfected with varying amounts of recombinant *Rosa26* BAC did not show an increase in luciferase activity in a dose dependent manner (Fig. 4A). This inconsistency could be explained by the prior observation that the optimal amount of transposon vector falls within a limited concentration range [35]. Higher or lower amounts of transposon vectors inhibit the development of the transposase-transposon complex, which is crucial for the transposon-mediated gene transfer [35]. To select the optimal condition between 8 μg and 64 μg of recombinant *Rosa26* BAC, we examined the toxicity based on the viability. Cells transfected with 64 μg of recombinant *Rosa26* BAC showed the lowest viability even though they exhibited the highest luciferase activity (Fig. S2 and Fig. 4A). However, the 8 μg condition, which exhibited the second highest luciferase activity, showed significantly higher viability compared to the 64 μg condition (Fig. S2 and Fig. 4A). Thus, 8 μg condition was chosen for subsequent experiments because the condition exhibited the second highest luciferase activity but less toxicity.

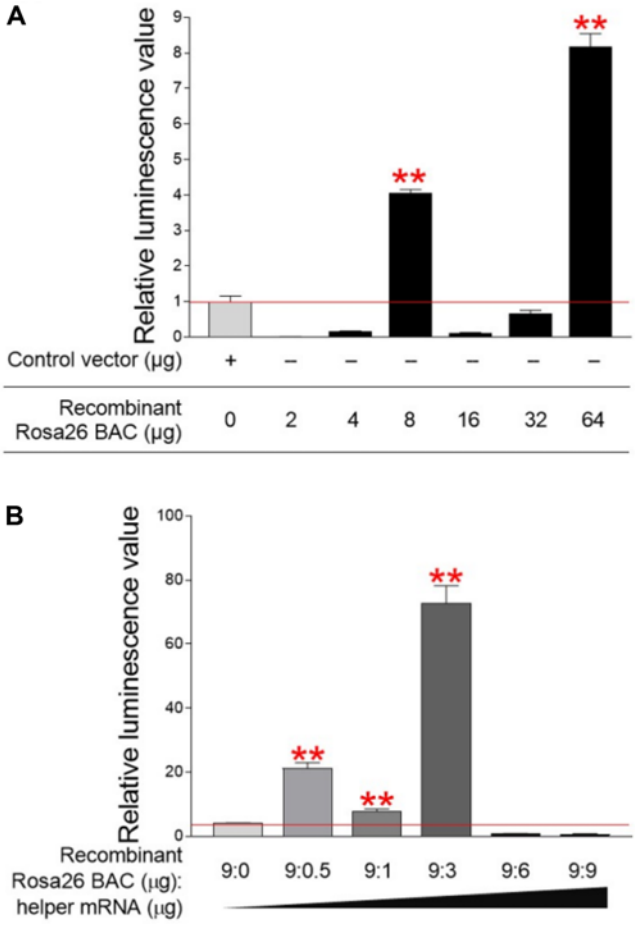

Fig. 4. Optimization of recombinant *Rosa26* BAC/helper mRNA to maximize protein productivity. (A) Relative luciferase activity of cells transfected with varying amounts of recombinant *Rosa26* BAC. **p < 0.01, Student's *t*-test. Means ± standard deviation (SD), N = 3. (B) Relative luciferase activity of transfected cells at varying weight ratios of recombinant *Rosa26* BAC and helper mRNA. **p < 0.01, Student's *t*-test. Means ± SD, N = 3. BAC: bacterial artificial chromosome.

Then, the amount of recombinant *Rosa26* BAC was fixed at 8 μg and the amount of helper mRNA was varied to determine the optimal ratio between recombinant BAC and helper mRNA. Cells transfected with 9:0.5, 9:1 or 9:3 ratios showed significantly increased luciferase activity compared to 9:0 ratio, although there was a difference in the level of increase (Fig. 4B). However, cells transfected with 9:6 or 9:9 ratios exhibited significantly decreased luciferase activity (Fig. 4B). Among the conditions, the 9:3 ratio achieved the highest yield and was chosen for an effective condition to maximize protein production (Fig. 4B).

4. Discussion

Developing strategies to boost protein productivity in mammalian cells is a prominent research goal in the field of biopharmaceuticals. Several approaches have been attempted to increase protein productivity, including vector engineering, metabolic engineering of host cells, and optimization of media components. Among them, the engineering and construction of new vectors is the easiest and most promising way to enable overproduction of the protein of interest [36,37]. To evaluate the protein productivity of established vector platforms, luminescence as a reporter protein has been used in various studies [38,39]. The luciferase reporter system is one of the most objective methods to monitor the protein production efficiency of a vector platform [40,41]. In this study, we developed a vector platform by incorporating the Tol2 transposon system into the *Rosa26* BAC system and measured luciferase activity to test its performance. This vector platform enhanced protein productivity in mammalian

cells. Furthermore, the impact of enhanced protein productivity was observed even in single clones produced from cell pools. Although the productivity-enhancing effects of this vector system have been confirmed, questions remain about limiting the molecular weight of the proteins it produces. The answer to this question can be inferred from the results of other studies. For example, genes encoding high molecular weight protein (β-galactosidase, 465.4 kDa) and low molecular weight proteins (super-folded green fluorescent protein [sf-GFP], 26.8 kDa) were integrated into the vector platform in *Bacillus subtilis* [42]. The vector platform produced 26 times the amount of large protein (β-galactosidase, 465.4 kDa) and 195 times the amount of small protein (sf-GFP) compared to the control vector [42]. Therefore, we suggest that even if our vector platform shows fold differences in the production of large and small proteins, it will not represent the weight limit of the proteins it produces. However, we admit that more research is needed to confirm our suggestion. To the best of our knowledge, this study provides the first demonstration that recombinant *Rosa26* BAC in combination with the Tol2 transposon system can overcome the shortcomings of conventional expression systems and consequently enable enhanced protein production in mammalian cells.

Tol2 transposon system has been utilized to increase transgene integration efficiency, which has improved protein productivity [22,43,44]. Despite the fact that transposon-mediated increase in protein productivity has attracted much attention, sustained expression of helper vectors can potentially damage the host genome and even decrease protein productivity [11]. Extending the relevance of these findings, helper vectors encoding Tol2 transposase can integrate into the genome, which can enable stable Tol2 transposase expression, resulting in destabilized effects on already integrated transgenes [10]. Tol2 transposase mRNA may be an effective alternative to helper vector, as a source of Tol2 transposase. It is possible that Tol2 transposase mRNA is reversely transcribed to synthesize cDNA, which is incorporated into the host genome, but this has rarely occurred. In this study, to circumvent the possible flaws of the transposon system, we used Tol2 transposase in the form of mRNA (helper mRNA) that would eventually be degraded in cells. Helper mRNA functioned as a transient source of Tol2 transposase to induce integration of recombinant *Rosa26* BAC in the genome. We propose that recombinant *Rosa26* BAC with helper mRNA serves as a novel expression platform for efficient protein production in mammalian cells.

The optimal molar ratio of the transposon vector and transposase is critical for the efficient formation of the transposon-transposase complex, which is important for the cut-and-paste mechanism of the transposon system [34]. For example, as the amount of SB transposase increases, the translocation activity of the SB transposon system decreases instead of reaching a plateau [45]. The significance of the optimal molar ratio is supported by the finding that the transposition activity of the PB transposon system decreased with increasing amount of PB transposase [46]. However, a general consensus on the optimal molar ratio has not been established, as opposite results have been observed with other transposon systems [47]. This discrepancy can be explained by the finding that an excess or lack in transposon vector quenched the formation of the transposase-transposon complex [35]. Because the optimal ratio of transposon vector and transposase falls within a small range, it should be studied at each transposon-mediated potential. In this study, we evaluated the amount of recombinant *Rosa26* BAC capable of increasing protein productivity and then determined the optimal molar ratio to maximize protein productivity by varying the helper mRNA concentration. The established ratio proved to be a more effective way of increasing protein productivity in the *Rosa26* BAC-mediated expression system.

5. Conclusion

We have developed an efficient expression platform for producing recombinant proteins. Evaluation of the effects of recombinant *Rosa26* BAC in combination with the Tol2 transposon system provided insight into the mechanisms leading to higher levels of protein production. Furthermore, the established optimal molar ratio between recombinant *Rosa26* BAC and helper mRNA maximized the productivity of the *Rosa26* BAC-based expression system. Thus, our findings imply that the novel methodologies suggested here to improve traditional expression systems might be applied to the production of therapeutic proteins in biopharmaceutics.

Acknowledgements

This research was supported by Research Assistance Program (2021) in the Incheon National University. This research was also supported by priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2020R1A6A1A0304195411).

Author Contributions

MUK, HWK, and JTP conceived of and designed the experiments. MUK, JYP, ESS, HL, YHL, and JJ performed

the experiments. MUK analyzed the data. MUK, HWK, and JTP wrote and edited the manuscript.

Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-021-0222-y) contains supplementary material, which is available to authorized users.

References

1. Ghaderi, D., M. Zhang, N. Hurtado-Ziola, and A. Varki (2012) Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. *Biotechnol. Genet. Eng. Rev.* 28: 147-175.
2. Swiech, K., V. Picanço-Castro, and D. T. Covas (2012) Human cells: new platform for recombinant therapeutic protein production. *Protein Expr. Purif.* 84: 147-153.
3. Rita Costa, A., M. Elisa Rodrigues, M. Henriques, J. Azeredo, and R. Oliveira (2010) Guidelines to cell engineering for monoclonal antibody production. *Eur. J. Pharm. Biopharm.* 74: 127-138.
4. Lai, T., Y. Yang, and S. K. Ng (2013) Advances in Mammalian cell line development technologies for recombinant protein production. *Pharmaceuticals (Basel)* 6: 579-603.
5. Canto, T. (2016) Transient expression systems in plants: potentialities and constraints. *Adv. Exp. Med. Biol.* 896: 287-301.
6. Liew, C. G., J. S. Draper, J. Walsh, H. Moore, and P. W. Andrews (2007) Transient and stable transgene expression in human embryonic stem cells. *Stem Cells* 25: 1521-1528. (Erratum published 2008, *Stem Cells* 26: 1094)
7. Zitzmann, J., C. Schreiber, J. Eichmann, R. O. Bilz, D. Salzig, T. Weidner, and P. Czermak (2018) Single-cell cloning enables the selection of more productive *Drosophila melanogaster* S2 cells for recombinant protein expression. *Biotechnol. Rep. (Amst.)* 19: e00272.
8. Tihanyi, B. and L. Nyitray (2020) Recent advances in CHO cell line development for recombinant protein production. *Drug Discov. Today Technol.* 38: 25-34.
9. Giraldo, P. and L. Montoliu (2001) Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res.* 10: 83-103.
10. Wilber, A., J. L. Frandsen, J. L. Geurts, D. A. Largaespada, P. B. Hackett, and R. S. McIvor (2006) RNA as a source of transposase for Sleeping Beauty-mediated gene insertion and expression in somatic cells and tissues. *Mol. Ther.* 13: 625-630.
11. Bire, S., D. Ley, S. Casteret, N. Mermod, Y. Bigot, and F. Rouleux-Bonnin (2013) Optimization of the piggyBac transposon using mRNA and insulators: toward a more reliable gene delivery system. *PLoS One.* 8: e82559.
12. Stone, N. E., J. B. Fan, V. Willour, L. A. Pennacchio, J. A. Warrington, A. Hu, A. de la Chapelle, A. E. Lehesjoki, D. R. Cox, and R. M. Myers (1996) Construction of a 750-kb bacterial clone contig and restriction map in the region of human chromosome 21 containing the progressive myoclonus epilepsy gene. *Genome Res.* 6: 218-225.
13. Mader, A., B. Prewein, K. Zboray, E. Casanova, and R. Kunert (2013) Exploration of BAC versus plasmid expression vectors in recombinant CHO cells. *Appl. Microbiol. Biotechnol.* 97: 4049-4054.
14. Zambrowicz, B. P., A. Imamoto, S. Fiering, L. A. Herzenberg, W. G. Kerr, and P. Soriano (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. U. S. A.* 94: 3789-3794.
15. Niwa, H., K. Yamamura, and J. Miyazaki (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene.* 108: 193-199.
16. Sparwasser, T. and G. Eberl (2007) BAC to immunology--bacterial artificial chromosome-mediated transgenesis for targeting of immune cells. *Immunology.* 121: 308-313.
17. Blaas, L., M. Musteanu, B. Grabner, R. Eferl, A. Bauer, and E. Casanova (2012) The use of bacterial artificial chromosomes for recombinant protein production in mammalian cell lines. *Methods Mol. Biol.* 824: 581-593.
18. Blaas, L., M. Musteanu, R. Eferl, A. Bauer, and E. Casanova (2009) Bacterial artificial chromosomes improve recombinant protein production in mammalian cells. *BMC Biotechnol.* 9: 3.
19. Jessen, J. R., C. E. Willett, and S. Lin (1999) Artificial chromosome transgenesis reveals long-distance negative regulation of rag1 in zebrafish. *Nat. Genet.* 23: 15-16.
20. Alattia, J. R., M. Matasci, M. Dimitrov, L. Aeschbach, S. Balasubramanian, D. L. Hacker, F. M. Wurm, and P. C. Fraering (2013) Highly efficient production of the Alzheimer's γ-secretase integral membrane protease complex by a multi-gene stable integration approach. *Biotechnol. Bioeng.* 110: 1995-2005.
21. Matasci, M., L. Baldi, D. L. Hacker, and F. M. Wurm (2011) The PiggyBac transposon enhances the frequency of CHO stable cell line generation and yields recombinant lines with superior productivity and stability. *Biotechnol. Bioeng.* 108: 2141-2150.
22. Balasubramanian, S., Y. Rajendra, L. Baldi, D. L. Hacker, and F. M. Wurm (2016) Comparison of three transposons for the generation of highly productive recombinant CHO cell pools and cell lines. *Biotechnol. Bioeng.* 113: 1234-1243.
23. Geurts, A. M., Y. Yang, K. J. Clark, G. Liu, Z. Cui, A. J. Dupuy, J. B. Bell, D. A. Largaespada, and P. B. Hackett (2003) Gene transfer into genomes of human cells by the sleeping beauty transposon system. *Mol. Ther.* 8: 108-117.
24. Balciunas, D., K. J. Wangensteen, A. Wilber, J. Bell, A. Geurts, S. Sivasubbu, X. Wang, P. B. Hackett, D. A. Largaespada, R. S. McIvor, and S. C. Ekker (2006) Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. *PLoS Genet.* 2: e169.
25. Ding, S., X. Wu, G. Li, M. Han, Y. Zhuang, and T. Xu (2005) Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell.* 122: 473-483.
26. Igarashi, H., K. Ikeda, H. Onimaru, R. Kaneko, K. Koizumi, K. Beppu, K. Nishizawa, Y. Takahashi, F. Kato, K. Matsui, K. Kobayashi, Y. Yanagawa, S. I. Muramatsu, T. Ishizuka, and H. Yawo (2018) Targeted expression of step-function opsins in transgenic rats for optogenetic studies. *Sci. Rep.* 8: 5435.
27. Sharan, S. K., L. C. Thomason, S. G. Kuznetsov, and D. L. Court (2009) Recombineering: a homologous recombination-based method of genetic engineering. *Nat. Protoc.* 4: 206-223.
28. Hwang, S. Y., M. U. Kuk, J. W. Kim, Y. H. Lee, Y. S. Lee, H. E. Choy, S. C. Park, and J. T. Park (2020) ATM mediated-p53 signaling pathway forms a novel axis for senescence control. *Mitochondrion.* 55: 54-63.

29. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* 21: 70-71.
30. Damdindorj, L., S. Karnan, A. Ota, E. Hossain, Y. Konishi, Y. Hosokawa, and H. Konishi (2014) A comparative analysis of constitutive promoters located in adeno-associated viral vectors. *PLoS One.* 9: e106472.
31. Suster, M. L., G. Abe, A. Schouw, and K. Kawakami (2011) Transposon-mediated BAC transgenesis in zebrafish. *Nat. Protoc.* 6: 1998-2021.
32. Izsvák, Z. and Z. Ivics (2004) Sleeping beauty transposition: biology and applications for molecular therapy. *Mol. Ther.* 9: 147-156.
33. Tharmalingam, T., H. Barkhordarian, N. Tejeda, K. Daris, S. Yaghmour, P. Yam, F. Lu, C. Goudar, T. Munro, and J. Stevens (2018) Characterization of phenotypic and genotypic diversity in subclones derived from a clonal cell line. *Biotechnol. Prog.* 34: 613-623.
34. Muñoz-López, M. and J. L. García-Pérez (2010) DNA transposons: nature and applications in genomics. *Curr. Genomics* 11: 115-128.
35. Hackett, P. B. (2007) Integrating DNA vectors for gene therapy. *Mol. Ther.* 15: 10-12.
36. Tran, D. T. M., T. T. P. Phan, T. T. N. Doan, T. L. Tran, W. Schumann, and H. D. Nguyen (2020) Integrative expression vectors with P*grac* promoters for inducer-free overproduction of recombinant proteins in *Bacillus subtilis*. *Biotechnol. Rep. (Amst.)* 28: e00540.
37. Mikiewicz, D., A. Plucienniczak, A. Bierczynska-Krzysik, A. Skowronek, and G. Wegrzyn (2019) Novel expression vectors based on the pIGDM1 plasmid. *Mol. Biotechnol.* 61: 763-773.
38. Muñoz-Fernández, G., J.-F. Montero-Bullón, J. L. Revuelta, and A. Jiménez (2021) New promoters for metabolic engineering of *Ashbya gossypii*. *J. Fungi (Basel).* 7: 906.
39. Panthu, B., T. Ohlmann, J. Perrier, U. Schlattner, P. Jalinot, B. Elena-Herrmann, and G. J. P. Rautureau (2018) Cell-free protein synthesis enhancement from real-time NMR metabolite kinetics: redirecting energy fluxes in hybrid RRL systems. *ACS Synth. Biol.* 7: 218-226.
40. Zhang, X., K. Xu, Y. Ou, X. Xu, and H. Chen (2018) Development of a baculovirus vector carrying a small hairpin RNA for suppression of sf-caspase-1 expression and improvement of recombinant protein production. *BMC Biotechnol.* 18: 24.
41. Jäckel, C., M. S. Nogueira, N. Ehni, C. Kraus, J. Ranke, M. Dohmann, E. Noessner, and P. J. Nelson (2016) A vector platform for the rapid and efficient engineering of stable complex transgenes. *Sci. Rep.* 6: 34365.
42. Zhou, C., B. Ye, S. Cheng, L. Zhao, Y. Liu, J. Jiang, and X. Yan (2019) Promoter engineering enables overproduction of foreign proteins from a single copy expression cassette in Bacillus subtilis. *Microb. Cell Fact.* 18: 111.
43. Balasubramanian, S., F. M. Wurm, and D. L. Hacker (2016) Multigene expression in stable CHO cell pools generated with the piggyBac transposon system. *Biotechnol. Prog.* 32: 1308-1317.
44. Narayanavari, S. A., S. S. Chilkunda, Z. Ivics, and Z. Izsvák (2017) Sleeping Beauty transposition: from biology to applications. *Crit. Rev. Biochem. Mol. Biol.* 52: 18-44.
45. Lohe, A. R. and D. L. Hartl (1996) Autoregulation of mariner transposase activity by overproduction and dominant-negative complementation. *Mol. Biol. Evol.* 13: 549-555.
46. Wu, S. C. Y., Y. J. J. Meir, C. J. Coates, A. M. Handler, P. Pelczar, S. Moisyadi, and J. M. Kaminski (2006) *piggyBac* is a flexible and highly active transposon as compared to *Sleeping Beauty*, *Tol2*, and *Mos1* in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 103: 15008-15013.
47. Kawakami, K. and T. Noda (2004) Transposition of the *Tol2* element, an *Ac*-like element from the Japanese medaka fish *Oryzias latipes*, in mouse embryonic stem cells. *Genetics*. 166: 895-899.