

Original Article

Efficient production of recombinant mAbs mediated by a MAR-enhanced transposon vector combined with blasticidin selection in CHO cells

Xiaoyin Wang^{1,2}, Xuelian Han¹, Ting Liu¹, Shiqi Zhang¹, Zimeng Han¹, Ruoyuan Feng¹, and Tianyun Wang^{1,2,*}

¹School of Basic Medical Sciences, Henan Medical University, Xinxiang 453003, China, and ²Henan Key Laboratory of Neurorestoratology and Protein Modification, the First Affiliated Hospital of Henan Medical University, Xinxiang 453003, China

*Correspondence address. Tel: +86-373-3029968; E-mail: wtianyuncn@126.com

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Abstract

Recombinant antibodies, primarily produced in Chinese hamster ovary (CHO) cells, are widely used to treat various diseases. For industrial production, a rapid and efficient method to screen stable, high-expressing clones is essential. However, conventional screening based on random integration is often cumbersome and labor intensive. This study establishes a novel strategy for generating stable, high-yielding clones by combining a MAR-based piggyBac (PB) transposon semitargeted integration system with blasticidin (BSD) selection. Compared to the random integrated vector pMAR-mAb, the MAR-PB system increases the titers (3.95- to 5.61-fold) and specific protein productivity (Qp; 4.28- to 6.07-fold) of four monoclonal antibodies in stable cell pools. When compared to PB-only vectors, the MAR-PB transposon system enhances the titers (by up to 2.50-fold) and Qp (1.96- to 2.77-fold), respectively. The increased antibody production correlates with elevated mRNA expression. Furthermore, this approach increases the proportion of high-expressing clones by more than 10-fold and significantly improves volumetric yield. Importantly, this approach promotes the long-term stability of recombinant mAb expression for over 60 generations. Transcriptome analysis reveals that the system modulates genes involved in DNA binding, transcriptional regulation, and protein binding. In conclusion, the MAR-based PB transposon system combined with BSD selection presents a significant improvement for efficiently generating high-yielding and stable CHO cell clones, offering a valuable tool for recombinant antibody production.

Key words CHO cell, piggyBac transposon, matrix attachment region, blasticidin, antibody

Introduction

The global biopharmaceutical industry is growing rapidly, with monoclonal antibodies (mAbs) as a key class of therapeutic proteins used to treat tumors, inflammation, autoimmune disorders and other diseases. The market is projected to reach \$856.1 billion by 2030 [1]. Mammalian cells, particularly Chinese hamster ovary (CHO) cells, are the primary expression system for their human-like post-translational modifications (PTMs), compatibility of suspension and adherent culture, and ability to facilitate correct protein folding [2-4]. However, traditional expression vectors lack homology to host DNA, and their random integration into host chromosomes causes cell clone heterogeneity. Additionally, inte-

gration into heterochromatic regions may silence the gene of interest (GOI), requiring extensive and labor-intensive screening to obtain stable, high-yielding cell clones [5].

Strategies to enhance protein of interest (POI) expression include genetic editing of cell lines [6,7], optimization of expression vectors [8-12], culture media [13-16] and cultivation processes [17,18]. Among these, selecting efficient expression vectors is an effective approach to increase POI expression in CHO cells [19,20].

Transposon semitargeted integration systems have been proven effective for the production of recombinant proteins [21,22]. As natural mobile genetic elements, transposons comprise a donor vector and a helper vector (transposase) [23]. Transposase

recognizes the inverted terminal repeats (ITRs) of the donor vector and uses a “cut-and-paste” mechanism to integrate the GOI into a highly transcriptionally active site, enhancing POI expression [24,25]. Commonly used transposons include Tol2 from the hAT gene family [26], Sleeping Beauty (SB) from the Tc1/mariner superfamily [27], and the insect-derived piggyBac (PB) [28]. Among these, the PB transposon system has broad activity and efficiently mediates transposition of DNA fragments up to 14 kb [29]. Co-transfection of donor and helper vectors enables efficient integration of GOIs in mammalian cells [30–33].

Additionally, chromatin regulatory elements (CREs) mitigate transgene silencing, with matrix attachment regions (MARs) known to prevent heterochromatin-mediated gene silencing. Ley *et al.* [34] evaluated a piggyBac transposon system containing human MARs and observed that MARX-29 increased protein expression by over 2-fold in CHO cells. Although combining regulatory elements with transposon vectors improves protein expression, an efficient, rapid method for screening stable, high-expressing cell clones remains crucial. In our previous study, we identified an efficient MAR consensus sequence that enhances transgene expression [35]. However, whether these favorable properties can be combined with the transposon system remains largely untested. Moreover, antibiotic selection is widely used to generate stable cell pools for protein expression [36,37]. Blasticidin S (BSD), a nucleoside antibiotic isolated from *Streptomyces coelicolor*, inhibits translation

by binding to the peptidyl transferase center of the large ribosomal subunit [38,39]. By utilizing the PB transposon system to mediate integration into transcriptionally active genomic loci, combining the boundary effect of MAR elements that shield genes from heterochromatic silencing, and employing BSD for stable cell line selection, we theoretically predict a significant enhancement in transgene expression. However, the applicability of BSD-resistance selection for generating stably transfected CHO cells, particularly when combined with both the transposon system and the MAR consensus sequence, remains unclear.

In the present study, we developed a novel and efficient method for generating stable, high-expressing CHO cell lines producing monoclonal antibodies by combining the boundary activity of MAR elements, semitargeted integration of PB transposons, and BSD resistance selection.

Materials and Methods

Plasmid construction

The original transposase plasmid (helper vector), the donor plasmid pPB-EGFP containing enhanced green fluorescent protein (EGFP), and four monoclonal antibody (mAb) donor plasmids (pPB-mAb1–4) were maintained in our laboratory [40] (Figure 1A–C). Each donor plasmid contained optimized 5′ and 3′ inverted repeat elements (5′ ITR and 3′ ITR) flanking the transposon sequence. The pMAR-mAb1–4 vector incorporated an efficient consensus MAR

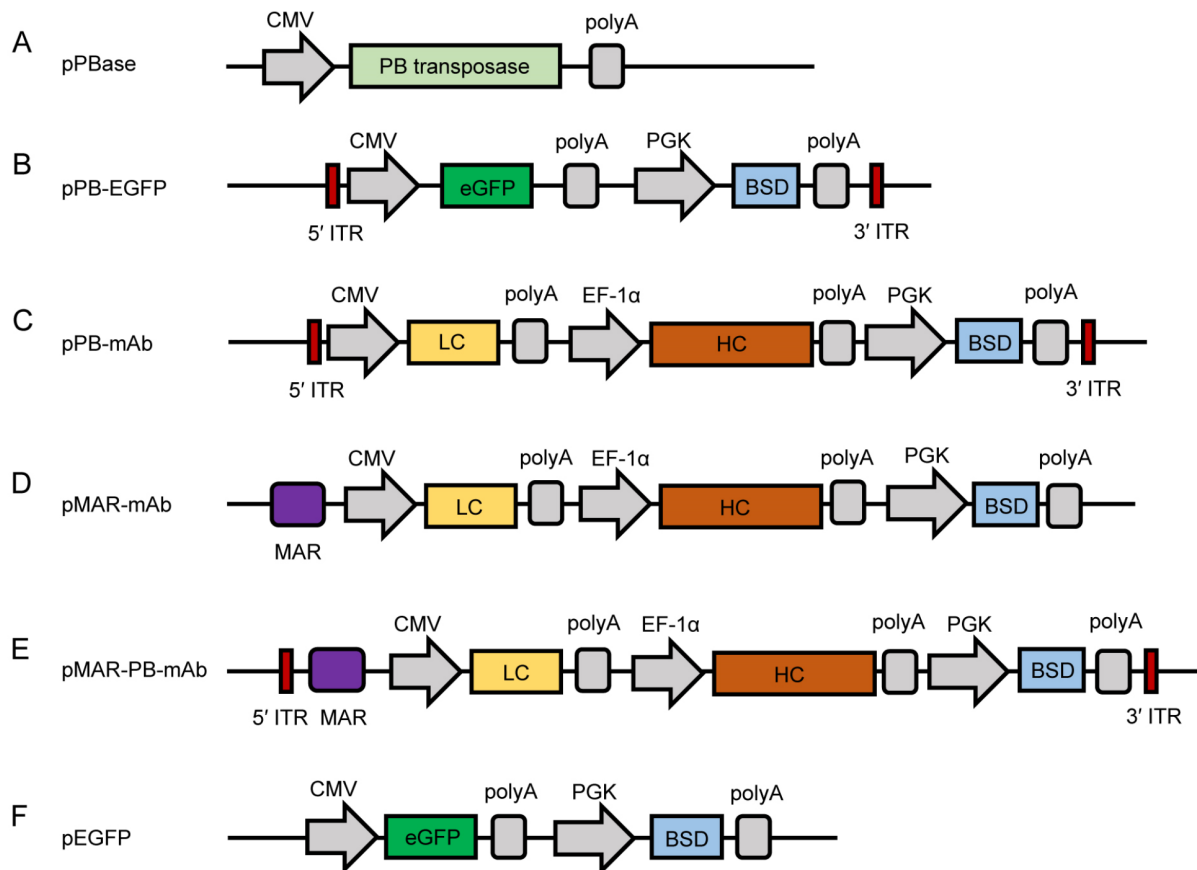


Figure 1. Representation of the MAR-based piggyBac vector maps for transposase and transposon used in this study BSD, blasticidin; CMV, human cytomegalovirus immediate early promoter; eGFP, enhanced green fluorescent protein; EF-1 α , human elongation factor 1 alpha promoter; HC, mAb heavy chain; ITR, inverted terminal repeats; LC, mAb light chain; mAb, monoclonal antibodies; MAR, matrix attachment regions; poly A, SV40 polyadenylation signal.

sequence, previously identified and characterized for its propensity to enhance transgene expression, located upstream of the expression cassette [35] (Figure 1D). To generate a series of MAR-based PB transposon vectors (pMAR-PB-mAb1-4), 5' ITR and 3' ITR sequences were synthesized, cloned and inserted into the pMAR-mAb vectors. The 5' ITR was introduced upstream of the CMV promoter, and the 3' ITR was ligated downstream of the poly A signal within the BSD expression cassette. This process yielded the four MAR-based PB transposon vectors: pMAR-PB-mAb1, pMAR-PB-mAb2, pMAR-PB-mAb3, and pMAR-PB-mAb4 (Figure 1E). Vector construction was performed by General Biol Co., Ltd. (Chuzhou, China). Each pMAR-PB-mAb plasmid comprised a light chain (LC) expression cassette under the CMV promoter, a heavy chain (HC) expression cassette under the EF-1 α promoter, a BSD selection cassette driven by the PGK promoter and a consensus MAR sequence upstream of the CMV promoter. The transposon vector pPB-mAb lacking MARs elements and pMAR-mAb and pEGFP vectors lacking ITR sequences served as controls (Figure 1C, D, F). The ITR and MARs sequences used in this study are provided in Supplementary Figure S1.

Cell culture

CHO-S cells obtained from Life Technologies (#A11557-01; Carlsbad, USA) were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. The cells were passaged every 2–3 days and seeded at a density of 1×10^5 cells/mL in 24-well plates the day before transfection.

Cell transfection

Plasmid transfection was performed using Lipofectamine 2000 (Biosharp, Beijing, China) according to the manufacturer's protocol. When cells in 24-well plates reached 70%–90% confluence, a mixture of 0.8 μ g plasmid DNA and 2 μ L Lipofectamine 2000 per well was used for transfection. Specifically, 0.8 μ g of DNA and 2 μ L of Lipofectamine 2000 were separately diluted in 50 μ L of DMEM/F12. Each solution was incubated at room temperature for 5 min. The diluted DNA and diluted Lipofectamine 2000 solutions were then combined and incubated at room temperature for 20 min to allow complex formation. Finally, the DNA-Lipofectamine complexes were added to each well. Control transfections were performed using corresponding random integration vectors lacking ITR sequences (pMAR-mAb, pEGFP) and transposon vectors lacking MARs elements (pPB-mAb).

Flow cytometry

Transfection efficiency was assessed using an inverted fluorescence microscope (Olympus, Tokyo, Japan). eGFP expression was observed and imaged using blue excitation light. Additionally, flow cytometry (Beckman Coulter Inc., Pasadena, USA) was employed to analyze the transfection rate and mean fluorescence intensity (MFI). Cells were trypsinized, re-suspended in PBS, and analyzed at a rate of ≤ 600 events per second, with approximately 10^5 cells per sample. Non-transfected cells served as a negative control. eGFP signals were detected using a 488 nm excitation laser and a 530/30 nm emission filter.

Screening of stable cell pools and cell clones

To determine the optimal BSD screening concentration, cells were

seeded at 1.25×10^5 cells/well in 24-well plates. Forty-eight hours post-transfection, cells were exposed to different concentrations of BSD (2.5, 5, 10, 15, and 20 μ g/mL). The medium was replaced every 2–3 days, and cell morphology was observed daily. The results showed that 15 μ g/mL BSD effectively eliminated all cells in the un-transfected group within approximately 7 days. This concentration was subsequently used for pressurized screening to generate stable cell pools. For the selection of stable cell pools and clones, the medium was replaced with DMEM/F12 supplemented with 10% FBS and 15 μ g/mL BSD (MDBio, Qingdao, China) 48 h post-transfection. Non-transfected cells were eliminated within 5–7 days. Surviving cells were subsequently maintained in 10 μ g/mL BSD-containing medium, which was changed every 2–3 days to derive stably transfected cell pools. Single-cell clones were isolated from the stable cell pools by limiting dilution seeded into 96-well plates and identified within 3–5 days. Upon reaching 70%–80% confluence, the cells were expanded into 24-well plates.

To assess recombinant protein expression, cells were inoculated at a density of 3×10^5 cells/mL in 125 ml shaker flasks and cultured in suspension with 30 mL of serum-free medium (SFM; Proteineasy Biotech Company, Xixiang, China). Samples were harvested when cell viability dropped below 50%, and recombinant protein expression was determined.

Western blot analysis

Supernatants from mAb-transfected cells were collected and mixed with 5 $\times$ loading buffer, boiled for 10 min, and separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated samples were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA), blocked with 5% milk for 2 h at room temperature, and incubated with horseradish peroxidase (HRP)-linked goat anti-mouse (for mouse origin antibody) or goat anti-human (for human origin antibody) secondary antibodies (Beyotime, Shanghai, China). Antigen-antibody complexes were visualized using ECL solution (Beyotime).

Enzyme-linked immunosorbent assay (ELISA)

After approximately 7 days of cell suspension culture, supernatants were harvested from cells transfected with plasmids containing different mAbs. mAb production was quantified using an ELISA kit (ELK Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instructions. Specific protein productivity (Qp) was calculated using the following formula:

$$Qp \text{ (pg/cell/day)} = \frac{\text{Final recombinant concentration (}\mu\text{g/mL)}}{\int_0^7 \text{VCD (}10^6 \text{ viable cells/mL)}}$$

qPCR analysis

Transgene copy number and mRNA expression were quantified using fluorescent quantitative PCR (qPCR). For copy number analysis, genomic DNA was isolated from stable cell pools. To assess mRNA expression, total RNA was extracted using TRIzol reagent (Vazyme, Nanjing, China) and reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Vazyme) following the manufacturer's protocol. The genomic DNA and cDNA were used as templates for qPCR with SYBR Green I fluorescent dye. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an

internal reference for mRNA analysis, and beta-2 microglobulin (*B2M*) was used for copy number analysis. The sequences of primers are shown in Table 1. Data acquisition was performed using an ABI 7500 SYBR qPCR instrument (Applied Biosystems, Foster, USA), and data were analyzed using 7500 Fast System SDS Software. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with normalization to *GAPDH* levels. Using NCBI BLAST software, the amplicon generated by the *B2M* primers showed one hit per haploid CHO genome after alignment to our CHO genome assembly. Given that CHO cells are near-diploid [41], *B2M* was estimated to be present at 2 copies per genome. The copy number of GOI was calculated as a ratio relative to the *B2M* reference gene. All experiments were performed in triplicate.

Transcriptomics analysis

Transcriptome analysis was performed by Shanghai Biotechnology Corporation (Shanghai, China) on three independent replicates of the pMAR-PB-mAb cell line and the control cell line (transfected with pMAR-mAb). Total RNA was extracted using the Super FastPure Cell RNA Isolation Kit (Vazyme). RNA-seq libraries were prepared with the VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina® (Vazyme) according to the manufacturer's instructions. Pooled libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, Inc., San Diego, USA) using a PE150 model. Gene expression levels were quantified using Cufflinks Suite algorithms and normalized to FPKM values. Differentially expressed genes were identified with a false discovery rate < 0.05, an absolute value of log2 (fold change) > 1, and a dispersion coefficient of 0.01. Official gene symbols for the identified genes were subsequently annotated using the STRING (<https://string-db.org>) and DAVID (<https://david.ncifcrf.gov>) databases for gene ontology (GO) analysis.

Table 1. Sequences of the primers used for qPCR in this study

Gene		Primer sequence (5'→3')
<i>mAb1</i>	LC	Forward: GTGGATCTGGTTCTGGGACT Reverse: AGGGAAAATAAACACTGATGGT
	HC	Forward: TATGCCGATAGTGTGAGGG Reverse: TTGATGGTGCCAGAGGGA
<i>mAb2</i>	LC	Forward: AGGACTACAGGTCCCCCTTC Reverse: CCTGGTCCGTCCAGCTATTC
	HC	Forward: TGACCATCAGCAAGGACACC Reverse: CAGGCTCAGGAAAGTAGCCC
<i>mAb3</i>	LC	Forward: ATTCCACCAACGTGTACCCC Reverse: GCTCAGGTGTAGGTGCAGT
	HC	Forward: TCAGCGACTACGCTGTGAAC Reverse: CCTTACCCAGGAAGCGTAG
<i>mAb4</i>	LC	Forward: CAATCACTGGGTGTTTGGCG Reverse: GCCATCCACCTTCCAATCCA
	HC	Forward: TGAATTGGGTGAGGCAGTCC Reverse: TGTCCTCGGCCCTCAGATTA
<i>GAPDH</i>		Forward: CGACCCCTTCATTGACCTC Reverse: CTCCACGACATACTCAGCAC
<i>B2M</i>		Forward: ACCACTCTGAAGGAGCCCA Reverse: GGAAGCTCTATCTGTGTCAA

Statistical analysis

Data were analyzed using SPSS 26.0 and are presented as the mean ± standard deviation (SD). Statistical comparisons were performed using one-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

Results

Optimization of the co-transfection ratio of donor and helper vectors to maximize antibody yield

To maximize PB transposon-mediated recombinant antibody yield, we first optimized the transposon to transposase ratio. Co-transfection was performed using transposon to transposase ratios of 1:1, 3:1, 5:1, 7:1, and 9:1. The transposon (donor) vector was pPB-EGFP, and cells transfected with a donor vector lacking ITR sequences served as a negative control.

Transfection efficiency was assessed 48 h post-transfection using an inverted fluorescence microscope (Figure 2A) and quantified by flow cytometry. A co-transfection ratio of 7:1 yielded the highest transfection efficiency (67.8%) compared to the control. Ratios of 1:1, 3:1, 5:1, and 9:1 showed either no improvement or only slight enhancement (Figure 2B).

Following selection, stable eGFP-expressing cell pools were obtained. The transposon to transposase ratio did not significantly affect cell growth characteristics (Figure 2C). However, eGFP stable expression was significantly higher at ratios of 3:1, 5:1, 7:1, and 9:1 compared to the control. Quantitatively, expression increased by 1.54-fold at 1:1, 2.59-fold at 3:1, 3.18-fold at 5:1, 4.35-fold at 7:1, and 2.51-fold at 9:1 (Figure 2D). Consequently, the 7:1 co-transfection ratio was selected for subsequent experiments.

MAR-based PB transposon combined with BSD enhances stable mAb expression

Stable CHO cell pools expressing mAbs were generated and inoculated at 3×10^5 cells/mL in 125-mL shaker flasks. Following approximately 7 days of suspension culture, mAb expression was analyzed by western blot analysis and ELISA. As shown in Supplementary Figure S2, viable cell density (VCD) and cell viability were not significantly affected, with similar growth trends observed among different mAb cell pools. However, compared to the randomly integrated vector pMAR-mAb, pMAR-PB-mAb significantly enhanced the stable expression of four different mAbs (Figure 3A). Specifically, the titer and Qp of mAbs 1-4 increased by 4.59-, 4.82-, 3.95-, and 5.61-fold for titer (Figure 3C,E) and 4.65-, 5.47-, 4.28-, and 6.07-fold for Qp (Figure 3D), respectively. Compared to the control pPB-mAb, pMAR-PB-mAb also showed significant improvements, with titer increases of 2.19-, 2.27-, 1.93-, and 2.50-fold (Figure 3B,F) and Qp increases of 2.33-, 2.54-, 1.96-, and 2.77-fold for mAbs 1-4 (Figure 3D), respectively.

MAR-based PB transposon combined with BSD selection influences mAb transgene copy number and mRNA level

To elucidate the mechanism by which MAR-based PB transposons regulate transgene expression, we performed qPCR to analyze mAb gene copy number and mRNA levels.

The results showed that cells transfected with pMAR-PB-mAb exhibited higher mAb gene copy numbers than those transfected with only the transposon plasmid pPB-mAb. As shown in Figure 4A-D, the MAR-PB transposon generated mAb cell lines (mAb1-4) with gene copy numbers ranging from 8.67 to 10.84 for the light

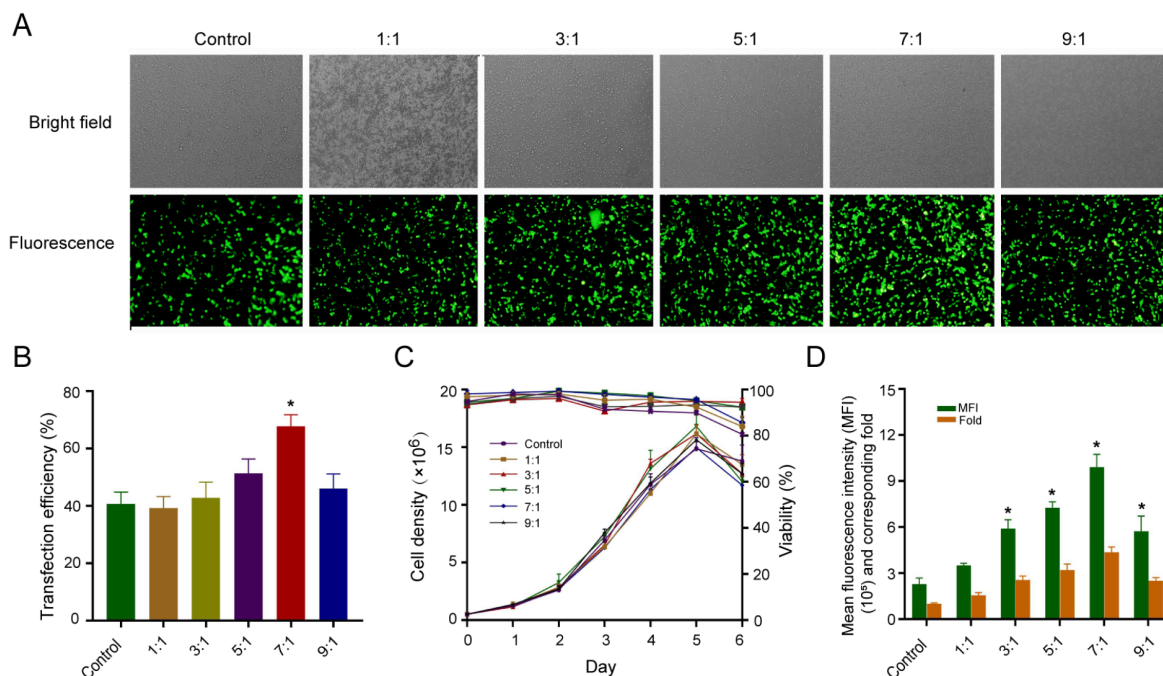


Figure 2. Effect of the transposon to transposase ratio on transfection efficiency and stable recombination protein expression in CHO cells (A) The fluorescence intensity was observed under a fluorescence microscope at 48 h post-transfection. (B) Transfection efficiency was analyzed at 48 h post-transfection by flow cytometry. (C) Viable cell density and viability. (D) eGFP stable expression and corresponding fold change. The results are expressed as the mean values obtained from three independent experiments; SEM is indicated. Compared with the control, * $P < 0.05$.

chain (LC) and 10.72 to 13.82 for the heavy chain (HC). In contrast, the control pPB-mAb yielded gene copy numbers ranging from 4.33 to 5.08 (LC) and 6.59 to 7.13 (HC). However, compared to the pMAR-mAb random integration vector, which resulted in higher copy numbers (10.37 to 12.06 for LC and 13.29 to 15.77 for HC), the MAR-based PB transposon demonstrated relatively lower mAb gene copy numbers.

In addition, as shown in Figure 4E,F, the MAR-PB-mAb vector demonstrated higher relative mRNA expression levels for both the LC and HC compared to the control pPB-mAb and pMAR-mAb. Specifically, expression ranged from 1.83- to 4.26-fold for the LC and 2.69- to 5.73-fold for the HC.

MAR-based PB transposon combined with BSD enhances the mAb-positive ratio and expression level of single clones

Single-cell clones were obtained from pMAR-PB-mAb and pMAR-mAb stable cell pools by limiting dilution. The pMAR-PB-mAb group exhibited a higher percentage of positive clones for mAbs 1-4: 37.5% (36/96), 36.5% (35/96), 33.3% (32/96), and 39.6% (38/96), compared to the MAR-mAb random integration control group with positive clone percentages of 31.2% (30/96), 27.1% (26/96), 29.2% (28/96), and 32.3% (31/96), respectively. These results indicate that the MAR-based PB transposon combined with BSD selection enhances the positive clone ratio of single-cell clones (Figure 5A).

To further assess the performance of the MAR-PB system, mAb-1 was selected for single-clone evaluation, yielding 36 positive clones from the MAR-PB-mAb group and 30 positive clones from the MAR-mAb control group. Quantitative assessment of mAb yields demonstrated a significant difference, with MAR-PB-mAb clones exhibiting production ranging from 25.0 to 103.3 $\mu\text{g/mL}$, exceeding

the 5.0 to 32.1 $\mu\text{g/mL}$ range observed for MAR-mAb clones (Figure 5B). The highest-producing MAR-PB-mAb clone showed a 3.5-fold increase in yield compared to the highest-producing control clone and a 24-fold increase compared to the lowest-producing control clone. Even the lowest-producing MAR-PB-mAb clone outperformed the lowest-producing control clone by 5-fold. Based on established criteria [42], MAR-PB-mAb clones were categorized as 83.3% high expression ($>30 \mu\text{g/mL}$), 16.7% medium producers (10–30 $\mu\text{g/mL}$), and 0% low expression ($<10 \mu\text{g/mL}$). In contrast, the majority of control clones (56.7%) were medium producers, with only 6.7% classified as having high expression (Figure 5C). These results clearly demonstrate that the MAR-PB system enables the more frequent and efficient isolation of high-expressing clones, with the top 10 clones all derived from the MAR-PB group and exhibiting expression levels exceeding 90 $\mu\text{g/mL}$.

MAR-based PB transposons combined with BSD selection enhance long-term stable mAb expression

Long-term stability was assessed by culturing four clones from each of the four mAb cell lines for 60 generations, with mAb productivity measured every 10 generations. The control group exhibited a significant decrease in mAb expression over time, with mAb1-1 dropping from 34.1 to 9.4 $\mu\text{g/mL}$, mAb2-1 from 25.6 to 5.3 $\mu\text{g/mL}$, mAb3-1 from 35.4 to 12.6 $\mu\text{g/mL}$, and mAb4-1 from 32.6 to 11.2 $\mu\text{g/mL}$. Conversely, MAR-PB clones maintained remarkably stable expression throughout, with mAb1-1 ranging from 103.3 to 90.8 $\mu\text{g/mL}$, mAb2-1 from 83.7 to 68.7 $\mu\text{g/mL}$, mAb3-1 from 90.9 to 78.2 $\mu\text{g/mL}$, and mAb4-1 from 105.5 to 92.4 $\mu\text{g/mL}$ (Figure 6). After 60 generations of culture, antibody expression in the MAR-PB clones retained 73.81%–84.39% of their initial expression after 60 generations, while control clones retained only 20.40%–40.07%.

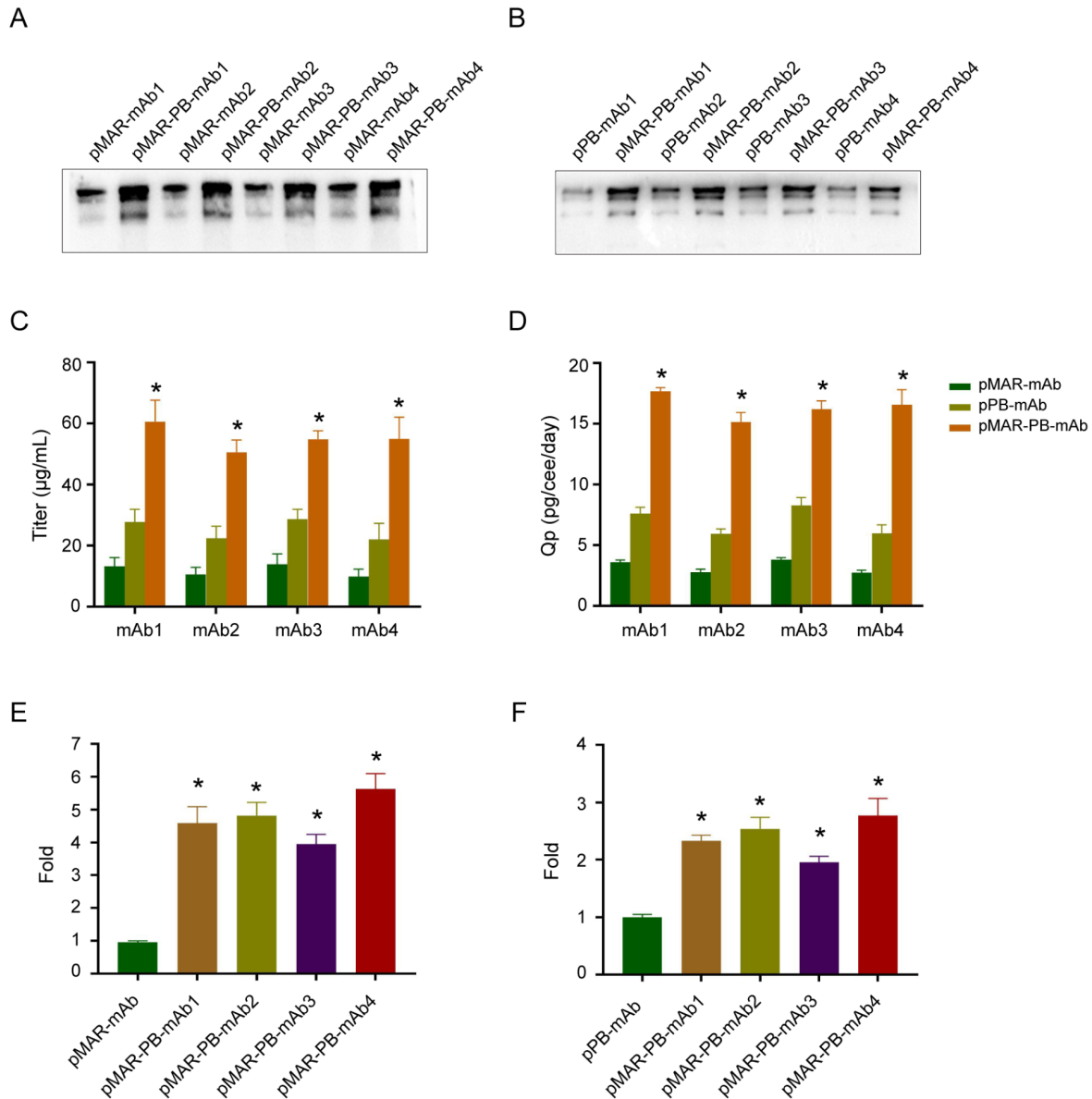


Figure 3. Effect of the MAR-PB transposon combined with BSD screening on the stable expression of four mAbs (A,B) Western blot results. (C) ELISA results. (D) Qp of mAbs. (E,F) Productivity fold of mAbs compared with control, * $P < 0.05$. The expression levels of pMAR-mAb, a randomly integrated vector (Figure 1D) lacking ITR elements, and pPB-mAb (PB-only vectors, Figure 1C) were used as controls. Error bars indicate the standard deviation of triplicate data. BSD, blasticidin; MAR, matrix attachment regions; PB, piggyBac.

These data demonstrate that the MAR-based PB transposon system combined with BSD selection substantially enhances the stable, long-term expression of recombinant mAb in CHO cells.

Transcriptomics reveals key genes that enhance stable antibody expression

Transcriptomic analysis of MAR-based transposon cell lines (pMAR-PB-mAb) and controls (pMAR-mAb) identified 980 differentially expressed genes ($q < 0.05$, $\log_2$ (fold change) > 1) (Figure 7). This analysis revealed 115 upregulated genes and 865 downregulated genes in the MAR-transposon-modified cells. GO enrichment analysis demonstrated significant involvement in molecular functions such as DNA binding, DNA-binding transcription factor activity, chromatin binding, transcriptional regulation, and protein

binding. Enriched biological processes included transcription regulation, protein secretion, and chromosome organization. Cellular components enriched were transcription regulator complex and nucleosome. These findings suggest that MAR-based transposon modification significantly impacts these cellular processes. Specifically, genes related to DNA binding include histones (H2A.V, H2B type 1, H2B type 2-F, H3.1, H3, and H4), Structural Maintenance of Chromosomes 2 (Smc2), and Neural Cell Adhesion Molecule 1 (Ncam1). Genes involved in the positive regulation of RNA polymerase II transcription included Myeloblastosis Oncogene-Like 2 (Mybl2), Zinc Finger Protein 394 (Zkscan14), Achaete-Scute Family BHLH Transcription Factor 2 (Ascl2), and Meis Homeobox 1 (Meis1). Additionally, genes encoding proteins involved in protein binding comprised Glutathione S-Transferase

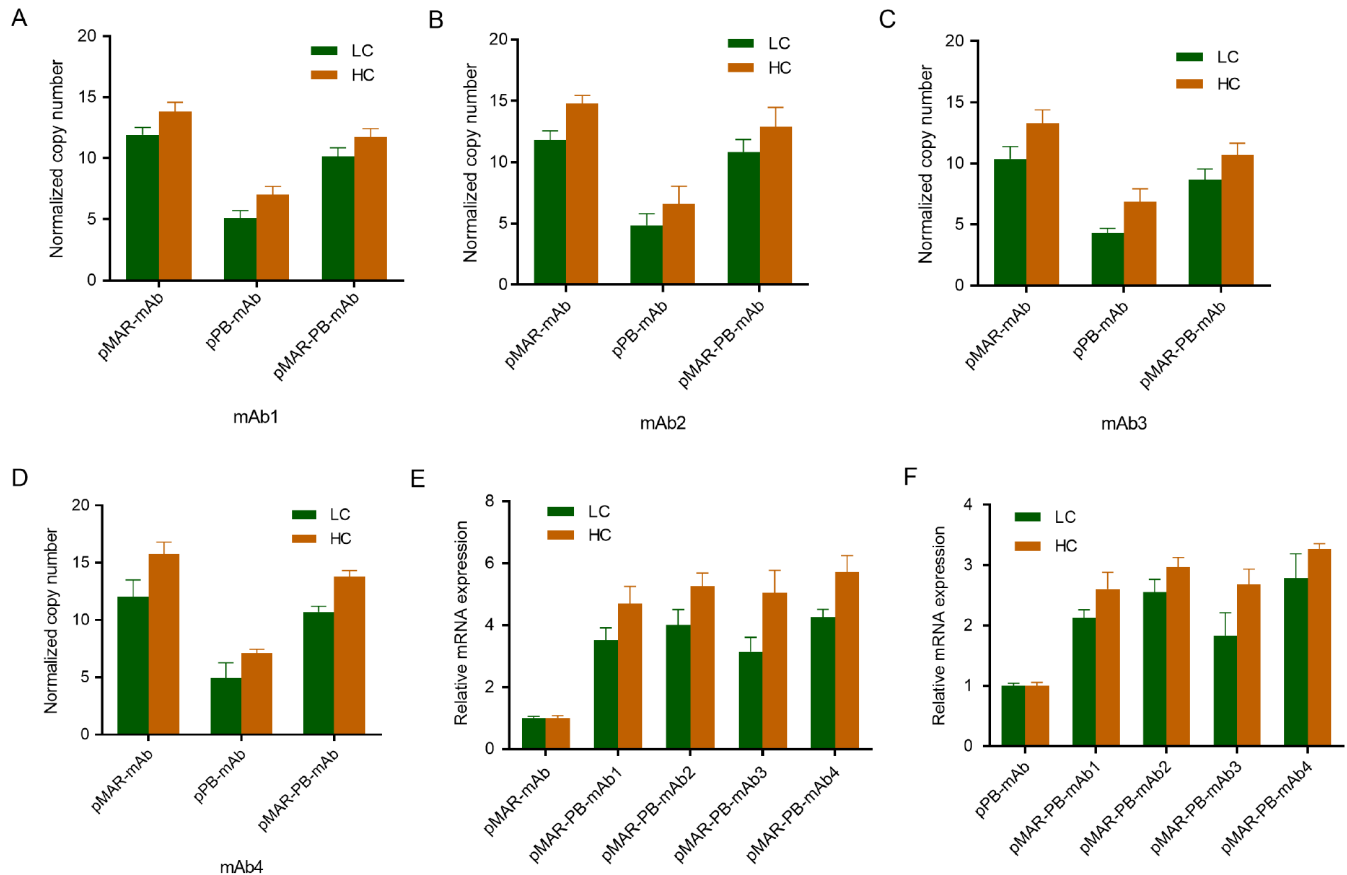


Figure 4. The copy number and mRNA expression levels of mAb1–4 in stably transfected CHO cells are evaluated by qPCR (A–D) Copy number. (E,F) mRNA levels. The results were calculated using the $2^{-\Delta\Delta Ct}$ method, and mRNA expression was normalized to the control, which was set as 1. The data presented are the mean $\pm$ standard deviation from three independent experiments.

Mu 6 (Gstm6), Protein Phosphatase 1 Regulatory Subunit 27 (Ppp1r27), Tumor Necrosis Factor Receptor Superfamily Member 26 (Tnfrsf26), Myosin, Heavy Chain 7B, Cardiac Muscle, Beta (Myh7b), Kelch Domain Containing 3 (Klhd3), Small Ubiquitin-Related Modifier 2, and Myosin XVb (Myo15m).

Discussion

Efficient and stable CHO cell screening systems are critical for the production of mAbs for clinical applications. Traditional random integration has several limitations, driving the exploration of alternative approaches. Transposon systems offer semitargeted transgene integration, preferentially inserting within or near transcriptionally active sites. Integrating regulatory elements into the transposon system further enhances transgene expression. Here, we demonstrate that the MAR-based PB transposon system combined with BSD selection effectively generates stable and high-producing mAb cell lines.

Excessive transposase can lead to re-excision of the GOI and reduced expression due to overexpression inhibition [43–45]. To prevent these issues, previous studies have recommended controlling the co-transfection ratio of transposons to transposases within a range of 5:1 to 30:1 [46]. In the present study, we optimized this co-transfection ratio and found that a 7:1 ratio is optimal in CHO cells for achieving higher transposition efficiency and transgene expression.

Matrix attachment regions (MARs) are *cis*-acting DNA elements that tether chromatin to the nuclear matrix [47], thereby organizing chromatin architecture. Prior studies have demonstrated that MAR elements can enhance transgene expression and reduce clonal variation in recombinant protein production [48–50]. However, previously reported MARs sequences vary considerably in length, often ranging from 200 bp to several kb, which complicates gene cloning and transfection procedures [51–54]. In a previous study, we identified a novel MARs consensus sequence that plays a critical role in enhancing both the level and stability of transgene expression [35]. In this study, we cloned it into the upstream of the piggyBac transposon vector expression cassette to construct the MAR-based PB vector and demonstrated a significant increase in the production of four mAbs. BSD, a protein synthesis inhibitor derived from actinomycetes, is frequently used as a selection marker in molecular biology [55]. In the present study, we found that combining the MAR-PB transposon system with BSD selection significantly increased stable mAb expression. This is consistent with previous reports showing that clonal cell lines generated using the transposon system exhibit higher and more stable volumetric productivity than those generated using a random integration system [56–61]. Prior research has shown that stable cell pools generated by PB transposon-based gene delivery can achieve up to 4-fold higher mAb titers than controls over ninety days [62], and Balasubramanian *et al.* [63] also demonstrated higher recombinant

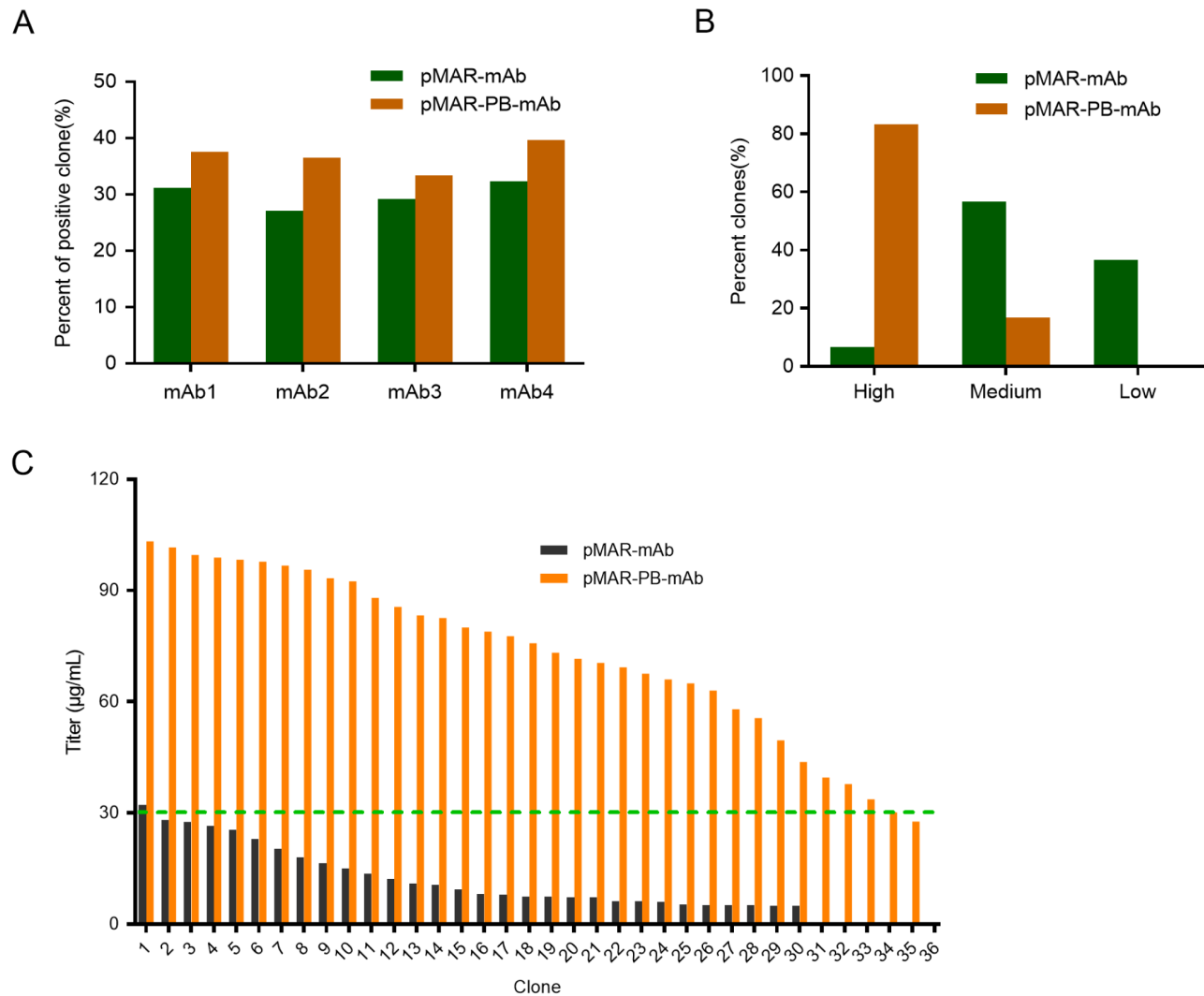


Figure 5. Comparison of mAb1 single clone expression (A) Percentage of positive clones. (B) Analysis of mAb1 volume yield from control and MAR-PB single clones by ELISA. (C) Percent clones in the high, medium, and low expression categories.

protein expression with the PB transposon system compared to the random integration system. Our results demonstrated that the combination of the MAR-PB transposon system and BSD selection increased the titer and Qp of the four mAbs by up to 3.95–5.61-fold and 4.28–6.07-fold, respectively. More importantly, this approach promoted long-term stability over 60 generations. Hydrophilicity and hydrophobicity profiles for the four antibodies were analyzed using the ProtScale tool (<https://web.expasy.org/cgi-bin/protscale/protscale.pl>). mAb4 displayed a pronounced characteristic of widespread and persistent hydrophilic regions with a limited proportion of hydrophobic segments. The abundance of hydrophilic regions and consequently high relative solubility are indicative of favorable conditions for accurate protein folding and efficient secretion [64], likely contributing to the observed differences in antibody expression.

Random transgene integration (RTI) typically yields a low proportion of industrially relevant clones, with only 1%–2% exhibiting suitable characteristics for recombinant mAb production [65,66]. In contrast, cell lines derived from the PB transposon system have shown an increased frequency of high-level expression

cells [67]. Consistent with these findings, approximately 83.3% of mAb1 clones generated using the MAR-PB system in this study were classified as high expression clones, with no low producers observed. These findings suggest that the MAR-based PB transposon combined with BSD selection facilitates the efficient isolation of high-level expression clones. Rajendra *et al.* [42] reported that combining the PB transposase system with the GS system in CHO-K1 cells generated cell pools with approximately 66% high-expressing clones, resulting in a 2–3-fold increase in volumetric productivity. While the GS system enhances screening, it is known for its labor-intensive nature and potential for phenotypic and genotypic instability. The MAR-PB system described herein offers a compelling alternative for mAb production. A remarkable 24-fold increase in productivity was achieved between the highest MAR-PB clone of mAb1 and the lowest control clone. Critically, MAR-PB cells exhibited remarkable long-term stability, exhibiting only a minor reduction (< 20%) in mAb expression after 60 generations, highlighting their potential for stable and efficient mAb production. While Rajendra *et al.* [42] employed a fed-batch process with multiple feeds, our study utilized a batch process. Implementing

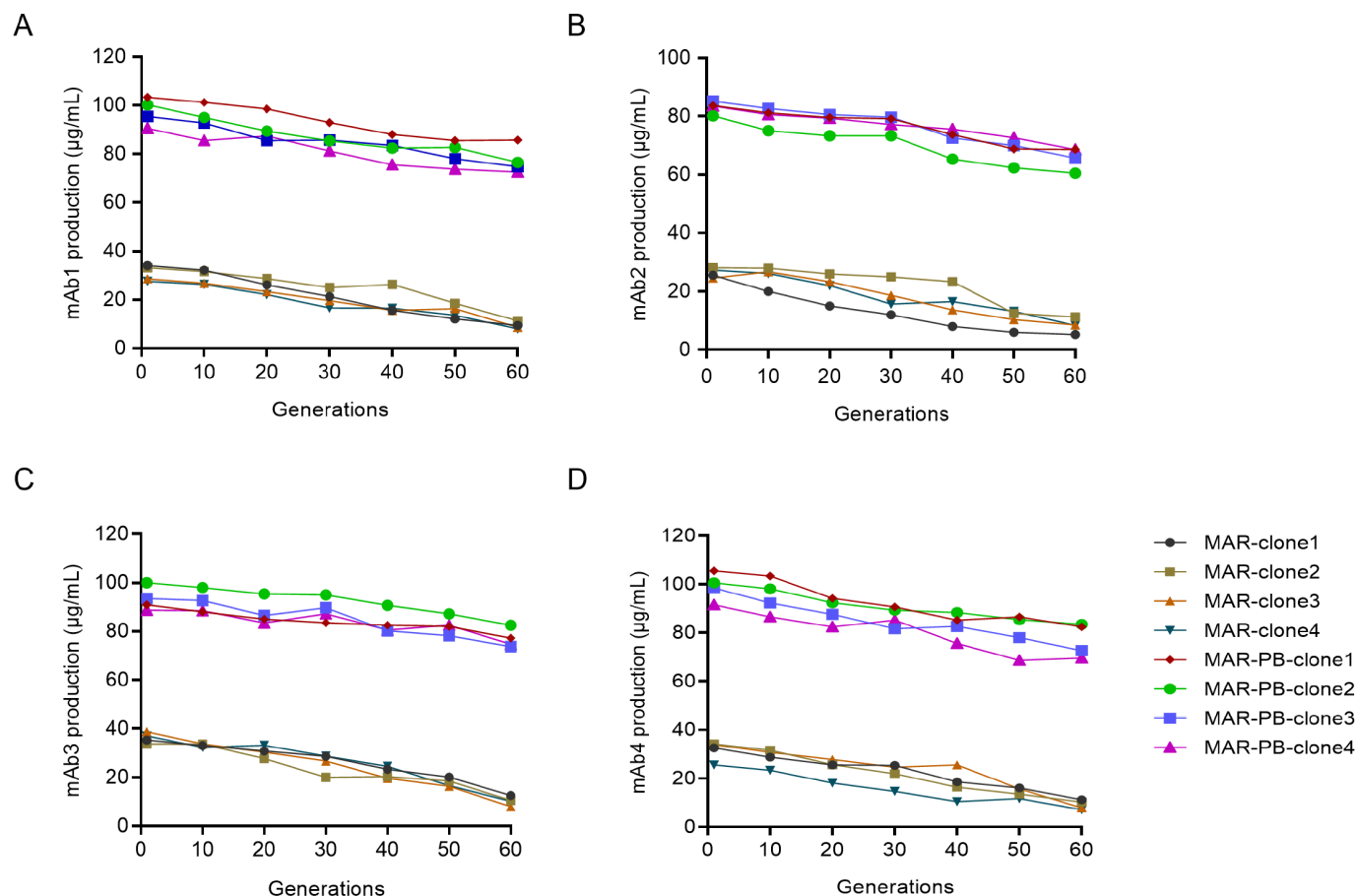


Figure 6. Long-term stable expression of four different mAbs (A) mAb1. (B) mAb2. (C) mAb3. (D) mAb4. Stability was tested for 4 clones per mAb per group to assess variability.

fed-batch coupled with process optimization, such as low-temperature cultivation, could yield even higher titers. Furthermore, Rajendra *et al.* [42] used expression vectors with insulator elements, where both HC and LC genes were driven by the CMV promoter and GS gene. In contrast, our study employed a vector strategy featuring a MARs element, a LC gene under the CMV promoter, an HC gene under the EF-1 α promoter, and a BSD selection cassette. Notably, the host cell line, selection methods, antibody gene sequences and selection strategies all differed.

The observed increase in transgene expression and long-term stable facilitation by transposon vectors and MARs elements is attributed to their tendency to integrate the GOI into transcriptionally active genomic loci and to modulate/maintain chromatin architecture. MARs serve as boundary or insulator elements by shielding genes from the spread of heterochromatin [68–70]. MARs also recruit transcription factors and chromatin remodeling enzymes, enabling local modifications to chromatin structure and helping to maintain an open chromatin state, which is essential for high gene expression [71–73]. Ley *et al.* [34] demonstrated that combining MARs with transposable vectors enhances transgene expression. However, their study utilized MAR 1–68 and MAR X-29 elements (approximately 3.6 kb in length) and selected cells with puromycin. In contrast, our study employed a shorter, consensus MAR sequence (approximately 1.1 kb) derived from 10 different MARs coupled with blasticidin for selection. Furthermore, while

Ley *et al.* focused on the positioning of MARs within vectors and evaluated recombinant protein expression with or without antibiotic selection, our research investigated the combined effects of MARs and transposon vectors with blasticidin on stable and long-term recombinant protein expression, including the distribution of single-cell clones exhibiting high, medium, and low expression. This approach is crucial for efficiently screening stable, high-expressing cells.

Transcriptome analysis revealed that MAR-based PB transposons enhance processes associated with DNA binding, transcriptional regulation, and protein binding. These observations suggest that epigenetic mechanisms, such as histone modification and chromosome conformation, may contribute to enhanced recombinant protein expression. Therefore, future studies should focus on investigating the epigenetic mechanisms underlying the observed effects.

To further assess the broad applicability of the MAR-based piggyBac transposon system with blasticidin selection, we investigated its impact on non-antibody recombinant protein productivity. Using recombinant human serum albumin (HSA) and recombinant human type III collagen, we observed that stable CHO cell lines generated from MAR-based PB transposon stable cell pools exhibited 2.58- to 3.29-fold higher volumetric productivity than those from MARs randomly integrated stable cell pools (Supplementary Figure S3). These findings demonstrate that this method

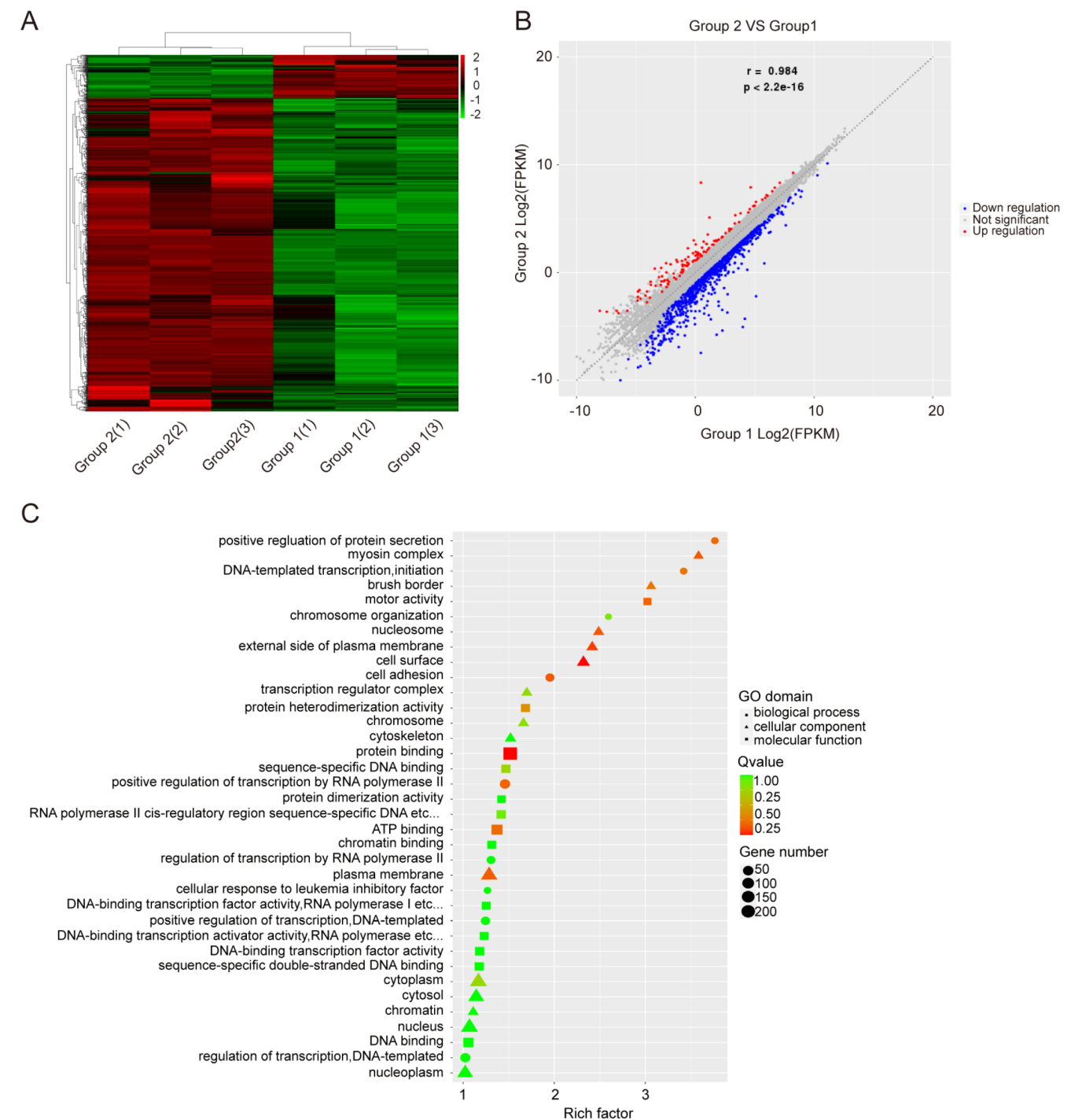


Figure 7. Analysis of gene expression differences between MAR-transposon cell lines and the control (A) Differential gene heatmap. (B) Expression correlation scatter plot. (C) GO functional classification of differentially expressed genes. Group 2 and group 1 represent transposon cell lines (pMAR-PB-mAb) and the control cell line (the cell line transfected with pMAR-mAb), respectively.

can be broadly applied to enhance non-antibody recombinant protein expression.

Stable clones generated via the MAR-based PB transposon system maintain high productivity and long-term stability, enabling the early and efficient production of representative drug substances for analytical, formulation, toxicology, and early clinical development. Furthermore, the MAR-based PB transposon stable clones represent

attractive candidates for supporting perfusion-based manufacturing processes due to their superior productivity and stability. However, detailed product quality assessments and regulatory considerations for MAR-based PB vectors in GMP production are prerequisites before their application in therapeutic protein manufacturing.

In conclusion, the expression and stability of recombinant antibodies are regulated by multiple factors, including the design

of expression cassettes, the choice of host cell line, the characteristics of mAb gene sequences, and the selection methodology employed. The findings of this study support the utility of the MAR-based PB transposon system combined with BSD selection for generating stable and efficient CHO cell pools and clones.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

The raw sequence data reported in this paper have been deposited in the Sequence Read Archive (SRA) under accession number PRJNA1420231.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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