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RESEARCH PAPER

# ROSA26 BAC-based system enables continuous high-yield protein production

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## Abstract

The mouse ROSA26 locus serves as a port to maintain stable expression of the transgene. Therefore, a bacterial artificial chromosome (BAC) containing the ROSA26 locus was used as a platform to produce proteins by incorporating transgenes with foreign promoters. However, the endogenous promoter of the ROSA26 locus, which allows stable high-level transgene expression, has not been applied to the ROSA26 BAC-based platform. In this study, we generated recombinant ROSA26 BAC by targeting "exon 1" or "intron 1" of the ROSA26 locus. Recombinant ROSA26 BAC (exon 1) had lower protein productivity compared to the control. However, recombinant ROSA26 BAC (intron 1) produced protein at a higher efficiency than the control group. The protein productivity induction effect by recombinant ROSA26 BAC (intron 1) was maintained up to a single clone, enabling stable protein production over a long period of time. Taken together, we established a ROSA26 BAC-based protein production system capable of producing protein at high yield over a long period of time.

**Keywords** ROSA26 BAC · Endogenous promoter · Intron 1

## 1 Introduction

One of the platforms used for protein production is based on plasmids [1]. Plasmid-based vectors continue to exist as extrachromosomally replicating episomes after transfection, enabling highest levels of gene expression between 24 and 96 h [1]. However, due to limited episomal stability, the expression level of the transgene gradually decreases [2–4]. To avoid these drawbacks, a process of selecting clones with desirable characteristics should be performed, which is both labor-intensive and time-intensive [5]. However, even after the establishment of a stably transfected clone, the chromatin surrounding the transgenic integration site, have a significant impact on the expression of the transgene (i.e., positional chromatin effects) [6]. Based on these findings, there were doubts about the use of the widely used plasmid-based vector systems for protein production [7, 8]. A new vector platform called bacterial artificial chromosome (BAC) has been used as a way to address positional chromatin effects. A genomic region of 150–350 kb can be found in BAC, which contains all cis-acting regulatory elements (insulators, enhancers, promoters, etc.) [9]. Therefore, BAC is considered a safe expression unit that allows stable transgene expression without disturbing the nearby chromatin where integration occurs [10].

Through gene trapping, the ROSA26 locus was discovered on mouse chromosome 6 [11]. The ROSA26 locus maintained long-term gene expression and exhibited resistance to gene silencing, allowing the transgene to be expressed constitutively in vivo. To date, more than 560 knock-in lines at the ROSA26 locus have been generated in mouse [12]. ROSA26 BAC containing the ROSA26 locus also has been alternatively used to generate animal models [13–15]. The transgene inserted into the ROSA26 locus in the ROSA26 BAC accurately mimicked the original

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expression pattern of ROSA26 locus and had little interference with the chromatin region surrounding the insertion site [14]. The proven utility of ROSA26 BAC has also been applied to produce recombinant proteins in mammalian cells [16–18]. The result that the ROSA26 BAC-based protein production platform improved protein production by 10 times compared to existing vector systems supports its importance as a protein production platform [18]. However, the ROSA26 BAC-based platform used a cytomegalovirus (CMV) immediate enhancer/β-actin (CAG) promoter instead of the endogenous promoter of the ROSA26 locus [18]. Exogenous promoters such as CMV and CAG do not have the characteristics of the endogenous promoter of the ROSA26 locus, which has the ability to maintain long-term gene expression [19]. Since maintaining gene expression for a long period of time is considered the most important factor in protein production [2, 20, 21], building a platform using the endogenous promoter of the ROSA26 locus has become the most urgent value in the biopharmaceutical industry.

ROSA26 locus consists of three exons (exon 1, 2 and 3) and two introns (intron 1 and 2) [11]. Among them, exon 1 contains the start codon of the ROSA26 gene. The region containing the start codon is commonly targeted to generate knock-in animals, because transgenes inserted in this region can be influenced by the endogenous promoter [22–24]. Intron 1 of the ROSA26 gene is the site where the promoterless β-galactosidase/neomycin resistance fusion gene (βgeo) was originally inserted in trapping experiments, allowing stable and ubiquitous expression of the inserted transgene [25–27].

In this study, we established two versions of recombinant ROSA26 BAC targeting exon 1 or intron 1 to evaluate the performance of the endogenous promoter of the ROSA26 locus. Among them, we found that the recombinant ROSA26 BAC targeting intron 1 enabled consistently high yields of protein production. Here, we propose an improved recombinant protein production platform using the endogenous promoter of the ROSA26 locus in mammalian cells.

## 2 Material and methods

### 2.1 Construction of targeting vector, control vector and helper vector

A recombinant plasmid was established by conventional cloning methods. A targeting vector against exon 1 of ROSA26 locus was designed where 5' exon 1 homology region (HR) and 3' exon 1 HR. The location of 5' exon 1 HR in the mouse genome was chr6:—strand 113054085–113054184. The location of 3' exon 1 HR in the mouse genome was chr6:—strand 113053585–113054084. A targeting vector against intron 1 of ROSA26 locus was designed where 5' intron 1 HR and 3' intron 1 HR. The location of 5' intron 1 HR in the mouse genome was chr6:—strand 113052993–113053478. The location of 3' intron 1 HR in the mouse genome was chr6:—strand 113052487–113052969. Utilizing the *CamR* targeting vector (131590; Addgene), the Tol2 transposon system was recombineered into the chloramphenicol resistance (*CamR*) region [17]. Specifically, the ampicillin resistance (*AmpR*) gene, the neomycin resistance (*NeoR*) gene, the 5′ and 3′ *CamR* HRs, and two inverted terminal repeats (ITRs) were all included in the construction of the *CamR* targeting vector. In the control vector, CMV promoter-driven luciferase gene was included [17]. Helper vectors designed to bind to ITRs encode the Tol2 transposase [28, 29]. Helper vectors were linearized and transcribed to produce helper mRNA using the MEGAscriptTM T7 Transcription Kit (AM1333; Thermo Fisher Scientific) according to the company's protocol [29].

### 2.2 BAC recombineering

ROSA26 BAC clone (RP24-85L15, BACPAC Resource Center) was used for BAC recombineering. When performing BAC recombineering, the λ red recombination approach was used [30]. Specifically, the λ red recombination method specifically regulates the expression of the lambda-red recombinase via a temperature-sensitive lambda repressor [31, 32]. The lambda-red recombinase system was added to a genetically engineered SW105 bacterial strain. This strain contains the PL operon encoding the lambda-red recombinase exo, bet, and gam, which are essential for the recombination process. The temperature-sensitive lambda repressor tightly regulates the PL operon (cI857). cI857 is activated at low temperatures (30–34 °C) and binds to the operator site, silencing recombinant gene transcription. A thermal shift to 42 °C reversibly inhibits cI857 activity, thereby activating the transcription of recombinant gene. Recombination then occurs.

### 2.3 Cell culture

CHO DG44 cells (A1100001; Thermo Fisher Scientific) were cultured as described in previous studies [29]. Specifically, CHO DG44 cells were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal bovine serum (SH30919.03; Hyclone), 10 mM sodium hypoxanthine, 1.6 mM thymidine (2,068,642; Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (SV30079.01; Hyclone). Cells were cultured at ambient air (20% $O_2$) with 5% $CO_2$. Using a Cedex HiRes Analyzer

(05650216001; Roche), cell density and viability were measured.

### 2.4 Transfections and cell line development

Control vector and recombinant ROSA26 BAC were transfected into cells at a DNA copy ratio of 1:1. Specifically, $5 \times 10^5$ cells were transfected with 0.3 μg control vector (size: 7,123 bps) or 6 μg recombinant ROSA26 BAC (size: 222,649 bps). 0.667 μg helper mRNA was transfected together with 6 μg recombinant ROSA26 BAC. Then, the cell line development process proceeded as previously described [29].

### 2.5 Measurement of luciferase activity

Luciferase activity was analyzed as described previously [17]. Briefly, luciferase activity was assessed using a luciferase assay kit (E1500; Promega). Cell counts were determined using a Cedex HiRes Analyzer. Cells ($2 \times 10^6$) were centrifuged at 200 × *g* for 2 min and washed twice with phosphate-buffered saline (PBS). Cells were shaken in 100 μL of PBS and 100 μL of 1 × cell culture lysis reagent. White 96-well plates (30,396; SPL Life Sciences) were used, and the lysed samples (100 μL) were added to each well. Then, 100 μL of luciferase assay reagent II was added to each well. Luciferase activity was measured on a VICTOR multilabel plate reader (2030-0050; PerkinElmer).

### 2.6 Quantitative polymerase chain reaction (qPCR)

qPCR using mRNA or genomic DNA was conducted as described previously [17]. More specifically, the following primers were used in qPCR: 5'-GCACCACCAACT GCTTAGC-3' (*GAPDH*-forward), 5'-AGTCTTCTGGGT GGCAGTGA-3' (*GAPDH*-reverse), 5'-AGGAGATACGCC CTGG-3' (*luciferase*-forward), and 5'-AATAACGCGCCC AACA-3' (*luciferase*-reverse).

### 2.7 Western blot analysis

Western blotting was done in accordance with earlier instructions [33]. HRP-conjugated anti-luciferase antibody (sc74548; 1:1,000 dilution; Santa Cruz Biotechnology) and HRP-conjugated anti-β-actin antibody (sc47778; 1:1,000 dilution; Santa Cruz Biotechnology) were utilized.

### 2.8 Determination of relative cell-specific productivity (Qp)

Relative Qp (pg/cell/day) in media was determined by calculating the relative activity of secreted luciferase protein in the medium. To quantify the amount of luciferase activity in the medium, 100 μL of medium samples were placed into each well of a white 96-well plate (30,396; SPL Life Sciences). After adding 100 μL of Luciferase Assay Reagent II to each well, luciferase activity was assessed using a VICTOR multilabel plate reader (2030-0050; PerkinElmer). Cell viability and density were measured using a Cedex HiRes Analyzer (05650216001; Roche). Qp was calculated according to the method proposed in a previous study [34].

### 2.9 Statistical analyses

Statistical analysis used Student's *t*-test or two-way ANOVA followed by Bonferroni's post hoc test.

## 3 Results

### 3.1 Establishment of recombinant ROSA26 BAC (exon 1)

To evaluate the performance of the endogenous promoter of the ROSA26 locus, a region within ROSA26 exon 1 containing the start codon of ROSA26 gene (chr6:—strand 113052487–113053478) was selected for targeting of the transgene (Fig. 1a). BAC targeting vector contained 5′ exon 1 HR and 3′ exon 1 HR (Fig. 1a). To assess the activity of the promoter, the luciferase gene was inserted between the 5′ exon 1 HR and 3′ exon 1 HR (Fig. 1a). The luciferase gene was placed followed by the splice acceptor sequence (SA) as found in the original gene trapping vector (Fig. 1a) [35]. To facilitate genomic integration of the ROSA26 BAC, the Tol2 transposon system was introduced into the *CamR* region (Fig. 1a). For incorporation of the Tol2 transposon system into the *CamR* region, a *CamR* targeting vector containing "5′ *CamR* HR & 3′ *CamR* HR" and a pair of ITR was used (Fig. 1a). After the BAC recombineering process, the recombination region was confirmed through DNA sequence analysis. Sequence analysis of the recombinant ROSA26 BAC (exon 1) demonstrated the presence of the recombinant sequence, providing evidence that homologous recombination was successfully achieved (Fig. 1b).

As a comparison group for the experiment, a control vector carrying a CMV promoter-driven luciferase gene was used [17]. Recombinant ROSA26 BAC (exon 1) or control vector were transiently transfected into cells. The luciferase activity in recombinant ROSA26 BAC (exon 1) was significantly lower than that in the control vector (Fig. 1c). These results indicate that the exon 1 region used to generate knock-in mice was not suitable for protein production in

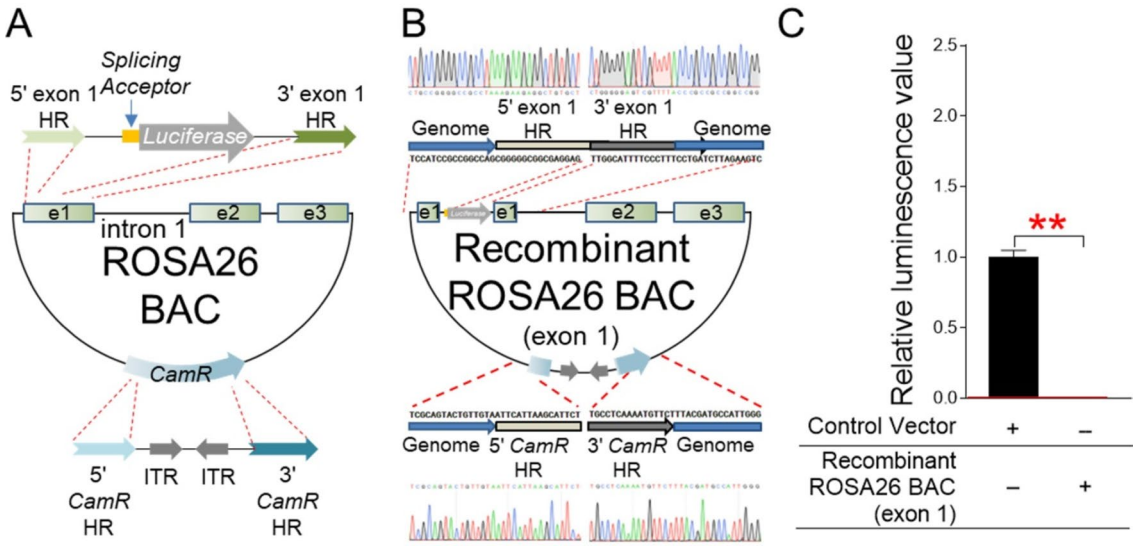


**Fig. 1** Establishment of recombinant ROSA26 BAC (exon 1). **a** A schematic representation of a BAC targeting vector and a *CamR* targeting vector. BAC targeting vector contained 5′ exon 1 HR and 3′ exon 1 HR. To assess the activity of the promoter, the luciferase gene was inserted between the 5′ exon 1 HR and 3′ exon 1 HR. The luciferase gene was placed followed by the splice acceptor sequence (SA) as found in the original gene trapping vector. *CamR* targeting vector contained "5′ *CamR* HR & 3′ *CamR* HR" and a pair of inverted terminal repeats (ITR). **b** Sequence analysis of the recombinant ROSA26 BAC (exon 1) demonstrated the presence of the recombinant sequence. **c** The luciferase activity in recombinant ROSA26 BAC (exon 1) was significantly lower than that in the control vector. $^{**}p < 0.01$, Student's *t*-test. Mean ± SD, $N = 3$. BAC: bacterial artificial chromosome, HR: homology region

mammalian cells. These results also mean that in order to create a protein production platform using ROSA26 BAC, a region other than exon 1 should be targeted.

### 3.2 Establishment of recombinant ROSA26 BAC (intron 1)

The intron 1 region in ROSA26 locus was also used frequently to generate knock-in mice [25–27]. Therefore, a region within ROSA26 intron 1 (chr6:—strand 113052487–113053478) was selected for targeting of the transgene (Fig. 2a). Specifically, BAC targeting vector contained 5′ intron 1 HR and 3′ intron 1 HR (Fig. 2a). To assess the activity of the promoter, the luciferase gene was inserted between the 5′ intron 1 HR and the 3′ intron 1 HR (Fig. 2a). SA was inserted in front of the luciferase gene (Fig. 2a). To facilitate genomic integration of the ROSA26 BAC, the Tol2 transposon system was introduced into the *CamR* region (Fig. 2a). After the BAC recombineering process, the recombination region was confirmed through DNA sequence analysis. Sequence analysis of the recombinant ROSA26 BAC (intron 1) demonstrated the presence of the recombinant sequence, providing evidence that homologous recombination was successfully achieved (Fig. 2b).

Recombinant ROSA26 BAC (intron 1) or control vector were transiently transfected into cells. The luciferase activity in recombinant ROSA26 BAC (intron 1) was significantly higher than that of the control vector (Fig. 2c). These results indicate that the intron 1 region was suitable as a targeting region for protein production in mammalian cells.

### 3.3 Underlying mechanism for increased protein production by recombinant ROSA26 BAC (intron 1)

We then investigated how recombinant ROSA26 BAC (intron 1) led to high-yield protein production. Because increased integration of the transgene into the genome is a prerequisite for high yield protein production [17, 29, 36], the integrated copy number of recombinant ROSA26 BAC (intron 1) into the genome was compared with that of the control vector. Compared with the control vector, the recombinant ROSA26 BAC (intron 1) significantly increased transformant integration by 1.57-fold (Fig. 3a). Next, we assessed whether increased transgene integration into the genome led to increased transgene expression. Indeed, compared with the control vector, recombinant ROSA26 BAC (intron 1) significantly increased transgene expression by 9.33-fold (Fig. 3b). Finally, Western blotting was performed to assess whether increased transgene expression led to increased protein production. Compared to the control vector, recombinant ROSA26 BAC (intron 1) markedly increased luciferase protein production by 5.7-fold (Fig. 3c).

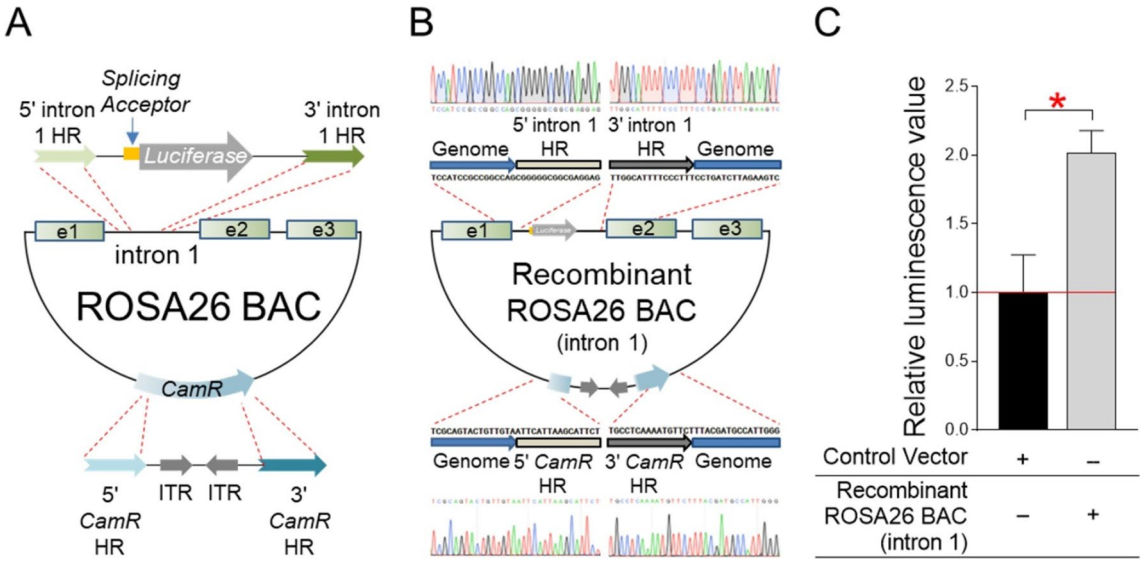


**Fig. 2** Establishment of recombinant ROSA26 BAC (intron 1). **a** A schematic representation of a BAC targeting vector and a *CamR* targeting vector. BAC targeting vector contained 5′ intron 1 HR and 3′ intron 1 HR. *CamR* targeting vector contained "5′ *CamR* HR & 3′ *CamR* HR" and a pair of inverted terminal repeats (ITR). *CamR* targeting vector contained "5′ *CamR* HR & 3′ *CamR* HR" and a pair of ITR. **b** Sequence analysis of the recombinant ROSA26 BAC (intron 1) demonstrated the presence of the recombinant sequence. **c** The luciferase activity in recombinant ROSA26 BAC (intron 1) was significantly higher than that of the control vector. $*p < 0.05$, Student's *t*-test. Mean ± SD, N = 3. BAC: bacterial artificial chromosome, HR: homology region

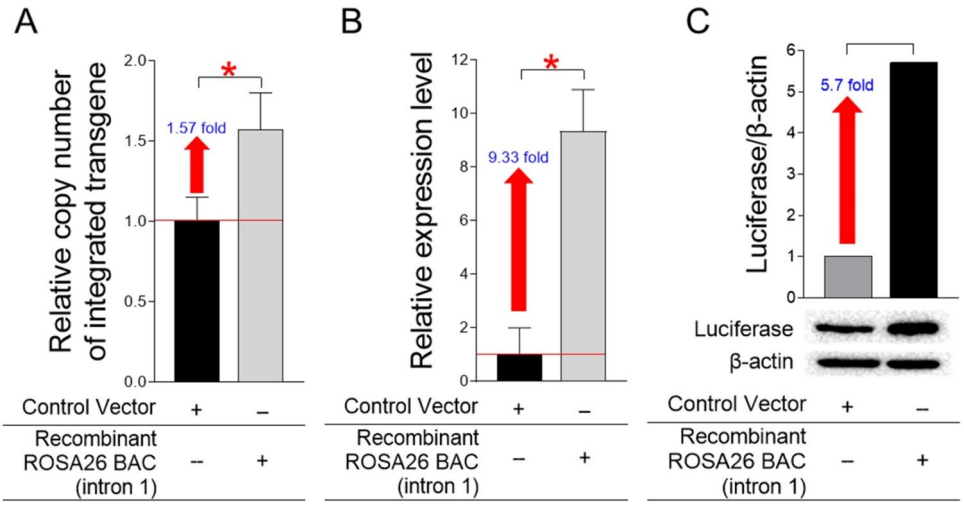


**Fig. 3** Underlying mechanism for increased protein production by recombinant ROSA26 BAC (intron 1). **a** The relative copy number of integrated transgene in cells transfected with control vector or recombinant ROSA26 BAC (intron 1). $*p < 0.05$, Student's *t*-test. Mean ± SD, $N = 3$. **b** The relative transgene expression in cells transfected with control vector or recombinant ROSA26 BAC (intron 1). $*p < 0.05$, Student's *t*-test. Mean ± SD, N = 3. **c** Western blot analysis to evaluate protein production in cells transfected with control vector or recombinant ROSA26 BAC (intron 1). BAC: bacterial artificial chromosome

### 3.4 Maintaining productivity-enhancing effect of recombinant ROSA26 BAC (intron 1) in single clones

Generating a single clone from a pool is an essential process to meet regulatory standards, as it ensures monoclonality and produces proteins consistently [37]. Therefore, a single cell isolation procedure was performed. For each group, thirty single clones were established (Fig. 4a). To evaluate the performance of each single clone, we used Qp, which quantifies the rate of protein production per cell and unit of time (pg/cell/day) [38]. Qp is one of the most important

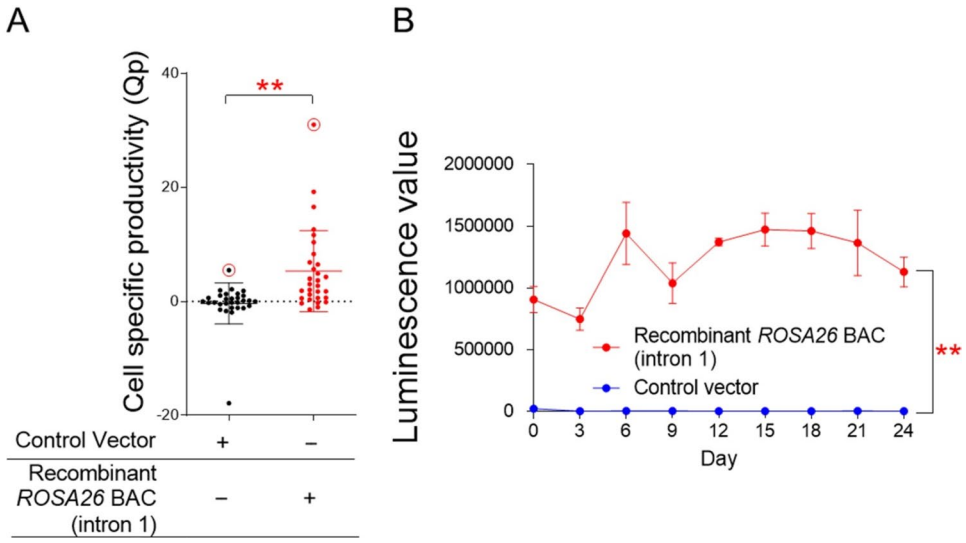


**Fig. 4** Suitability of recombinant ROSA26 BAC (intron 1) for long-term protein production. **a** Maintaining productivity-enhancing effect of recombinant ROSA26 BAC (intron 1) in single clones. Each dot represents the cell-specific productivity (Qp) of single clones. The horizontal bar indicates the average of Qp for each condition. $^{**}p < 0.01$, Student's *t*-test. Mean ± SD, N = 30. **b** To perform batch culture-based stability tests, luciferase activity was measured for 24 days. $^{**}p < 0.01$, two-way ANOVA followed by Bonferroni's post test. Mean ± SD, N = 3. BAC: bacterial artificial chromosome

parameters used when comparing the performance of protein production platforms [39, 40]. Single clones generated with the recombinant ROSA26 BAC (intron 1) showed significantly higher Qp compared to clones generated with control vector (Fig. 4a). These findings demonstrate that the productivity-enhancing effects of the recombinant ROSA26 BAC (intron 1) persisted in single clones. The maintenance of increased productivity even in single clones suggests that the recombinant ROSA26 BAC (intron 1) could be a next-generation platform in protein production.

### 3.5 Suitability of recombinant ROSA26 BAC (intron 1) for long-term protein production

The urgent research goal in the field of biopharmaceutics is to develop a system capable of continuous and efficient protein production [2, 20, 21]. Therefore, we investigated whether recombinant ROSA26 BAC (intron 1) could maintain high levels of protein production for long periods of time. The suitability of recombinant ROSA26 BAC (intron 1) was assessed in batch culture experiments for 24 days. During 24 days of culture, recombinant ROSA26 BAC (intron 1) showed significantly higher luminescence compared to the control vector (Fig. 4b). These findings suggest that recombinant ROSA26 BAC (intron 1) is a suitable platform for biopharmaceutical production that requires long culture periods.

## 4 Discussion

Increasing protein production in mammalian cells is one of the most interesting research goals in the biopharmaceutical industry [41, 42]. Various strategies have been tried to enhance protein productivity, including modification of media components, host cell engineering, and vector engineering [43, 44]. Among them, the most efficient way to increase protein productivity is to modify vectors and apply them to protein production [20, 45–47]. ROSA26 BAC containing the ROSA26 locus has recently attracted attention as a platform for producing recombinant proteins in mammalian cells [16–18]. For example, recombinant ROSA26 BAC was constructed for production of human IgG1 constant region [18]. The control vector produced 0.5 pg/cell/day of human IgG1 constant region in the supernatant, whereas the recombinant ROSA26 BAC produced 5.7 pg/cell/day [18]. Another recombinant ROSA26 BAC was constructed for the scFc antibody (fusion of the single-chain fragment variable [GenBank: CAA01551] to the human IgG1 Fc region [GenBank: CAA49866]) [10]. The control vector produced 0.6–9.5 pg/cell/day of scFc antibody in the supernatant, whereas the recombinant ROSA26 BAC produced 10–30 pg/cell/day [10]. However, the ROSA26 BAC-based expression method uses exogenous promoters such as CMV and CAG, instead of the endogenous promoter of the ROSA26 locus [10, 16–18]. The endogenous promoter of the ROSA26 locus is worthy of application in protein production platforms because it enables sustained, high-efficiency gene expression. In this study, we established two ROSA26 BAC-based protein production platforms using the endogenous promoter of the ROSA26 locus. First, we targeted exon 1 (chr6:—strand 113,052,487–113053478) containing the start codon of the ROSA26 gene, because targeting the start codon region is a commonly used strategy to generate knock-in animals [22–24]. Therefore, the recombinant ROSA26 BAC (exon 1) inserted the start codon of the luciferase gene into the original start codon position of the ROSA26 gene, so that the expression of the luciferase gene was influenced by the endogenous ROSA26 promoter. However, the recombinant ROSA26 BAC (exon 1) did not

induce the production of luciferase protein. This may be due to the nature of the ROSA26 region encoding noncoding RNA that is transcribed but not translated [35]. Second, we targeted intron 1 of the ROSA26 locus (chr6:—strand 113,052,487–113053478) because insertion of a promoterless βgeo into intron 1 resulted in constitutive expression of βgeo under the influence of the endogenous ROSA26 promoter [35]. Thus, intron 1 in ROSA26 locus is the target region used to generate knock-in animals in mice and other species [25–27]. The recombinant ROSA26 BAC (intron 1), targeting intron 1 of the ROSA26 locus, induced 5.7-fold higher protein production compared to the control vector. Furthermore, the productivity improvement effect achieved by recombinant ROSA26 BAC (intron 1) was maintained up to single clones. This study is the first to use the intron 1 region of the ROSA26 locus as an endogenous promoter to increase protein production in mammalian cells. Here we propose to use the recombinant ROSA26 BAC (intron 1) as a next-generation protein production platform. This platform will serve as a driving force to increase the price competitiveness of biopharmaceuticals in response to the needs of the biopharmaceutical industry that requires high productivity.

Maintaining consistent protein production over long periods of time is one of the most challenging goals in biopharmaceutical production when the use of antibiotic selection is not applicable [2, 20, 21]. Various attempts have been made to achieve a sustainable production, but they have not been effective. For example, the plasmid vector-based protein production platform failed to achieve a consistently high level of protein production because the expression of gene significantly decreased with each generation [48–50]. To address these potential shortcomings, the use of site-specific integration (SSI) for cell line development has been proposed [51]. Integration of the transgene into a genomic hotspot that allows consistent expression via SSI allowed the generation of stable isogenic clones that maintain consistent protein production over long periods of time [51–54]. However, SSI can only integrate at most two copies of the transgene into the host genome, resulting in low protein productivity [51, 55]. In this study, we found that protein productivity improvement by the recombinant ROSA26 BAC (intron 1) was observed up to single clones. Extending the relevance of these findings, recombinant ROSA26 BAC (intron 1) continued to maintain higher luciferase activity than the control vector even over 24 days. Ultimately, our findings indicate that recombinant ROSA26 BAC (intron 1) is the most suitable platform for biopharmaceutical production that requires prolonged culture periods where antibiotic selection is not applied.

In summary, we created a recombinant ROSA26 BAC (intron 1)-based protein production platform utilizing the endogenous promoter of the ROSA26 locus. This platform increased the expression of the transgene and ultimately increased protein production. Additionally, the effect of this platform on increasing protein production remained constant over long culture times. Therefore, our results suggest that this new platform can ultimately be applied to large-scale biopharmaceutical production by solving unresolved problems in the biopharmaceutical industry.

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**Author's contributions** MUK Writing—original draft, Writing—Review and Editing, Investigation. MKS Investigation. JHP Investigation. JHY Investigation. YJL Investigation. DK Investigation. BS Investigation. YHL Investigation. MK Investigation. YB Writing—Review. HWK Writing—Review. JTP Writing—original draft, Writing—Review and Editing.

**Data availability** The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** Neither ethical approval nor informed consent was required for this study.

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