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Development of predictable and high-yield CHO cell line by site-specific integration

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Abstract
Chinese hamster ovary (CHO) cell is a widely used cell line for the production of therapeutic proteins. Customarily, CHO host cell line is established through random integration, which requires multiple rounds of screening to identify the optimal producer. In contrast, site-specific integration (SSI) technology can boost cell line development efficiency by directing gene of interest (GOI) to certain genomic loci that supports sustained and stable expression. In this study, by utilizing AI algorithms and robotic systems, we have identified several CHO host cell lines carrying marker gene by Bxb1-mediated SSI technology. After the substitution of marker gene with different types of GOI at specific sites, some of cases delivered a titer exceeding 15 g/L. Thanks to the uniformity of SSI cell lines, protein titer and quality of monoclonal cells can be predicted based on the performance of corresponding minipools. Different monoclonal cells originated from the same minipool also showed consistent protein quality. Furthermore, it was observed that the monoclonal cells obtained by SSI demonstrated consistency in protein titer, quality and genetic stability after 26 consecutive passages (approximately 90 days). In summary, we have successfully developed an effective platform for the construction of SSI cell lines, which allows the rapid and efficient expression of various proteins while maintaining consistency in stability and product quality.

Keywords CHO cell line, Site-specific integration (SSI), Bxb1 integrase

Introduction

Over decades, FDA-approved antibody drugs and antibody-related medications are on the rise. The top ten biopharma deals of 2024 amounted to over $ 31 billion, according to data as of the end of November 2024. Each deal on the 2024 list is worth over $ 2 billion, with several potentially reaching two or even three times that amount [1, 2]. The biopharmaceutical market has seen a growing demand for recombinant protein-based biotherapeutics [3–5]. Among mammalian cells, CHO cell has emerged as a major expression system in biopharmaceutical development and biopharmaceutical industry. As CHO cell allows protein folding and post-translational modifications, the CHO cell-expressed proteins are compatible to proteins in humans in terms of molecular structure, physicochemical properties and biological functions [6].

The traditional approach to develop recombinant CHO cell line is to randomly integrate foreign genes into the genome, which is time-consuming, laborious and unpredictable [4]. In recent years, different SSI approaches have been employed to achieve stable expression of GOI [7], including CRISPR/Cas9 [8], transposon [9, 10],

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site-specific integrase Bxb1/*att* [11, 12], Cre/*loxP* [13], Flp/*FRT* [14], ϕC31 [15, 16], and RNA-guided combination of Cas9 and Bxb1 [15]. Theoretically, the insertion of foreign gene at specific site ensures its continual and predictable expression [7] and avoids strong position effects due to differences in local chromatin structure and interference from neighboring genomic environments. The transfection efficiency of Bxb1 is significantly higher than other methods [16]. It catalyzes gene cleavage and integration with high efficiency and precision by recognizing the homologous sequence *attP* (phage attachment site) and *attB* (bacterial attachment site). At the same time, the Bxb1-catalyzed integration is irreversible [11, 17] and no accessory proteins or supercoiled DNA templates is required. More importantly, it lacks pseudo-sequences in the mammalian genome [18], therefore has higher safety and reliability.

In this study, by leveraging AI algorithms and robotic systems, we have successfully developed a CHO-SSI platform based on the Bxb1 recombinase-mediated cassette exchange (RMCE) system, which facilitates rapid screening of stable and predictable recombinant CHO cell lines and improves the titer and quality of protein of interest. Experiment results indicated that recombinant cell lines expressing different types of protein exhibited high and stable protein expression and consistent quality in stability testing. In addition, the titer and quality of the final selected monoclonal cells can be predicted based on minipool results. These findings suggested that the CHO-SSI platform has the potential to develop cell lines for biopharmaceutical purposes in an efficient and convenient manner.

Methods

Plasmid construction

The landing pad containing plasmid pLanding1.1 was constructed by Gibson assembly using 2× Gibson Master Mix (NEB, E2611S). All essential fragments were synthetized by GenScript Biotech, including EGFP gene (GenBank: MN623123.1) flanked by sequence *attP*/*GA* and *attP* [19], and hygromycin resistance gene (GenBank: CP132301.1). Neomycin resistance gene (GenBank: CP132301.1), CMV (hCMV) promoter (GenBank: MT267310.1), SV40 sequence (GenBank: OQ579021.1), Ori sequence (GenBank: MN922300.1) and ampicillin resistance sequence (GenBank: OQ982570.1) were amplified from pcDNA3.3 plasmid using PrimeSTAR MAX DNA polymerase (Takara Bio, R045B). Plasmid pLanding1.1 was treated with *Ssp*I for linearization to developing CHO-SSI host cell by electroporation.

Vectors containing GOI were constructed to be pDonor plasmid, in which the *attB*-pUC57-Mini plasmid [19] containing *attB/GA*, *attB* and *Bam*HI and *Not*I cleavage sites was synthesized by GenScript Biotech. Fragments containing multiple cloning sites (MCS) were amplified from pCGS3 plasmid (Sigma) with *Bam*HI and *Not*I cleavage sites using Prime STAR MAX DNA polymerase. Following the digestion of *attB*-pUC57-Mini plasmid and pCGS3 plasmid by *Bam*HI and *Not*I, the resultant fragments were ligated by T4 DNA ligase.

The codon optimization and synthesis of GOI sequence was completed by GenScript Biotech. Certain cleavage sites in the MCS of pDonor plasmid was selected for endonuclease digestion. GOI was then inserted to the MCS of pDonor plasmid and ligated by T4 ligase. Linearized pDonor plasmid was prepared for electroporation by treating with *Fsp*I.

The codon optimization of Bxb1 nucleic acid sequence (GenBank: AHB17669.1) was conducted by GenScript Biotech.

Cell culture

CHO-K1 (Sigma) and selected CHO-SSI host cells were passaged and cultured with Subculture Medium that consists of 99% Ex-Cell Advanced CHO Fed-batch Medium (Sigma, 24366 C-10 L) and 1% GlutaMAX (Gibco, 35050-061). The cells were either subcultured every 3 days at a seeding density of 5×10^5 cells/ml or every 4 days at a seeding density of 3×10^5 cells/ml. Monoclonal CHO-SSI host cells were cultured with Cloning Medium which contains 75% EX-CELL CHO Cloning Medium, 1% GlutaMAX, 5% ClonaCell-CHO ACF Supplement and 20% CHO-K1 cell culture supernatant when in 96-well plates. The cells were cultured in CO_2 static incubator (ESCO, CLM-240B-8-NF) at 37℃, 5% CO_2 when they were in cell culture plates, and cultured in CO_2 shaker incubator (Crystal Technology, IS-RDS6C5) at 37℃, 5% CO_2, 120 rpm when they were in bioreactor tubes or shake flasks.

Transfection

CHO-K1 cells or CHO-SSI host cells were counted after thawing and cultured for at least two passages. Cell viability should exceed 95% and cell density should be $2–5 \times 10^6$ cells/ml before electroporation. 3×10^6 cells were then counted and centrifuged at 1,000 rpm for 5 min. Supernatant was discarded and the cell pellet was washed once with 1× PBS. Cell pellet was then resuspended with 100 µl transfection reagent R (Invitrogen, MPK10096). A total of 3.4 pmol DNA was mixed with the cell suspension for further electroporation. Electroporation was performed using 1575 V, 10 ms and 3 pulse (MPK5000, Invitrogen). After electroporation, the cells were cultured for two days in medium consists of 75% Ex-Cell Advanced CHO Fed-batch Medium (Sigma, 24366 C-10 L), 20% EX-CELL CHO Cloning Medium (Sigma, C6366-500 ml) and 5% ClonaCell-CHO ACF Supplement (STEMCELL Technologies, 03820). For development of protein production cell lines in CHO-SSI cells, a total of 3.4 pmol DNA containing Bxb1 containing plasmid and linearized pDonor plasmid was pre-mixed and introduced into CHO-SSI host cells by electroporation. To develop producing cell line by random integration, only a total of 3.4 pmol linearized pDonor plasmid was mixed with CHO-K1 cells and transfected by electroporation.

Antibiotic screening

The CHO-K1 fluorescent cell pools with plasmid pLanding1.1 was recovered for two days. After that, minipools were constructed in 96-well plates at 8000 cells/well and screened with Geneticin (G418, Thermo Fisher, 10131). The cell growth was monitored during the incubation period, and no further pressurization was required after expansion.

After transfecting the pDonor plasmid carrying GOI into CHO-SSI host cells, the minipools were constructed as what was described above. Hygromycin (Thermo Fisher, 10687010) was used for antibiotic screening, and no antibiotics was required after expansion culture.

AI-assisted CHO-SSI monoclonal cell selection

The dataset for Al model training data consisted of cell images captured with 40x objectives using the Invitrogen, EVOS M7000 microscope, including both green fluorescence and non-fluorescence images as illustrated in Figure S1. The training process was carried out in two steps: single-cell image extraction followed by expression prediction.

In the first step, a YOLOv5 [20] model was finetuned to detect and localize individual single cells within the microscope images. This step is necessary because cells often grow in clusters, and such aggregated cells are not suitable for single-cell extraction and monoclonal isolation. In the second step, an AI model was trained to predict protein expression titer of individual single-cells. For this step, non-fluorescence images of single-cells were, as illustrated in Figure S1A and S1C were used as input and the fluorescence intensity calculated from the corresponding fluorescence images, as illustrated in Figure S1B and S1D were used as the ground-truth label. In this case, the fluorescence intensity was treated as a proxy for protein expression titer, with higher fluorescence intensity values representing higher titer levels. The network architecture was based on ResNet50 [21], which was used to extract representative features from each single-cell image. These extracted features were then passed through a final linear layer to generate a continuous prediction of the corresponding fluorescence intensity.

During the selection of CHO-SSI monoclonal cells, a total of 1.5×10^5 viable cells were suspended in 150 μl medium containing 80% Ex-Cell Advanced CHO Fed-batch Medium and 20% EX-CELL CHO Cloning Medium, mixed with 1.2 ml semi-solid medium (Molecular Devices, K8840) in a 35 mm petri dish, and incubated at 37℃ for 1 h. Then, non-fluorescence cell images were captured using the Invitrogen, EVOS M7000 microscope with the same setting as before. Our AI model automatically detected single cells in these images and predicted their expression titers. These single-cells were then ranked according to their predicted titers, and the highest ranked single-cells were transferred from petri dish to 96-well plate containing 200 μl Cloning Medium through micromanipulation robotic system. The 96-well plates were cultured in incubator at 37℃, 5% CO2 for 14 days and cell images were captured on day 0, 1, 7 and 14.

Whole-genome sequencing

Whole-genome sequencing was performed by Sequanta Technologies (Shanghai, China). Approximately 5×10^6 CHO-SSI cells were collected for sequencing with 75× read depth. The experiment included genomic DNA (gDNA) extraction, DNA quality testing, library preparation, sequencing and data analysis. The raw data obtained via whole-genome sequencing were then analyzed using bioinformatics approaches, and the reference CHO genome was GCF\U 003668045.3\U CriGri-PICRH-1.0, see https://www.ncbi.nlm.nih.gov/assembly/GCF_000223135.1/.

Minipools and monoclonal cells construction

For site-specific integration, GOI-transfection pools were imaged using a microscope (Invitrogen, EVOS M7000) in various fields of view after recovery from electroporation. The fluorescence intensity of every single cell in the image was obtained using our proprietary cell detection algorithm. Meanwhile, the fluorescence intensity outside the region of interest was also obtained by our proprietary algorithm, based on which the average fluorescence intensity of the background region (i.e. the fluorescence threshold) was calculated. The proportion of non-fluorescent cells is measured by the number of single cells that have a fluorescence intensity below or equal to the fluorescence threshold when the total number of single cells is taken into counting. Subsequently, the non-fluorescent cells were sorted into 3 to 5 minipools, screened by Hygromycin B resistance. After a comprehensive assessment of the cell growth, titer, protein qualities, etc., the top 1 was utilized for monoclones screening by limited dilution with 3 plates (60 clones/plate).

In the case of random integration, GOI-transfection pools were separated to 50 plates of minipools after electroporation recovery. And top 3 or 5 minipools were utilized for monoclones screening by limited dilution with at least 50 plates (96 clones/plate).

Fed-batch culture

The cells were initially seeded into 125 ml shake flask with an initial culture volume of about 30 ml at a viable cell density of about 5×10^5 cells/ml. The cells were cultured with Subculture Medium at 36.5 ± 0.5℃, 5% CO_2, 120 rpm, 80% RH. Feed medium Cell Boost 7a (Hyclone, SH31026.07) and Cell Boost 7b (Hyclone, SH31027.01) were supplemented in ratios of 3%/0.3% of initial culture volume on day 3. Starting on day 5, Cell Boost 7a and Cell Boost 7b were added to the culture every two days in ratios of 5%/0.5% of initial culture volume. Cell viability and viable cell density were measured by cell counter (Countstar, IC1000). Glucose and lactate levels were detected by biosensor analyzer (SIEMAN, M-100). Protein titer was determined by biomolecular interaction analyzer (Sartorius, Octet R8). Samples were harvested on day 14. Cell growth and metabolic data were detected on day 3, 5, 7, 9, 11, 13 and 14. Protein titer check was performed on day 7, 9, 11, 13 and 14.

Stability assessment

The CHO-SSI host cell lines or GOI-expressing cell lines were subcultured every 3 or 4 days for 26 consecutive passages (approximately 90 days). Part of the cells were cryopreserved at designated passages while the others were used to proceed with further experiments. For CHO-SSI host cell lines, the average fluorescence intensity and proportion of fluorescent cells were calculated by calculation software (GBB, Main). CHO-SSI host cell lines with a < 20% variance in fluorescence intensity from P1 to P26 and a < 5% proportion of non-fluorescent cells were identified as host cell line candidates, whose EGFP copy number was analyzed subsequently. The stability of GOI-expressing cell lines were evaluated based on titer, protein quality and gene copy number at selected passages.

Gene copy number analysis

Relative gene copy number was determined by relative quantification using Applied Biosystems® 7500 Fast Real-Time PCR System. The gDNA of all cell lines in this study was extracted using FastPure Cell/Tissue DNA Isolation Mini Kit (Vazyme, DC102-01), and reactions were performed with 2× Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Q712-02). Relative standard curve was constructed with P1 gDNA, and the gDNA of other passages were then diluted within the linear range of the relative standard curve for quantitative reactions. The relative GOI copy number in the gDNA of other passages was calculated based on the Pfaffl method [19], with reference to the GOI copy number of P1 - and B2M gene was used as the internal reference.

Purification of protein of interest

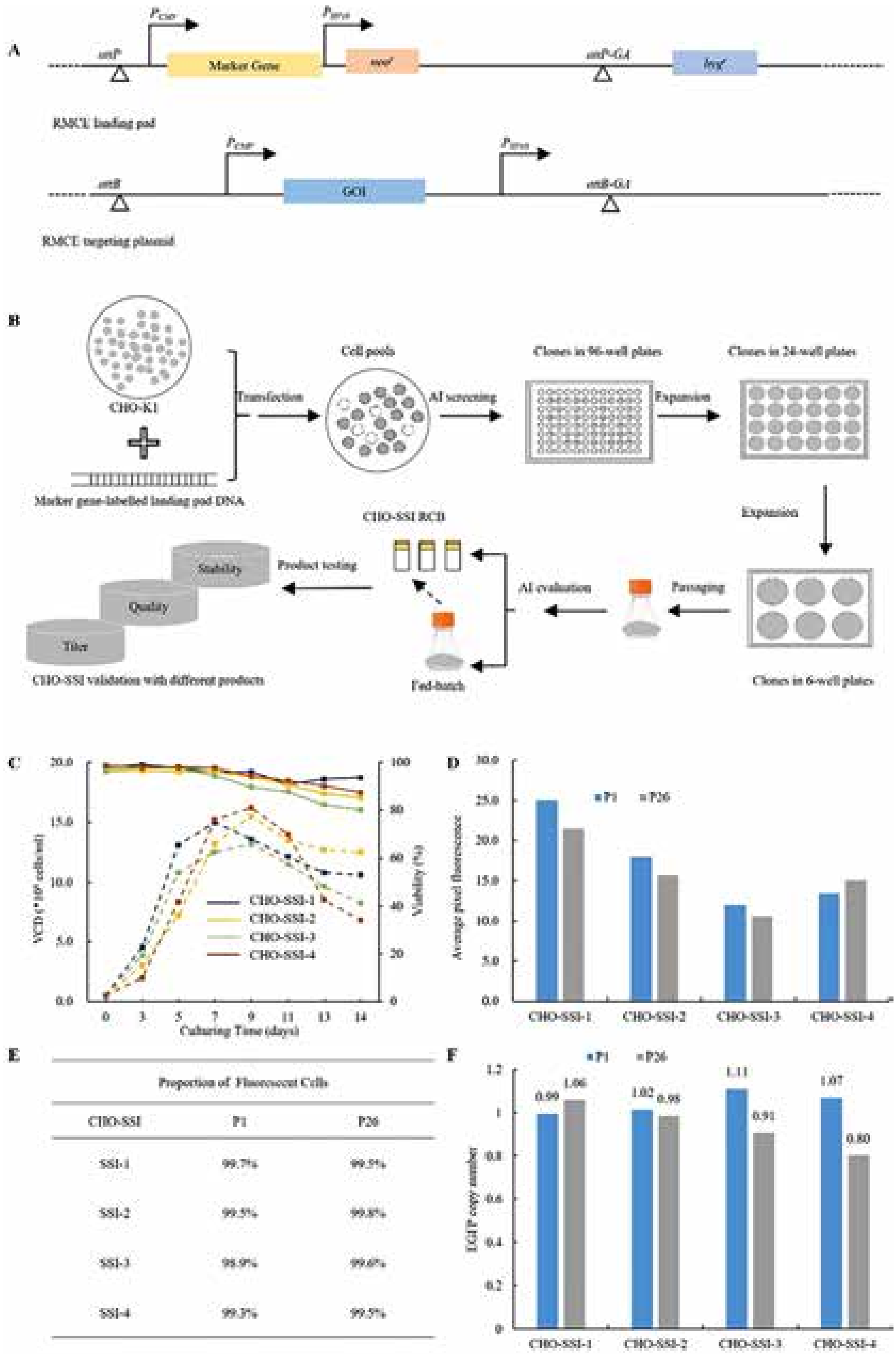
Supernatant at harvest was centrifuged at 3,000 rpm for 10 min (Thermo, SORVALL ST16) to remove cell pellets, and centrifuged at 9,000 rpm for another 30 min. The resultant supernatant was filtered through 0.22 μm filter (Millipore, SLGVR33RB), and loaded to ready-to-use HiTrap MabSelect SuRe Protein A column (Cytiva, 11003494), which was prepacked with 5 ml MabSelect SuRe Protein A resin (Cytiva, HiTrap MabSelect SuRe). The Protein A column was regenerated with 0.5 M NaOH and then equilibrated with 1× PBS prior to use. The protein of interest was eluted by elution buffer (50–100 mM acetate buffer/glycine-HCl buffer/citrate buffer, depending on the properties of the protein of interest) at a pH of 3.0. The sample solution was neutralized by Tris-HCl and filtered through 0.22 μm filter. Sample concentration was measured by ultra-micro spectrophotometer (KAIAO, K5600C). Purified samples were stored at -20℃ for further analysis.

Purity measurement of protein of interest: size exclusion chromatography

Size exclusion chromatography was performed to measure the purity of the protein of interest through high-performance liquid chromatography system (Waters, Arc) equipped with UV detector or ADA detector. For monoclonal antibodies, column: TOSOH TSKgel G3000SWXL HPLC column; mobile phase A: aqueous solution containing 0.1 M sodium sulfate and 0.1 M phosphate (with a pH of 6.5); mobile phase B: acetonitrile; ratio of mobile phase A and mobile phase B: 80:20; flow rate: 0.8 ml/min; column temperature: 25℃. For normal bispecific antibodies, column: Sepax BioMix SEC column; mobile phase: aqueous solution containing 1.5 M NaCl and 0.1 M phosphate (with a pH of 7.0); flow rate: 0.5 ml/min; column temperature: 25℃.

Glycan profiling of protein of interest

The protein was exchanged into 20 mmol/L, pH 6.5 citrate buffer through 10 KDa ultrafiltration centrifugal filters. N-glycan in glycoprotein was hydrolyzed by N-glycosidase F (PNGase F) (NEB, P0705S) at 37℃ for 2 h. The hydrolyzed N-glycan was recovered through PVDF microplate (Agilent, 200945-100) and vacuum-dried. The hydrolyzed N-glycan was then labeled with 2-aminobenzamide (2-AB) (Sigma, A89804-5G) at 65℃ for 2 h and fluorescence was detected by hydrophilic interaction chromatography (Waters, Hi-Class) for subsequent analysis. By comparing the retention time of N-glycan controls (Agilent), the glycoforms, glycoform distribution and glycoform proportion were determined.



CHO-SSI	P1	P26
SSI-1	99.7%	99.5%
SSI-2	99.5%	99.8%
SSI-3	98.9%	99.6%
SSI-4	99.3%	99.5%

Fig. 1 (See legend on next page.)

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Fig. 1 CHO-SSI host cell lines selected through AI algorithm. **(A)** Bxb1-mediated recombinase-mediated cassette exchange (RMCE). Maps of representative landing pad elements and target vector for RMCE. *P*, promoter; *CMV*, human cytomegalovirus; *SV40*, simian virus 40; marker gene, enhanced green fluorescent protein (EGFP) was adopted in this study; *neo*^{r}, neomycin resistance gene, conferred resistance to G418; *attP*, phage attachment site; *attB*, bacterial attachment site; *attP-GA* or *attB/GA*: with "GA" as the central dinucleotide, as opposed to the wild type that with "GT" as the central dinucleotide; *hyg*^{r}, hygromycin resistance gene; GOI, gene of interest. **(B)** CHO-SSI screening workflow. Transfect CHO-K1 cells with linearized pLanding1.1 plasmid and EGFP-expressing cell pools are generated. Conduct antibiotic screening with G418. Sort out monoclonal CHO-SSI host cells with robotic system and AI algorithm. Culture the monoclonal CHO-SSI host cells in 96-well plates for 14 days, and scale up monoclonal CHO-SSI host cells with stable fluorescence expression to 24-well plates and 6-well plates sequentially. Scale up further to construct RCB and proceed with growth evaluation in shake flasks. **(C)** Growth assessment of different CHO-SSI host cell lines through 14-day fed-batch culture. **(D-F)** Stability assessment of CHO-SSI host cell lines, including the EGFP fluorescence intensity at designated passages, proportion of EGFP-expressing fluorescent cells and EGFP copy number, B2M (Beta-2-microglobulin) was used as the internal control

Charge variation analysis of protein of interest: iCIEF method

Charge variation analysis of the protein of interest was performed by ProteinSimple whole-column imaging detection capillary electrophoresis instrument. First, the electrode environment in the capillary was established by adding cathode solution and anolyte solution into the electrode tank of cIEF cartridge (ProteinSimple). Then, the samples and reagents were mixed to 100 µl solution and loaded into the capillary. After a 1-minute prefocus at 1500 V followed by a 5 to 10-minute focus at 3000 V, the pH gradient of the electrolyte solution gradually formed. Each solute migrated in the capillary until their isoelectric point (pI) was reached. At this point, they focused in a zone and exhibited a neutral pH. The entire capillary stabilized when charge isomers of the protein of interest focused in their respective zones. Eventually, the signals of different pIs were converted into different peaks, based on which different charge isomers and their relative proportion were determined.

Results

AI algorithm-assisted host cell line selection

As illustrated in Fig. 1A, the vector pLanding1.1 primarily consists of homologous sequences that can be recognized by Bxb1 integrase (*attP* and *attP*-GA), marker gene EGFP and *neo*^{r} gene that conferred resistance to antibiotics G418. In addition, a promoterless *hyg*^{r} gene is incorporated at the 3' end of *attP*-GA. The vector pLanding1.1 is randomly integrated to the host cell genome, and the integration site is marked and referred to as landing pad. The pDonor plasmid includes the Bxb1 RMCE donor cassette (*attB* and *attB/GA*) and multiple cloning site (MCS) for GOI insertion. To circumvent random insertion, an SV40 promoter is incorporated at the 5' end of *attB/GA* to activate *hyg*^{r} gene on landing pad and hygromycin screening when expected integration occurs. The non-fluorescent cells whose EGFP cassette is fully replaced by GOI are identified as positive recombinant cell lines for further culture [22].

The entire screening process for CHO-SSI host cells is illustrated in Fig. 1B. The vector pLanding1.1 was first electroporated into CHO-K1. Following a two-day recovery, minipool was constructed through antibiotic selection using G418. The minipool with the highest proportion of fluorescent cells was then expanded and subcultured until viability exceeded 90%. Subsequent to this, the optimal monoclonal cells were selected based on algorithm and sorted out by robotic system. Following a 14-day culture in 96-well plates, monoclonal cells with stable EGFP expression were expanded for RCB construction and cell growth evaluation. Cells exhibited a $<90\%$ viability or a $>5\%$ non-fluorescence rate were discarded.

The above identified CHO-SSI host cell lines were then evaluated through 14-day fed-batch culture. As shown in Fig. 1C, the peak viable cell density (VCD) of CHO-SSI-1 was approximately 1.5×10^7 cells/ml, and the viability of all four CHO-SSI host cell lines exceeded 80% on day 14. Subsequently, a 90-day cell passaging experiment (26 passages) was carried out to validate CHO-SSI host cell line stability. The average fluorescence intensity of CHO-SSI-1 was 25, the highest among all the four CHO-SSI host cell lines. The average fluorescence intensity of CHO-SSI-2 was 18, 33% and 26% higher compared to CHO-SSI-3 and CHO-SSI-4, respectively (Fig. 1D). It was found out that among all the four CHO-SSI host cell lines, the EGFP fluorescent variance between P1 and P26 was within 20% (Fig. 1D), and the variance of fluorescent cell proportion was less than 1% (Fig. 1E), indicating high fluorescence stability. Furthermore, the copy number of EGFP at P1 and P26 was measured to validate CHO-SSI cell line stability. As shown in Fig. 1F, the copy number of all CHO-SSI cell lines exhibited stability during consecutive passaging. In the stability testing on all the four CHO-SSI host cell lines, CHO-SSI-1 and CHO-SSI-2, with high EGFP fluorescence intensity and cell growth ability, were selected as the host cell lines for subsequent product testing.

Protein expression testing on CHO-SSI host cell lines

After transfecting different GOIs into the CHO-SSI host cell lines, the resultant cell pools contained both fluorescent and non-fluorescent cells (Fig. 2A). The proportion of non-fluorescent cells was generally greater than 60% after enrichment (Fig. 2B).

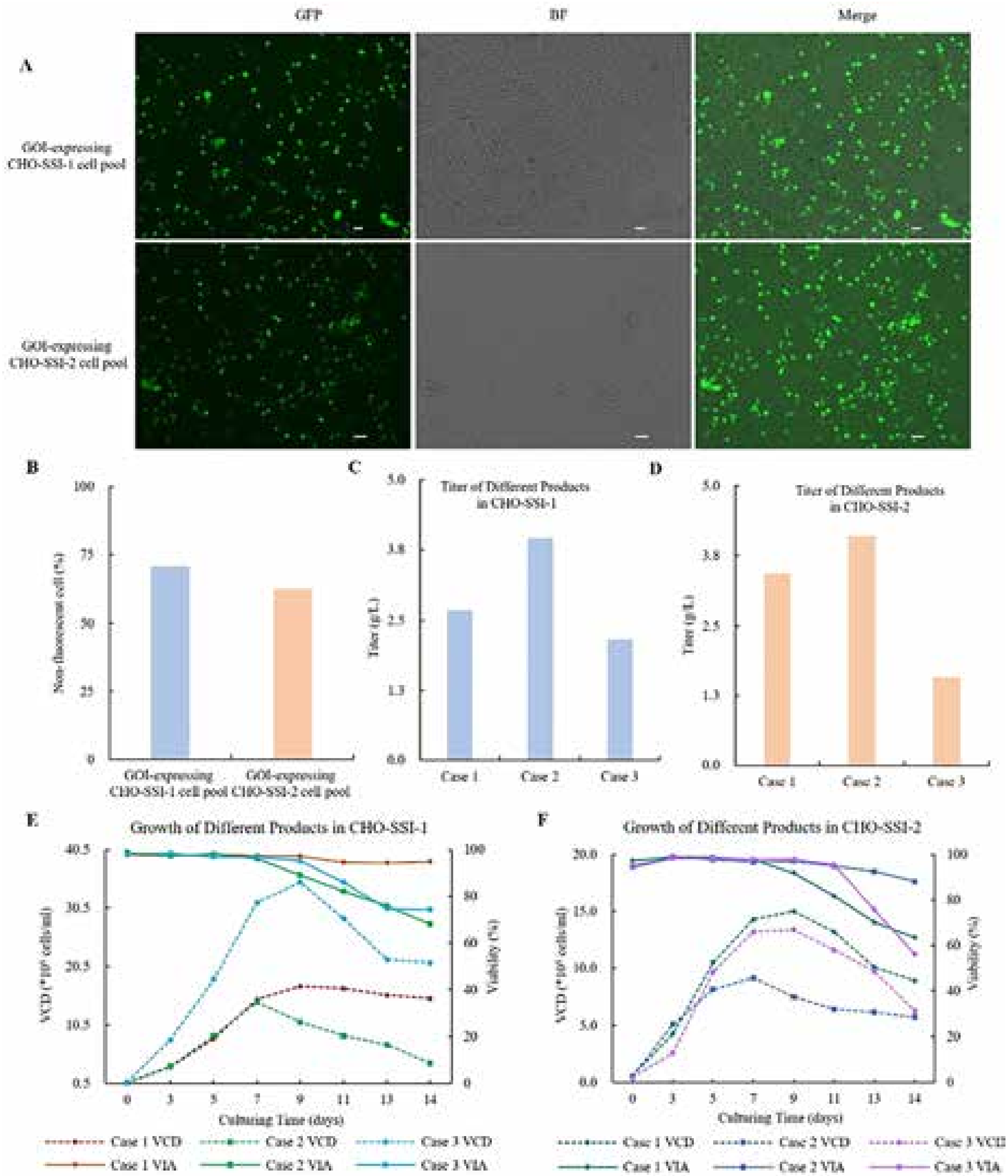

Fig. 2 CHO-SSI host cell lines can be applied to the expression of different proteins. **(A)** Images of minipools after transfecting different GOIs into CHO-SSI host cell lines. The images were on the magnification of 10× with a scale of 50 μm. GFP, Green Fluorescent Protein. BF, Bright Field. **(B)** Proportion of non-fluorescent cells in minipools. **(C-D)** Titer of monoclonal cells from 3 different cases in both CHO-SSI-1 (C) and CHO-SSI-2 (D) at day 14 of fed-batch culture. **(E-F)** Monoclonal cell growth assessment of different cases in CHO-SSI-1 (**E**) and CHO-SSI-2 (**F**) during 14 days of fed-batch culture

Two different monoclonal antibodies (mAbs, case 1 and case 2) and a bispecific antibody (bsAb, case 3) were expressed in CHO-SSI-1 and CHO-SSI-2 respectively. For the two monoclonal antibodies, after 14-day fed-batch culture, the titer came in at 2.7 g/L (case 1) and 4.0 g/L (case 2) in CHO-SSI-1 (Fig. 2C), while the titer in CHO-SSI-2 reached 3.4 g/L and 4.1 g/L, slightly higher than that in CHO-SSI-1 (Fig. 2D). The titer of the bispecific antibody (case 3) in both CHO-SSI-1 and CHO-SSI-2 was comparable, which was 2.2 g/L and 1.6 g/L,

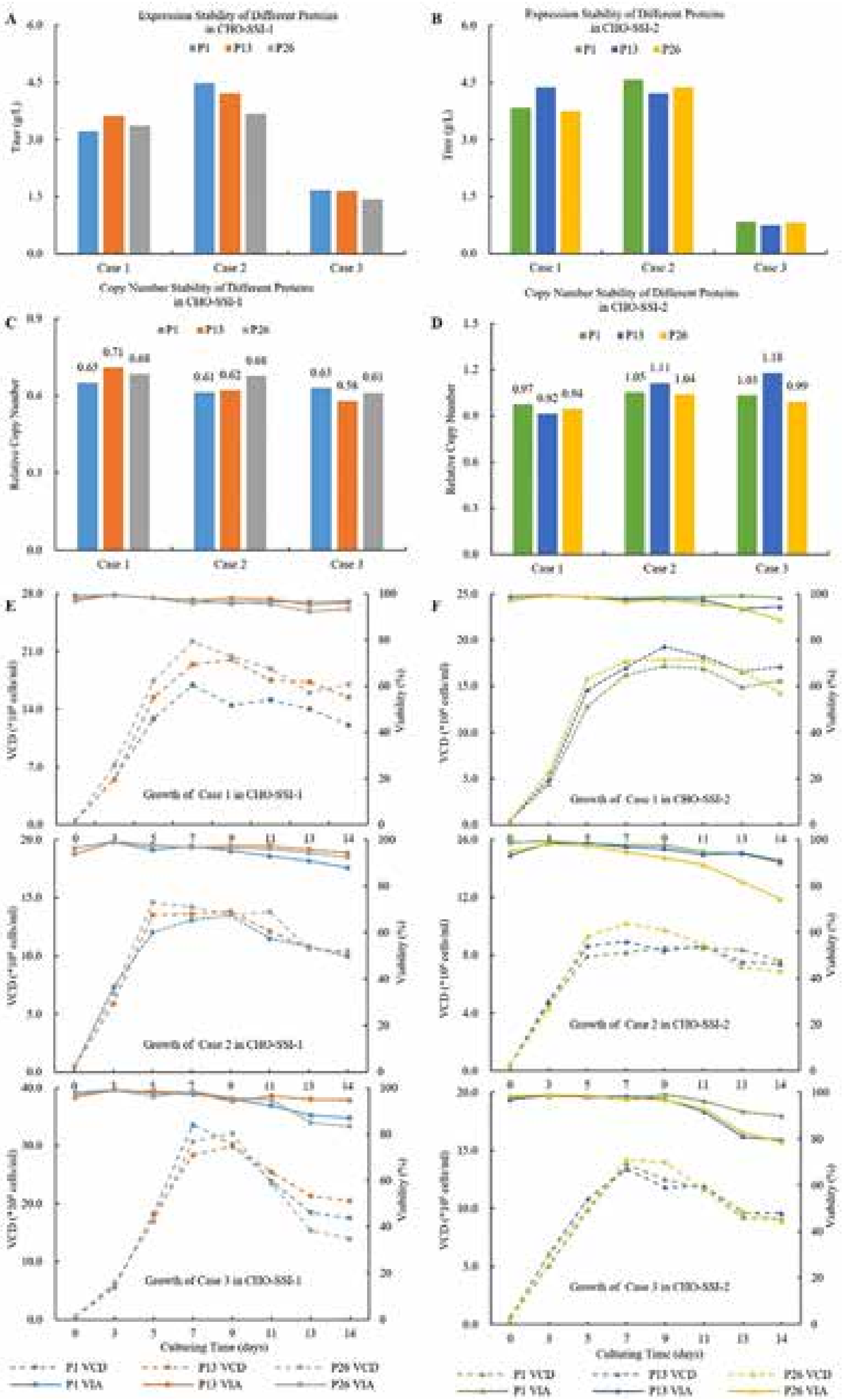

Fig. 3 (See legend on next page.)

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Fig. 3 Stability analysis of recombinant monoclonal cell lines derived from CHO-SSI-1 and CHO-SSI-2. The recombinant monoclonal cell lines were cultured in shake flasks for 90 consecutive days. Following this step, generation of P1, P13 and P26 for different cases were fed-batch cultured for 14 days to evaluate protein titer, copy number and cell growth. **(A-B)** Titer of three cases in CHO-SSI-1 (**A**) and CHO-SSI-2 (**B**) at P1, P13 and P26. **(C-D)** Gene copy number analysis result of stability assessment. Heavy chain copy number of each case in CHO-SSI-1 (**C**) and CHO-SSI-2 (**D**) at generations of P1, P13 and P26 were assessed, B2M was the internal reference. **(E-F)** Cell growth of three cases in CHO-SSI-1 (**E**) and CHO-SSI-2 (**F**) at P1, P13 and P26

respectively (Fig. 2C and D). It was noteworthy that the peak VCD for case 1 and case 2 in CHO-SSI-1 during cell growth assessment was 1.4×10^7 cells/ml and 1.7×10^7 cells/ml, respectively. This was about 2 times lower than the peak VCD of around 3.5×10^7 cells/ml exhibited by case 3 (Fig. 2E); while in CHO-SSI-2, the peak VCD of case 1, case 2 and case 3 was 1.5×10^7 cells/ml, 9.2×10^6 cells/ml, and 1.3×10^7 cells/ml, all lower than that in CHO-SSI-1. These results have proven that cells showed different cell growth profile when integrated with different GOIs.

Monoclonal cells expressing different proteins showed stability

Stability assessment was conducted based on the abovementioned six recombinant cell lines (three different proteins expressed on two CHO-SSI cell lines, respectively). The fed-batch culture data showed that when passaged to either P13 or P26, the titer of all the six recombinant cell lines was similar to that on P1 (Fig. 3A and B), and the titer variance across different passages was within 20%. Looking into fed-batch culture data, one of the monoclonal antibody cases on CHO-SSI-1 saw a titer decrease of up to 18% in P26 (Fig. 3A), and another monoclonal antibody case on CHO-SSI-2 saw a titer decrease of 7% (Fig. 3B). This was also in line with the gene copy number analysis results (Fig. 3C and D). In terms of cell growth, despite the differences in monoclonal cells expressing different products, all products showed consistent growth trend across all the passages in the stability testing (Fig. 3E and F).

Regarding quality assessment, we have adopted iCIEF for charge variation, UPLC for glycan profile, and SEC-HPLC for protein quality. Results showed that across all the passages in the stability testing, the charge variation (Fig. 4A-D), glycan profile (Fig. 4E-H) and quality (Fig. 4I-L) of the proteins of interest indicated high consistency. Based on the abovementioned data, the recombinant cell lines derived from the integration of CHO-SSI host cell lines and different proteins of interest have proven stability in product quality across all the passages in the stability testing.

The predictability and consistency in CHO-SSI host cell lines

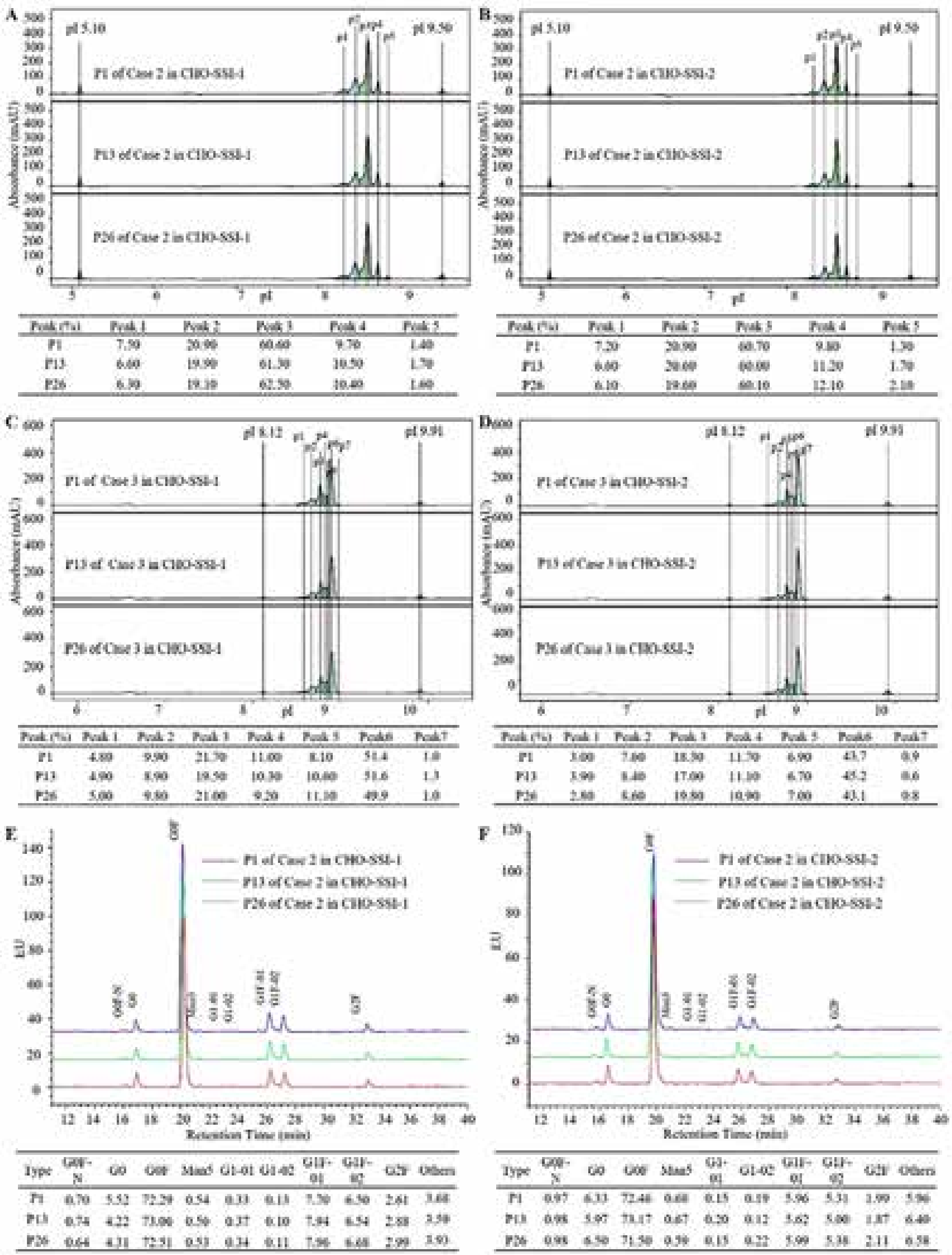
On our CHO-SSI platform, we proceeded with monoclonal cell sorting and fed-batch culture on the selected minipools. It was shown that titer of monoclonal cell can be predicted based on minipool titer to some extent (Fig. 5A). In addition, on CHO-SSI-1, the charge variation (Fig. 5B) and glycan profile (Fig. 5D) between minipools and monoclonal cells showed mild variance, which was also observed on CHO-SSI-2 (Fig. 5C and E). Similarly, the minipools and monoclonal cells showed high similarity in protein purity (Fig. 5F and G). It can be concluded that the minipools and monoclonal cells derived from our CHO-SSI platform have shown consistency in protein quality. Therefore, with our CHO-SSI platform, protein quality assessment can be advanced to the minipool stage, which will significantly accelerate the process of cell line development. On a separate note, it was observed that different monoclonal cells derived from either CHO-SSI-1 or CHO-SSI-2 and expressing the same protein shared approximately the same charge variation (Fig. 6A and B), glycan profile (Fig. 6C and D) and protein purity (Fig. 6E and F).

While cases are less satisfactory as for the protein quality among minipool and monoclones that were integrated randomly (Fig. S2). Herein, we applied the randomly integrated case 2 minipool or monoclones to explore protein quality. As shown in Figure S2, the charge variation (Fig. S2A) and glycan profile (Fig. S2B) between minipool and the top 3 monoclonal cells showed a lack of consistency. Also, a difference of more than 15% was seen in the main peak between minipools and monoclones as for the protein purity (Fig. S2C).

The diversity in random integration leads to unpredictability in protein titer and quality, which requires heavy workload and cost before identifying an ideal product cell line. Therefore, it is important to be able to predict protein titer and quality in the course of cell line screening.

CHO-SSI host cell line demonstrated universality

In addition to in-house testing, the CHO-SSI host cell lines have been applied in the product R&D of multiple pharmaceutical companies, IVD companies, universities, research institutes, among others. The results showed that different types of proteins have exhibited high titer on the CHO-SSI platform, including monoclonal antibodies, bispecific antibodies, difficult-to-express proteins (viruses, antigens, scFv, nanobodies) and non-human antibodies, such as murine, ovine and canine antibodies (Fig. 7). The titer of multiple monoclonal antibodies exceeded 10 g/L (some even hit 15 g/L); several bispecific antibodies presented with a titer of more than 6 g/L (some even exceeded 12 g/L); for difficult-to-express



Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
P1	7.50	20.90	60.60	9.70	1.40
P13	6.60	19.90	61.30	10.50	1.70
P26	6.30	19.10	62.50	10.40	1.60

Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
P1	7.20	20.90	60.70	9.80	1.30
P13	6.60	20.60	60.00	11.20	1.70
P26	6.10	19.60	60.10	12.10	2.10

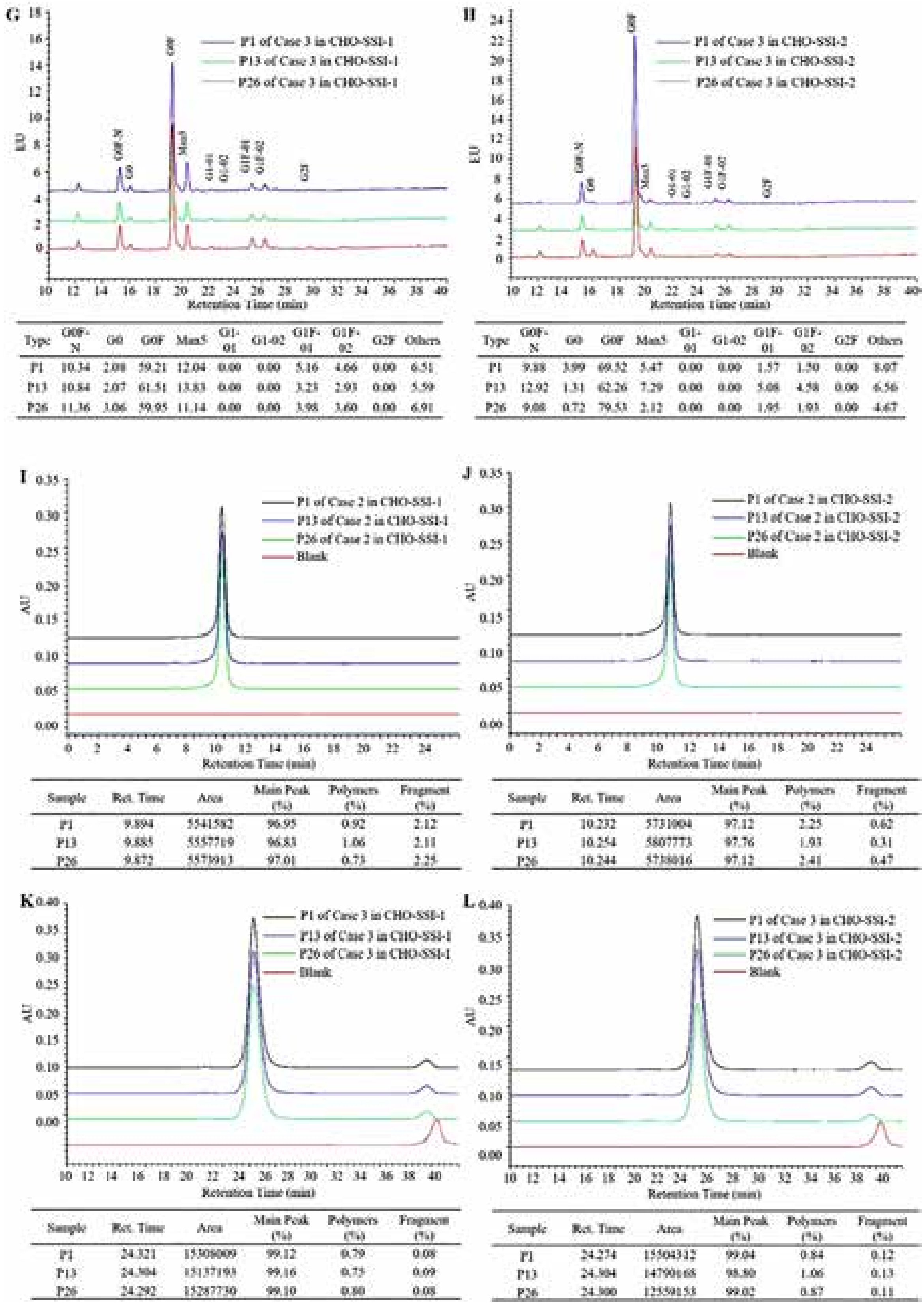
Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak6	Peak7
P1	4.80	9.90	21.70	11.00	8.10	51.4	1.0
P13	4.90	8.90	19.50	10.30	10.60	51.6	1.3
P26	5.00	9.80	21.00	9.20	11.10	49.9	1.0

Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak6	Peak7
P1	3.00	7.00	18.30	11.70	6.90	43.7	0.9
P13	3.90	8.40	17.00	11.10	6.70	45.2	0.6
P26	2.80	6.60	19.80	10.90	7.00	43.1	0.8

Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	0.70	5.52	72.29	0.54	0.33	0.13	7.30	6.50	2.61	3.68
P13	0.74	4.22	73.06	0.50	0.37	0.10	7.84	6.54	2.88	3.50
P26	0.64	4.31	72.51	0.53	0.34	0.11	7.96	6.68	2.99	3.93

Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	0.97	6.33	72.46	0.68	0.15	0.19	5.96	5.31	1.99	5.96
P13	0.98	5.97	73.17	0.67	0.20	0.12	5.62	5.00	1.87	6.40
P26	0.98	6.50	71.50	0.59	0.15	0.22	5.99	5.38	2.11	6.58

Fig. 4 GOI-expressing CHO-SSI-1 and CHO-SSI-2 monoclonal cell lines protein quality stability analysis. **(A-D)** Charge variation of case 2 in CHO-SSI-1 (**A**) and CHO-SSI-2 (**B**), and case 3 in CHO-SSI-1 (**C**) and CHO-SSI-2 (**D**); p refers to peak. **(E-H)** Glycan profile of case 2 in CHO-SSI-1 (**E**) and CHO-SSI-2 (**F**), and case 3 in CHO-SSI-1 (**G**) and CHO-SSI-2 (**H**). **(I-L)** Purity of case 2 in CHO-SSI-1 (**I**) and CHO-SSI-2 (**J**), and case 3 in CHO-SSI-1 (**K**) and CHO-SSI-2 (**L**)



G

Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	10.34	2.08	59.21	12.04	0.00	0.00	5.16	4.66	0.00	6.51
P13	10.84	2.07	61.51	13.83	0.00	0.00	3.23	2.93	0.00	5.59
P26	11.36	3.06	59.95	11.14	0.00	0.00	3.98	3.60	0.00	6.91

H

Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	9.88	3.99	69.52	5.47	0.00	0.00	1.57	1.50	0.00	8.07
P13	12.92	1.31	62.26	7.29	0.00	0.00	5.08	4.58	0.00	6.56
P26	9.08	0.72	79.53	2.12	0.00	0.00	1.95	1.93	0.00	4.67

I

Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
P1	9.894	5541582	96.95	0.92	2.12
P13	9.885	5557719	96.83	1.06	2.11
P26	9.872	5573913	97.01	0.73	2.25

J

Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
P1	10.232	5731004	97.12	2.25	0.62
P13	10.254	5807773	97.76	1.93	0.31
P26	10.244	5738016	97.12	2.41	0.47

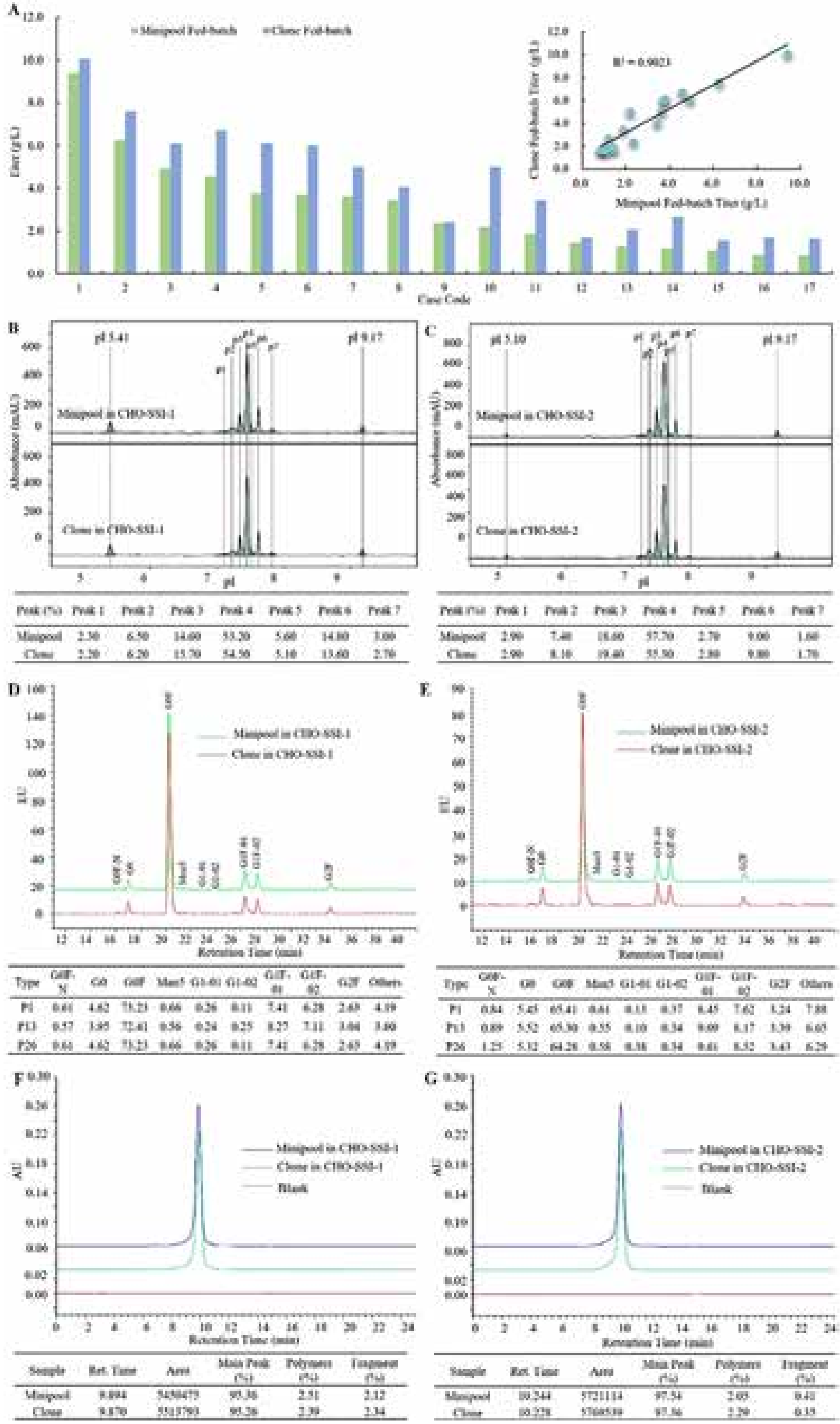
K

Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
P1	24.321	15308009	99.12	0.79	0.08
P13	24.304	15137193	99.16	0.75	0.09
P26	24.292	15287730	99.10	0.80	0.08

L

Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
P1	24.274	15504312	99.04	0.84	0.12
P13	24.304	14790168	98.80	1.06	0.13
P26	24.300	12559153	99.02	0.87	0.11

Fig. 4 (continued)



Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
Minipool	2.30	6.50	14.60	53.20	5.60	14.80	3.00
Clone	2.20	6.20	15.70	54.50	5.10	13.60	2.70

Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
Minipool	2.90	7.40	18.60	57.70	2.70	9.00	1.60
Clone	2.90	8.10	19.40	55.50	2.80	9.80	1.70

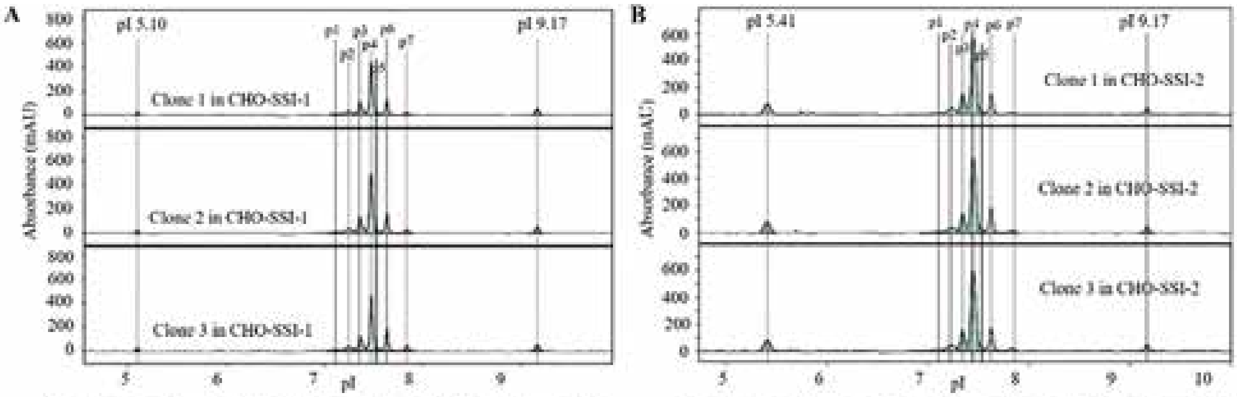
Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	0.61	4.62	73.23	0.66	0.26	0.11	7.41	6.28	2.63	4.19
P13	0.57	3.95	72.41	0.56	0.24	0.25	8.27	7.11	3.04	3.60
P26	0.61	4.62	73.23	0.66	0.26	0.11	7.41	6.28	2.63	4.19

Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	0.84	5.45	65.41	0.61	0.13	0.37	8.45	7.62	3.24	7.88
P13	0.89	5.52	65.30	0.55	0.10	0.34	9.09	8.17	3.39	6.65
P26	1.25	5.32	64.26	0.58	0.58	0.34	8.61	8.52	3.43	6.29

Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
Minipool	9.894	5450475	95.36	2.51	2.12
Clone	9.870	5515793	95.26	2.39	2.34

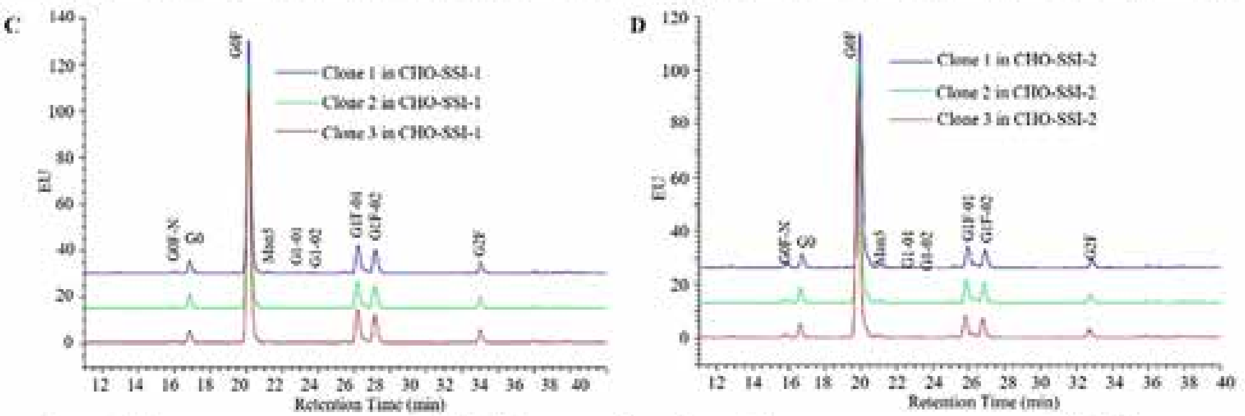
Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
Minipool	10.244	5721114	97.54	2.05	0.41
Clone	10.228	5769539	97.36	2.29	0.35

Fig. 5 Predict monoclonal cell titer and quality between minipool and its monoclonal cells. **(A)** Correlation between minipool titer and monoclonal cell titer in 14-day fed-batch culture. **(B-C)** Charge variation of minipool and monoclonal cell expressing the same product in CHO-SSI-1 (**B**) and CHO-SSI-2 (**C**); p refers to peak. **(D-E)** Glycan profile of minipool and monoclonal cell expressing the same product in CHO-SSI-1 (**D**) and CHO-SSI-2 (**E**). **(F-G)** Purity of minipool and monoclonal cell expressing the same product in CHO-SSI-1 (**F**) and CHO-SSI-2 (**G**)



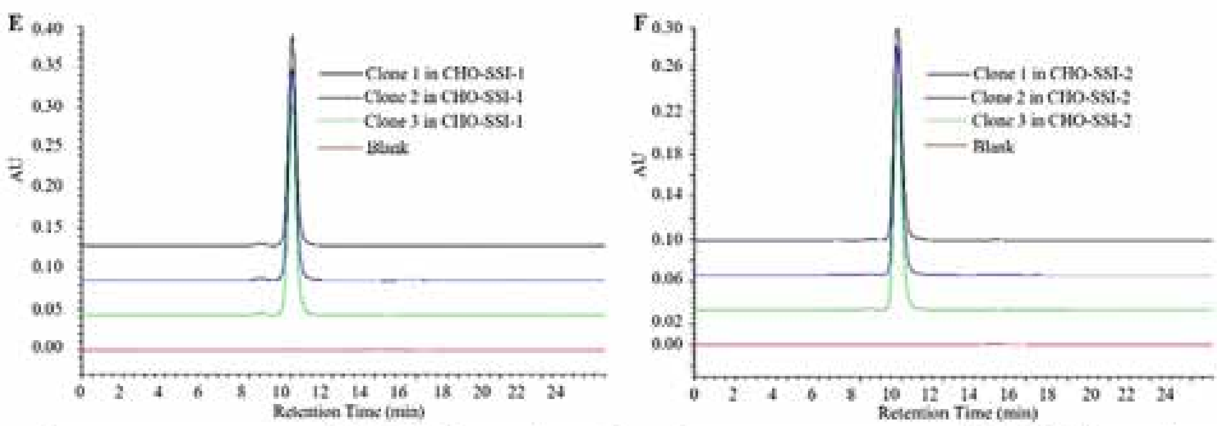
Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
Clone 1	3.10	7.50	18.40	47.70	3.60	15.50	4.30
Clone 2	3.20	8.00	17.00	47.40	4.80	15.50	4.10
Clone 3	3.30	7.50	17.10	44.20	3.60	18.30	6.00

Peak (%)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
Clone 1	2.70	8.20	16.10	53.80	5.40	11.60	2.20
Clone 2	2.20	7.20	15.30	52.90	5.30	14.40	2.80
Clone 3	2.30	7.20	15.40	54.80	5.20	12.80	2.20



Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	0.61	4.62	73.23	0.66	0.26	0.11	7.41	6.28	2.63	4.19
P13	0.57	3.95	72.41	0.56	0.24	0.25	8.27	7.11	3.04	3.60
P26	0.61	4.62	73.23	0.66	0.26	0.11	7.41	6.28	2.63	4.19

Type	G0F-N	G0	G0F	Man5	G1-01	G1-02	G1F-01	G1F-02	G2F	Others
P1	0.84	5.45	65.41	0.61	0.13	0.37	8.45	7.62	3.24	7.88
P13	0.89	5.52	65.30	0.55	0.10	0.34	9.09	8.17	3.39	6.65
P26	1.25	5.32	64.28	0.58	0.38	0.34	9.61	8.52	3.43	6.29



Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
Clone 1	9.801	5423601	96.19	3.36	0.45
Clone 2	9.800	5842813	96.21	3.19	0.60
Clone 3	9.799	5721807	97.08	2.74	0.18

Sample	Ret. Time	Area	Main Peak (%)	Polymers (%)	Fragment (%)
Clone 1	9.877	5137934	95.47	3.26	1.26
Clone 2	9.869	5231292	95.73	2.98	1.30
Clone 3	9.872	5172593	95.24	3.48	1.29

Fig. 6 Quality consistency analysis on monoclonal cells derived from CHO-SSI host cell lines. **(A-B)** Charge variation of different monoclonal cells derived from CHO-SSI-1 (**A**) and CHO-SSI-2 (**B**) and expressing the same product; p refers to peak. **(C-D)** Glycan profile of different monoclonal cells derived from CHO-SSI-1 (**C**) and CHO-SSI-2 (**D**) and expressing the same product. **(E-F)** Purity of different monoclonal cells derived from CHO-SSI-1 (**E**) and CHO-SSI-2 (**F**) and expressing the same product

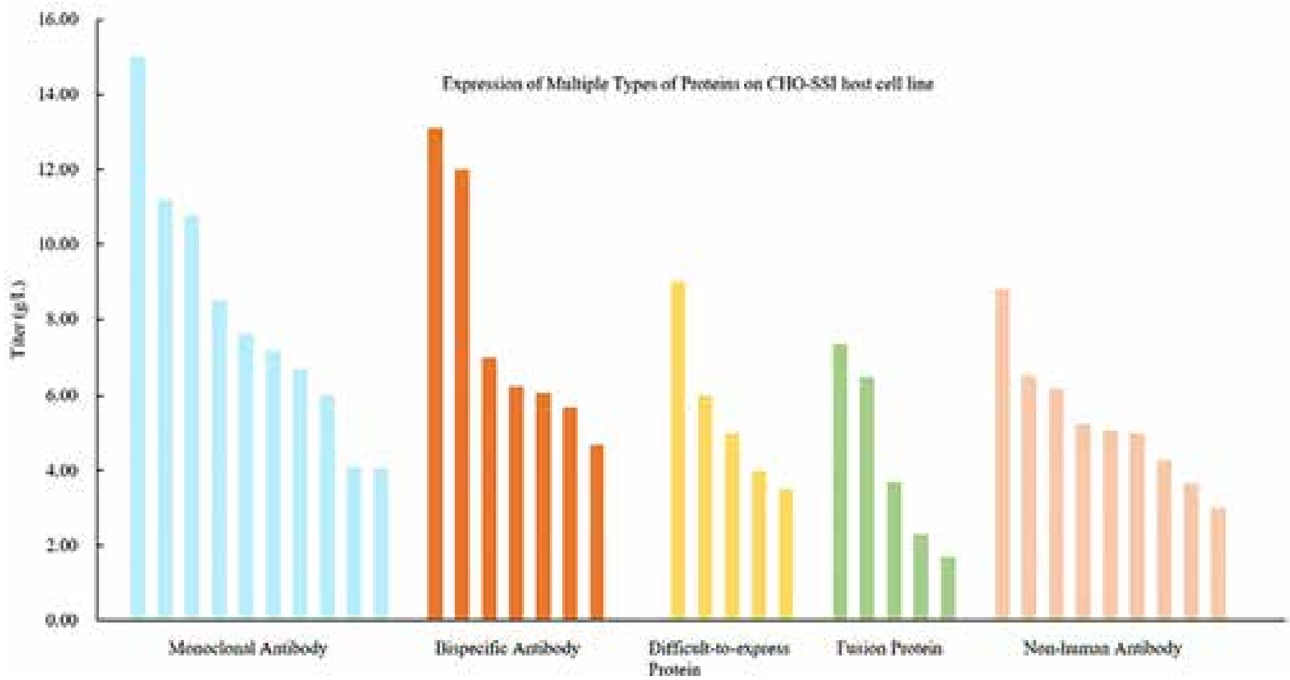


Fig. 7 Expression of multiple types of protein on CHO-SSI host cell line. Different types of protein were expressed on the CHO-SSI platform, including IgG1 monoclonal antibodies, IgG4 monoclonal antibodies, symmetric and asymmetric bispecific antibodies, difficult-to-express proteins (including viral and parasitic antigens, scFv, and nanobodies), fusion proteins, and non-human antibodies (including murine, ovine, and canine antibodies)

proteins, fusion proteins and non-human antibodies, there were also cases with titer over 5 g/L.

Discussion

CHO cell line is the most popular host cell line for protein-based drug production. Different from *E. coli* or yeast that allows stable expression of genes in the plasmids, the stable expression of exogenous protein in CHO cells can only be achieved through integrating the GOI into the CHO cell genome. Random integration is a widely-adopted cell line development method, through which the GOI is transfected into CHO cells for large-scale, product titer and quality-oriented screening. However, the inherent deficiency of this technology – low recombination efficiency and random insertion on CHO cell genome – may lead to numerous repetitive works. The transposon technology is also applied in CHO cell line development, which has significantly improved the recombination efficiency of GOI on the genome but yet to ensure stable cell growth state. The integrase-mediated SSI was put forward in recent years. However, there are very few successful cases so far, as CHO cell genome safe harbors are difficult to be identified.

Genomic safe harbors for GOI expression should have high genetic activity and the ability to accommodate foreign DNA with long-term stability. Identifying safe harbors is thus one of the major pain points in SSI technology platform development. To identify suitable sites for SSI, we have first developed AI algorithms with over one billion cell images and biochemical data, including titer and genetic stability. Then, we combined AI with robotic system to screen and select monoclonal CHO host cell lines with genomic safe harbors labelled with EGFP landing cassettes using a high-throughput platform with a screening capacity of 150,000 cells/batch. The candidates, all monoclonal CHO-SSI host cells, were further validated through the expression of monoclonal antibodies, bispecific antibodies and fusion proteins. CHO-SSI-1 and CHO-SSI-2 were tagged as safe harbor 1 (sh1) and 2 (sh2) on the genome, with high genetic stability and protein titer.

CHO-SSI-1 host cell line is single site single copy, and the site is located on chromosome 3 (NC\U 048596:34375010) between spliceosome RNA U6 and probable global transcription activator SNF2L2. The gap is about 209,314 bp in length without known genes, so integration of foreign genes at this site would not disrupt the expression of other genes. Likewise, CHO-SSI-2 host cell line carries only one copy located on chromosome 2 (NC\U 048595:28250698), and the site is located in the ninth intron of Mtf1 gene (metal regulatory transcription factor 1), which would not affect its transcription and splicing. The EGFP-expressing CHO-SSI-1 and CHO-SSI-2 host cell lines were presented with stable fluorescence intensity (Fig. 1D). The EGFP genetic stability on CHO-SSI-1 and CHO-SSI-2 genome after 90 days of passaging was monitored through real-time PCR. The results showed that the gene loss rate was less than

15% over 90 days of passaging compared to P1 (Fig. 1F), indicating the EGFP-labelled sites in CHO-SSI-1 and CHO-SSI-2 were with high genetic stability. After replacing the EGFP genes in CHO-SSI-1 and CHO-SSI-2 with GOI, the titer of monoclonal antibodies was greater than 6 g/L in most cases, and some even hit a high 15 g/L. In fed-batch culture, the doubling time of CHO-SSI-1 and CHO-SSI-2 were about 20 h, similar to wild-type CHO-K1 (about 22 h), indicating the two integration sites have not made significant impacts on CHO cell phenotype and physiology.

Different from random integration that features diversity in charge variation and glycosylation, on our CHO-SSI platform, the monoclonal cells expressing the same GOI saw mild variance in antibody charge variation and glycosylation (Fig. 6A-D). Results from cell stability assessment showed that both the protein titer variance and GOI loss were less than 20% when comparing P1, P13 and P26. All data have proved that the cell lines developed through SSI exhibited high consistency and stability.

The data of all cases has indicated that the product titer and antibody quality of CHO-SSI-1 and CHO-SSI-2, which were empowered by SSI, can be predicted ahead of schedule, which is worthy to note. The minipool titer was close to monoclonal cell fed-batch titer (Fig. 5A), while the charge variation and glycosylation modifications (Fig. 5B-E) of minipools and monoclonal cells also showed high similarity. It can thus be inferred that the final monoclonal cell performance can be predicted based on minipool results with high accuracy.

Random integration leads to a high degree of diversification in cell physiology, which requires extensive screening and validation to obtain a stable monoclonal cell. In SSI, the host cell hot spot is first marked with EGFP, then the EGFP is replaced by GOI. As the replacement will result in the absence of fluorescence in cell lines, the cell line screening workload will be significantly reduced, cell lines with GOI will be easier to be identified, and the cell line development process will be accelerated. Furthermore, with various monoclonal cell sorting instruments (FACS, Namocell, VIPS, Cytena, etc.), the CHO-SSI platform is applicable to a variety of cell line development processes (minipool, bulk pool or other faster screening processes), making it highly universal. With the CHO-SSI platform, the screening workload is reduced, high consistency in minipool and monoclonal cell culture performance is observed, and monoclonal cell performance can be predicted based on minipool performance. In SSI, minipool functions uniquely as a tool to investigate into monoclonal cell quality attributes ahead of schedule. Unlike natural monoclonal antibody, bispecific antibody is lab-created. Meanwhile, the folding of tertiary and quaternary structures of bispecific antibody is also different from natural antibody. SSI enables to design different construction models of light chains and heavy chains on the marked hot spots on the genome, which can increase bispecific antibody titer and reduce miss-pairing rate. Different plasmid constructions have been studied on the CHO-SSI platform to express bispecific antibodies, and the titer of monoclonal cell exceeded 10 g/L (Fig. 7). In addition, we also saw high titer on the CHO-SSI cell lines in the expression of other types of therapeutic antibodies and non-human antibodies for in vitro diagnostics, as illustrated in Fig. 7.

Conclusion

In traditional development of biopharmaceuticals, uncertainty lies in the cell line development through random integration, leading to highly labor-intensive screening and validation. In contrast, the cell line development by SSI has significantly reduced labor input. On the CHO-SSI platform, only a small number of approximately 10 minipools require enrichment in early stages. Only one to two 96-well plates are needed in the clone stage (containing 60 to 120 monoclonal cells), followed by the selection of 24 monoclonal cells in the 6-well plate stage for amplification. From this stage, 5 to 10 monoclonal cells are further selected for fed-batch assessment to identify monoclonal cells with high and stable titer and consistent quality. Meanwhile, with our CHO-SSI platform, process development, quality characterization and API studies etc. can be conducted at minipool stage. The advantages of the CHO-SSI platform are clear: it can significantly reduce labor and material input, shorten development timeline and cut costs by at least 50%.

Abbreviations

CHO	Chinese hamster ovary
SSI	Site-specific integration
EGFP	Enhanced green fluorescent protein
GOI	Gene of interest
RMCE	Recombinase-mediated cassette exchange
MCS	Multiple cloning site
VCD	Viable cell density
mAbs	Monoclonal antibodies
bsAb	Bispecific antibody

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13036-025-00610-z.

Supplementary Material 1

Author contributions

Wanjun Lan, Xiaoli Yang, Juanjuan Yao and Lung JR Lin designed the outline and drafted, edited, revised the manuscript. Xiaoli Yang and Juanjuan Yao generated the figures. Wanjun Lan and Xiaoli Yang performed most of the experiments. Juanjuan Yao detected the gene copy number. Wenzheng Li and Weihai Chen participated in the AI-assisted cell selection. Qi Shen conducted the protein purification experiments. Jiangping Li, Kang Li and Huiling Li performed the protein SEC, glycan profiling and iCIEF experiments and analyzed experiment data. Yujia Chen revised the manuscript. Liang Chen and Kingsley Leung contributed to conceptualization and revised the manuscript. Both authors reviewed and approved the final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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