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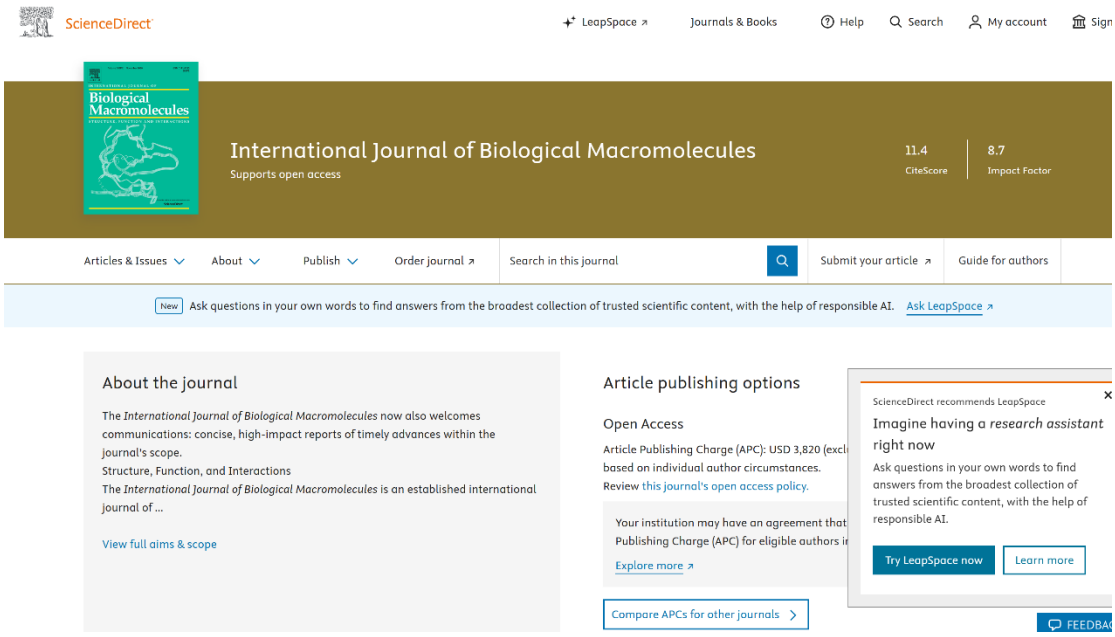

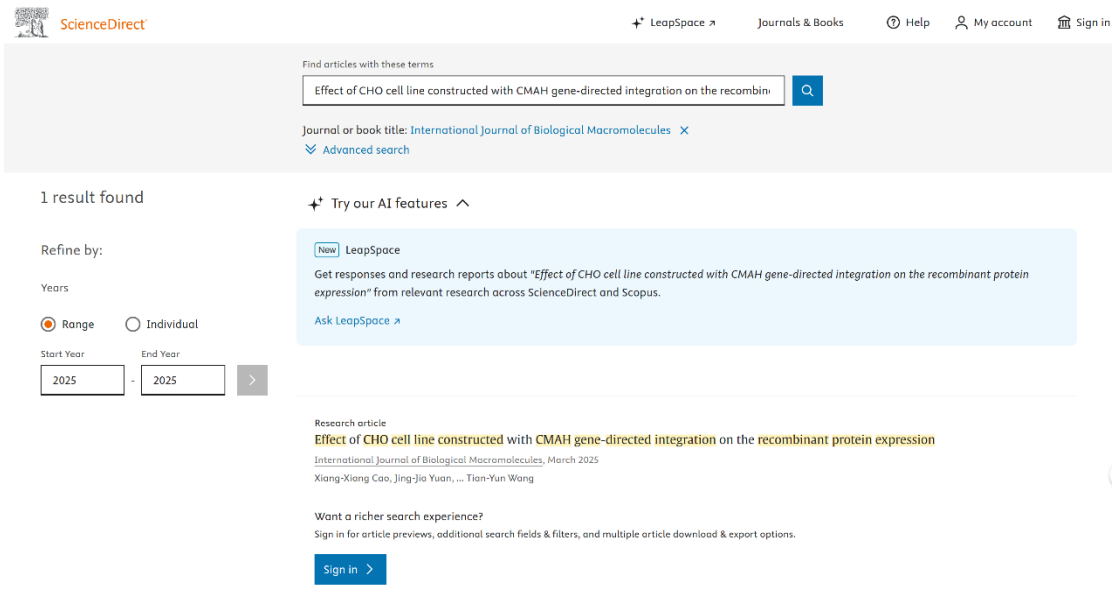

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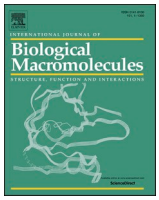



Effect of CHO cell line constructed with CMAH gene-directed integration on the recombinant protein expression

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ABSTRACT

Chinese hamster ovary (CHO) cells are the most widely used platform for recombinant therapeutic protein (RTP) production. Traditionally, the development of CHO cell lines has mainly depended on random integration of transgenes into the genome, which is not conducive to stable long-term expression. Cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH) is expressed in CHO cells and produces *N*-hydroxyacetylneuraminic acid, which may cause a human immune response. However, the effects of transgene integration at the CMAH site on RTP expression in CHO cells remain unclear. In this study, we selected *CMAH* gene, which is lacking in humans, as the target site to construct recombinant CHO cell line using the CRISPR/Cas9 technique. Erythropoietin (*EPO*) and *EGFP* integration at the CMAH site resulted in more stable expression levels and lower heterogeneity than random integration. In addition, the proportion of N-glycosylation levels in the EPO glycoside of CMAH integration site also changed. In conclusion, CMAH site integration improved the stability of RTP expression in CHO cells.

1. Introduction

Chinese hamster ovary (CHO) cells are the predominant platform used in the biopharmaceutical industry, and almost 70 % of approved recombinant therapeutic proteins (RTPs) are produced by CHO cells [1–3]. Despite the widespread use of these cells, the long-term expression of transgenes is unstable, and post-translational modifications (PTMs) of proteins, such as glycosylation, are not completely consistent with human native forms [4,5]. Therefore, effective strategies for improving protein expression, stability and glycosylation should be explored. Host cell proteins (HCPs) may induce immune responses to products [6] and affect the stability of RTP expression [7]. Notably, human cells lack the cytidine monophosphate N-acetyineuraminic acid hydroxylase (*CMAH*) gene, and *N*-hydroxyacetylneuraminic acid (Neu5Gc) produced by CMAH in CHO cells may also cause an immune response and affect the activity of pharmaceuticals [8,9]. However, the effect of the site-specific integration of transgenes at the CMAH location in the CHO cell genome on the expression of RTPs is unclear. In recent years, gene editing technology has been widely used in cell line engineering to regulate glycosylation, increase productivity [10], eliminate problematic HCPs [11], and facilitate site-specific transgenic integration [12,13], opening up new avenues for engineering CHO cell lines and improving transgenic expression stability.

Traditionally, CHO cell line development techniques are based on the random integration of genes of interest (GOIs) and selected markers into the genome [14–16]. This method requires considerable time and cannot accurately control the integration site, resulting in a position effect. Moreover, the expression levels of GOIs decrease over generations [17–19]. In addition, screening for stable, high-yield cell line clones is time-consuming and laborious [20,21]. Compared to the random integration, site-specific integration of targeted genes can effectively avoid the abovementioned problems [22,23]. There are two approaches to site-specific integration, one of which is mediated by site-specific recombinases, such as Flp-FRT, Bxb1, Cre-loxP, and phiC31/R4 [24–27]. However, this approach has limitations, as requires the establishment of cell lines for targeted gene integration and is suitable for a limited number of specific genomic regions [19,28]. Another

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Abbreviations

CHO	Chinese hamster ovary
RTP	recombinant therapeutic protein
GOI	gene of interest
CMAH	cytidine monophosphate N-acetyineuraminic acid hydroxylase
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/Cas9 nuclease
EPO	erythropoietin
PTM	post-translational modification
HCP	host cell protein
TALEN	transcriptional activator-like nuclease
ZFN	zinc finger nuclease
sgRNA	single guide RNA
BSD	blasticidin
RT-qPCR	real-time polymerase chain reaction
MFI	median fluorescence intensity
PVDF	polyvinylidene fluoride
UPLC	ultra-performance liquid chromatography
HDR	homologous-directed repair

method mainly depends on engineered nucleases, including transcriptional activator-like nucleases (TALENs), zinc finger nucleases (ZFNs), and the CRISPR/Cas9 system [29–32]. These nucleases cause double-strand DNA breaks at target sites in the genome to activate intracellular DNA repair mechanisms and repair GOIs with homologous recombination reparation at these sites [11].

The CRISPR/Cas9 technique improves the specificity of the Cas9 nuclease using a single guide RNA (sgRNA) that fuses CRISPR RNAs (crRNAs) with trans-activated crRNA (tracrRNA) to guide Cas9 protein to recognize target sites [11]. In contrast to TALEN and ZFN-based technologies, CRISPR/Cas9 technology is based on simple base pairing between an engineered sgRNA and the target genome positions, which results in higher targeting efficiency and a lower cost. This also avoids intricate protein design and assembly [33–35]. CRISPR/Cas9 has been successfully used for target gene insertion in various cell lines for mechanistic analysis and industrial production [36,37], including in CHO cell line construction, to enhance transgenic expression and long-term stability [13].

Our laboratory has been committed to screening for efficient expression vector elements [15,38–41], optimizing serum-free cell medium [42–45], and constructing cell lines using CRISPR/Cas9 technology [12,13], to enhance transgenic expression and long-term expression stability. In this study, we constructed a CRISPR/Cas9-mediated site-specific integration at the *CMAH* locus in the CHO cell line, and investigated the effect of *CMAH*-gene-directed integration on recombinant protein expression stability and glycosylation.

2. Materials and methods

2.1. Cell culture and transfection

CHO cells (Life Technologies, Carlsbad, CA, USA; #A11557-01) were cultured in a constant temperature incubator at 37 °C with 5 % CO_2 using DMEM/F12 medium containing 10 % fetal bovine serum (WISENT, Nanjing, China) and 1 % penicillin and streptomycin (ProteinEasy, Xinxiang, China). The cells were seeded in 24-well plates overnight at a density of 1.5×10^5 cells/well. When cell confluence reached 75 % the following day, transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), as previously reported [46]. Briefly, the total amount of transfected plasmid was 1 μg per well, and the transfection ratio for the donor vector and the Cas9 vector was 1:1.5. After transfection for 48 h, the cells were screened and cultured in medium supplemented with 15 μg/mL blasticidin (BSD; Sangon Biotech, Shanghai, China) for approximately 1 week, after which, 10 μg/mL puromycin (Beyotime, Shanghai, China) was added and the cells were incubated for a further 2 weeks. After 3 weeks of pressure screening, monoclonal cells wereobtained using the limited dilution method, and samples with only one cell per well were considered monoclonal. The monoclonal cells were subsequently cultured in a serum-free CD medium suspension (ProteinEasy, Xinxiang, China). Only clones positive for both 5′/3′ junction polymerase chain reaction (PCR) and Western blot tests were selected for further study. Cell growth and viability were monitored using a cell counter (Countstar, Shanghai, China).

2.2. gRNA design and screening

The CHO cell genome database in NCBI was used to search the sequence of the *CMAH* locus (Gene ID: 100689391). Site-specific 5′ and 3′ homologous arms were constructed on both sides of the GOIs (EGFP, EPO). The online software zIFIT sgRNA (http://zifit.partners.Org/ZiFiT/Introduction.aspx) was used to design two pairs of CMAH sgRNAs (Table 1) based on the encoding frame 8 of the *CMAH* gene sequence. The sgRNA primers were as follows; sense, 5′-AAACACCG-gRNAsense-3′, and antisense, 5′-CTCTAAAAC-gRNAanti-sense-3′. The primers were then annealed to obtain double-stranded sgRNAs. Subsequent experiments were carried out and the sgRNAs were cloned into the CRISPR/Cas9 system containing red fluorescent markers (Beijing Weishang Lide Company, Beijing, China; #vk001-04). The Cas9 expression vector (pCAG-T7-Cas9+gRNA-pGK-Puro-T2A-mCherry) contained Cas9 elements activated by the CAG promoter, the purinomycin-resistance gene and mCherry markers were linked by the 2A peptide, and sgRNA clone sites activated by the *U6* promoter. The sticky ends TTTGTGGC and GTTTAGAG were designed at the 5′ and 3′ end of the U6 promoter, respectively. These recombinant vectors that simultaneously expressed the sgRNA and the Cas9 protein were amplified in *Escherichia coli* DH5α cells and finally purified for cell transfection using an EndoFree Maxi Plasmid Kit (ProteinEasy, Xinxiang, China). The extracted sgRNAl and sgRNA2 plasmids were transfected into CHO cells and fluorescence was detected after 24 h of transfection. sgRNA1 had a high transfection efficiency and was, therefore, selected for subsequent experiments.

2.3. Junction PCR primers designing

PCR primers spanning the 5′ and 3′ homologous arms were designed as shown in Table 2, and were amplified using junction PCR. The PCR products were detected using agarose gel electrophoresis and verified by sequencing.

2.4. Vector construction

Vector construction was performed as previously reported [47], and the MAR expression vector was used as the vector skeleton. *EGFP* and *EPO* genes were cloned into a vector, containing BSD-resistance genes and various regulatory elements to constitute the donor vector (Fig. 1A). sgRNA1 with a high cutting efficiency at the CMAH site was selected, and cloned into the Cas9 vector to construct the CRISPR/Cas9 system (Fig. 1B).

Table 1
The sequence of sgRNAs.

sgRNAs	Sequence (5′-3′)
sgRNA1-F	AAACACCGgtggattgcaccagacccaa
sgRNA1-R	CTCTAAAACttgggtctggtgcaatccac
sgRNA2-F	AAACACCGgcaccagacccaatgggggga
sgRNA2-R	CTCTAAAACtcccccattgggtctggtgc

Table 2
Junction PCR primers design.

Name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Production size
5′ junction PCR primers	AAGAGAAGACAGTATACTATGC	CCACCTCAGTGATGACGAGA	905 bp
3′ junction PCR primers	CTGTTGAAGCTCTTAAGCC	GAGACAATGGATACAAATGC	987 bp

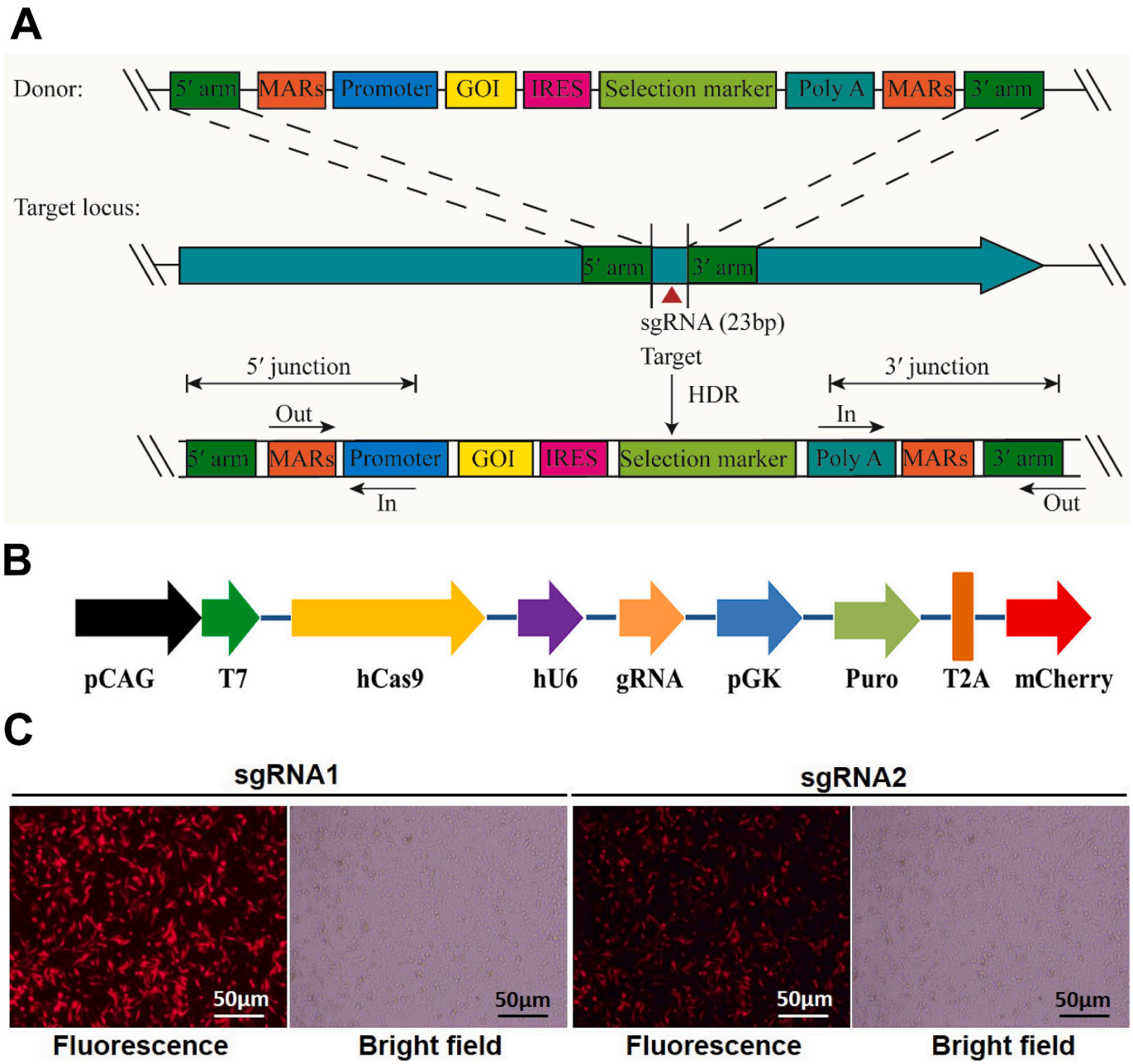

Fig. 1. Donor vector and CRISPR/Cas9 system model. (A) Schematic of site-specific integration. The donor vector consisted of 5′- and 3′-homologous arms, the MAR sequence, a promoter, GOIs, internal ribosome entry site (IRES), a selection marker and a poly A element. The specific site (red triangle) of CMAH was cleaved by Cas9, resulting in double-bond disconnection, and HDR-mediated directed reparation, which enabled the directed integration of the expression frame into the target site. (B) The CRISPR/Cas9 system. Nuclease Cas9, sgRNA, and various components were inserted into the pCAG skeleton to construct the CRISPR/Cas9 system. (C) Fluorescence intensity and the corresponding bright-field images after instantaneous transfection with Cas9-sgRNA for 24 h.

2.5. Genomic DNA extraction and 5′/3′ junction PCR

The 5′/3′ junction PCR primers were designed based on the sequence of the *CMAH* site. The 5′ junction PCR forward primer 5′-AAGAGAAGACAGTATACTATGC-3′ and reverse primer 5′-CCACCTCAGTGATGACGAGA-3′; and 3′ junction PCR forward primer 5′-CTGTTGAAGCTCTTAAGCC-3′ and reverse primer 5′-GAGACAATGGATACAAATGC-3′. Stable CHO cell pools and monoclonal cells were used to extract genomic DNA using a commercially available extraction kit (Biyuntian, Shanghai, China), and the extracted DNA was used as the amplification template. The PCR conditions were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and 5

min for 72 °C. The PCR products were detected using agarose gel electrophoresis and verified using sequencing.

2.6. Real-time polymerase chain reaction

Total RNA was extracted from cell lines that were transfected with different vectors, and reverse transcription was performed using a cDNA synthesis kit (Nearshore Protein Technology Co., Ltd.) according to the manufacturer's instructions. mRNA was measured by real-time polymerase chain reaction (RT-PCR) using the primers listed in Table 3 and a qPCR kit (Vazyme, Nanjing, China) [48,49]. The following PCR cycling conditions were employed: 95 °C for 2 min, followed by 40 cycles at 95 °C for 30 s and 55 °C for 30 s. After a further 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C, the PCR amplification products were subjected to melting curve analysis. All experiments were performed at least three times, and *GAPDH* was used as an internal control.

2.7. Protein expression and stability assay

The median fluorescence intensity (MFI) of the cell lines stably expressing EGFP was observed with a confocal microscopy [46], and protein expression stability was analyzed using a Guava EasyCyte™ 8HT flow cytometer and Flow Jo software (version 7.6; Tree Star, Ashland, OR, USA). Transiently and stably EPO-expressing CHO cells were suspended for 8 days, and an equal volume of supernatant was collected for sample preparation. Western blotting was also performed to identify the EGFP protein levels of cell lines with random integration (EGFP-21, EGFP-31) and site-specific integration (EGFP-3, EGFP-23). Briefly, the cells were collected by centrifugation, and protein extraction buffer (Beyotime, Shanghai, China, #P0013C) containing a protease inhibitor cocktail was used to lyse the cells. The lysates were then shaken for 1.5 h at 4 °C in a rotary, and then centrifuged for 20 min at 4 °C with 12,000*g*. The proteins were quantified using BCA kit (Beyotime, Shanghai, China, #P0010S) and equal amounts of proteins (15 μg for EGFP, 25 μg for EPO and 5 μg for GAPDH) were separated with 10 % SDS-PAGE gels and then proteins were transferred onto polyvinylidene fluoride (PVDF) membranes via electroblotting and then blocked by 5 % milk without fat [50,51]. The membranes were incubated overnight at 4 °C with a 1:2000 dilution of His-tagged antibody (UW, Beijing, China), EGFP (Thermo Fisher Scientific, Waltham, MA, USA, #MA1-952), GAPDH (CST, Danvers, MA, USA, #97166) respectively and a 1:5000 dilution of goat anti-mouse antibody for 1 h at room temperature (Affinity, Jiangsu, China). Images of the protein bands were captured using a chemiluminescence imaging system equipped with a charge-coupled device camera (Tanon, Shanghai, China).

2.8. Protein glycosylation

The samples (1 mg/mL) were centrifuged and replaced by replacement buffer. PNGaseF enzyme was then added and the samples were incubated at 37 °C for 17 h. For glycoside recovery and labeling, samples before and after sugar cutting were placed in glacial acetic acid to recover the supernatant. The concentrated supernatant was labeled with 2-AB and incubated at 37 °C for 17 h. After labeling, the supernatant was separated using hydrophilic chromatography. Ultra-performance liquid chromatography (UPLC) was used to detect glycotype differences, and the labeled supernatant was separated using hydrophilic chromatography. The percentage of N-glycosides detected in the sample was calculated using the area normalization method using an ACQUITY UPL®BER Amide 1.7 μm 2.1 × 100 mm UPLC column with the following formula:

$$\%\text{Peak Area X} = \text{Area of Peak X}/\text{Total Peak Area} \times 100\%$$

Table 3
The primers of qRT-PCR.

Primers name	Sequences (5′-3′)
CMAH-F	TGGTCATTCAAGCTGGAGGGTTTG
CMAH-R	TCTGGTGCAATCCACTGTGTTGAG
EGFP-F	TTCAAGGACGACGGCAACTACAAG
EGFP-R	CCTTCAGCTCGATGCGGTTCAC
EPO-F	GCCTGACCACCCTGCTGAGG
EPO-R	TCCTGAAGGTATCGGCGGTGATG
GAPDH-F	CAAAGGCACAGTCAAGGCTGA
GAPDH-R	TGGTGAAGACGCCAGTAGATT

2.9. Off-target prediction

Four bases of the first 20 bp and one of the first 13 bp after NGG were set as mismatched bases. The CCTOP CRISPR/Cas9 online prediction system (http://crispr.cos.uni-heidelberg.de) was used to predict the potential missing sites in the sgRNA designed in the experiment. Primers were designed according to potential off-target sites, and PCR was performed. The amplification products were sequenced to verify the occurrence of off-target events. We used CRISPR/Cas9 technology to edit genes in CHO cells, which did not involve ethical issues or human gene therapy. Therefore, this experiment was only required to predict potential off-target sites without the need for expensive whole-genome sequencing. A specific analysis of the potential off-target sites is shown in Table 4. There were many potential off-target sites, and the site (LOC100751614) was randomly selected for this experiment, and two cloned strains expressing EGFP were randomly selected for verification.

2.10. Statistical analysis

All data are reported as means ± standard deviation. Statistically significant differences were determined using least significant difference multiple comparison test of one-way analysis of variance using SPSS 17.0 GraphPad Prism 5 (GraphPad Software Inc., San Diego, Ca, USA). $p < 0.05$ was considered statistically significant, whereas $p < 0.01$ indicated an extremely significant difference for all data.

3. Results

3.1. Development of homologous-directed repair-mediated site-specific integration in CHO cells

We developed a targeted integration system in CHO cell lines mediated by CRISPR and homologous-directed repair (HDR) to achieve controlled and accurate transgenic integration. The MAR expression vector constructed in our laboratory was used as the backbone [46,47] and EGFP and EPO were incorporated into the vector. The vectors contained BSD-resistance genes and various regulatory elements that constituted the two donor vectors (Fig. 1A). As linear plasmids are more favorable for HDR than cyclic plasmids [52], we constructed donor plasmids with 5′- and 3′-homologous arms and linearized the insert fragments to improve the efficiency of HDR-mediated target integration. The human genome lacks the *CMAH* gene, and coding region 8 of *CMAH* gene sequence was selected as the editing and insertion site; two pairs of sgRNAs were designed using online software to construct the vectors (Fig. 1B). The two sgRNA vectors were transfected into CHO cells under the same conditions, and the fluorescence measurements were performed after 24 h (Fig. 1C). Based on its higher transfection efficiency, sgRNA1 was selected for subsequent experiments.

3.2. Precise integration of transgenes at the CMAH locus in CHO cells

CHO cells were co-transfected with codon-optimized Cas9, highly efficient sgRNA1, and donor vectors. The co-transfected cell pools (EGFP and EPO) were screened using BSD and purinomycin for 3 weeks, and then 5′/3′ junction PCR was performed on the two stable cell pools to confirm specific integration at the CMAH site (Fig. 2A). Sequencing of

Table 4
Potential off-target sites and information.

Coordinates	target_seq	PAM	Gene name	Gene id
NW_003613881.1:1101541-1101563	GTGGATTG[CACCAGACCCAA]	TGG	CMAH	100689391
NW_003614901.1:183482-183504	G**AT**G**C**TA**G**[CACCAGACCCAA]	AGG	Casz1	100770088
NW_003614901.1:183647-183669	G**AT**G**C**TA**G**[CACCAGACCCAA]	AGG	Casz1	100770088
NW_003613591.1:4029804-4029826	**A**TGA**A**TTG[**CT**CCAGACCCAA]	TGG	Ropn1	100757652
NW_003614179.1:185510-185532	GT**CT**ATA**A**[CACCAGACCCAA]	AGG	NA	NA
NW_003615214.1:55431-55453	**A**TGGA**AG**G[**A**ACCAGACCCAA]	AGG	NA	NA
NW_003616050.1:147882-147904	G**AC**G**C**TTG[CA**G**CAGACCCAA]	GGG	LOC100751614 Casc3	100751614
NW_003614892.1:82651-82673	**TT**GG**T**TTG[**GT**CCAGACCCAA]	GGG		100773741
NW_003613945.1:714870-714892	GTG**CA**G**T**G[**AC**CCAGACCCAA]	GGG	Exoc4	100750685
NW_003614658.1:429325-429347	GT**TG**C**T**TG[**TAA**CAGACCCAA]	AGG	NA	NA
NW_003613646.1:3293080-3293102	GTGG**TG**TG[**AG**CCAGACCCAA]	GGG	Ptprj	100765394
NW_003614000.1:540609-540631	**C**TG**T**ATTG[C**CA**CAGACCCAA]	AGG	LOC100764363	100764363
NW_003614448.1:217798-217820	**TA**GGAT**GG**[CACCA**T**ACCCAA]	TGG	NA	NA
NW_003615183.1:174790-174812	**A**TGGAT**CC**[CACC**GG**ACCCAA]	GGG	Frmd6	100759547
NW_003613600.1:2022960-2022982	GTG**CC**TTG[CA**AA**AGACCCAA]	GGG	NA	NA
NW_003614462.1:724431-724453	GTG**CC**TTG[CA**TA**AGACCCAA]	GGG	Cbr3	100689021
NW_003614143.1:131640-131662	**C**TGGA**G**TG[CA**GC**TGACCCAA]	GGG	LOC100757128	100757128
NW_003613624.1:1572778-1572800	GTGGA**AGA**[CACCA**T**ACCCAA]	GGG	Elavl4	100773765
NW_003613610.1:2873892-2873914	**TT**GGAT**GG**[CAC**TA**CACCCAA]	GGG	Ano2	100775014
NW_003614572.1:76198-76220	**AGG**CATTG[CACCAGAC**A**CAA]	CGG	Terf2	100762546

the 5′/3′ junction PCR products was then performed to confirm site-specific integration of EGFP and EPO in the stable cell pools (Fig. 2B) and positive clones were further verified by 5′/3′ junction PCR. According to the sequencing results, 6 out of 44 cells expressing EGFP showed EGFP-targeted integration, with an integration efficiency of 13.6 % (Supplementary Fig. 1A and B). Of the 52 monoclonal cells expressing EPO protein, 10 positive cells were identified using 5′/3′ junction PCR, and the integration efficiency was 19.2 % (Supplementary Fig. 1C, D).

3.3. *CMAH*-site integration did not affect growth characteristics of CHO cells

To further investigate the effect of site-specific integration on growth characteristics, we analyzed the growth ability of CHO cells with site-specific integration at the *CMAH* site (EGFP-3 and EGFP-23), and random integration (EGFP-21 and EGFP-31), and wild-type CHO-S cell. The cells were cultured at the same seeding density and suspended for 8 days. They were sampled and counted every 24 h, and 0.08 % trypan blue staining was used to determine the viable cell density (VCD) and viability (Fig. 3). The results showed that the VCD of wild-type, site-specifically integrated, and randomly integrated cells reached the highest levels on the 6th day and then began to decline, and the VCD of wild-type CHO-S cells was the highest (1.17×10^7 cells/mL). In addition, the VCD of wild-type CHO-S cells on the 8th day was 5.42×10^6 cells/mL and was lower than that of site-specific integrated EGFP-3 cells (6.23×10^6 cells/mL); however, there was no significant difference. Our results showed that there was no significant difference in VCD or cell viability among site-specifically integrated, randomly integrated, and wild-type CHO cells, indicating that the *CMAH*-site integration of target genes did not affect the growth characteristics of CHO cells.

3.4. *CMAH*-site integration did not improve the transgene expression

RT-PCR was performed to analyze the expression levels of CMAH and its transgenes in the stable integrated clones. The mRNA expression levels in wild-type CHO cells were set to 1, and the CMAH mRNA expression levels of the cell line EPO-41 with integration at the *CMAH* site were close to 0. Moreover, the mRNA levels of the cell lines EGFP-3, EGFP-23, and EPO-19 with site-specific integration were close to 0.5, indicating that full allelic knockdown occurred in EPO-41 cells, whereas only a single allelic knockdown occurred in the cell lines EGFP-3, EGFP-23, and EPO-19 (Fig. 4A, B). Additionally, EGFP mRNA levels were analyzed and the level in the EGFP-3 cell line with site-specific integrated EGFP-3 was normalized to 1. The results showed that the EGFP mRNA expression levels in the EGFP-23 cell line with site-specific integration and the EGFP-21 cell line with random integration were higher than those in cell line EGFP-3, whereas the mRNA expression levels in the cell line EGFP-31 with random integration were lower than those in the EGFP-3 cell line. Moreover, the mRNA expression levels of EPO in EPO-19 cells with site-specific integration were normalized to 1. The expression levels were higher in cells with site-specific integration EPO-41 cells and random integration (EPO-7 and EPO-15 cells) than those in EPO-19 cells (Fig. 4C, D). Then, we analyzed the expression levels of EGFP and EPO proteins with random integration and site-specific integration cells by Western blot. The results showed that the expression levels of EGFP and EPO proteins were similar to those of mRNA (Fig. 4E, F). Moreover, site-specific integration did not significantly increase the protein expression levels of EGFP and EPO compared with random integration. Based on the expression levels of EGFP and EPO, integration at the CMAH site did not lead to excessive expression of transgenes, and the CMAH site was not suitable for improving protein expression. However, the integration of transgenes at the CMAH site was more effective than random integration and the heterogeneity of protein expression was reduced between cell lines.

3.5. Targeted integration enhanced transgene expression stability

We further evaluated the effects of CMAH-site integration by analyzing the productivity and stability of the CHO cell lines. The expression levels were determined in EGFP-3 and EGFP-23 cells with site-specific integration and EGFP-21 and EGFP-31 cells with random integration. The cell line EGFP-21 showed the strongest green fluorescence, followed by EGFP-23 cells, whereas the EGFP-3 and EGFP-31 cell lines showed weak fluorescence (Fig. 5A). Then, we cultured these cell lines for 25 generations and the expression levels of EGFP were detected every five generations. Flow cytometry revealed that the MFI of EGFP-21 cells was the highest and turned to decline after the 15th generation (Fig. 5B), whereas, that of EGFP-31 was the lowest and it began to decline after 20 generations. Compared to the fluorescence levels in cells with random integration cells (EGFP-21 and EGFP-31 cells), the fluorescence levels in those with site-specific integration (EGFP-3, EGFP-23) were more stable in the subculture. Of note, the difference in fluorescence intensity between cells with random integration (EGFP-21, EGFP-31) was larger than that between cells with site-specific integration (EGFP-3, EGFP-23), confirming that expression heterogeneity was low in cells with site-specific integration. The results showed that site-specific integration of transgenes at the CMAH site improved protein

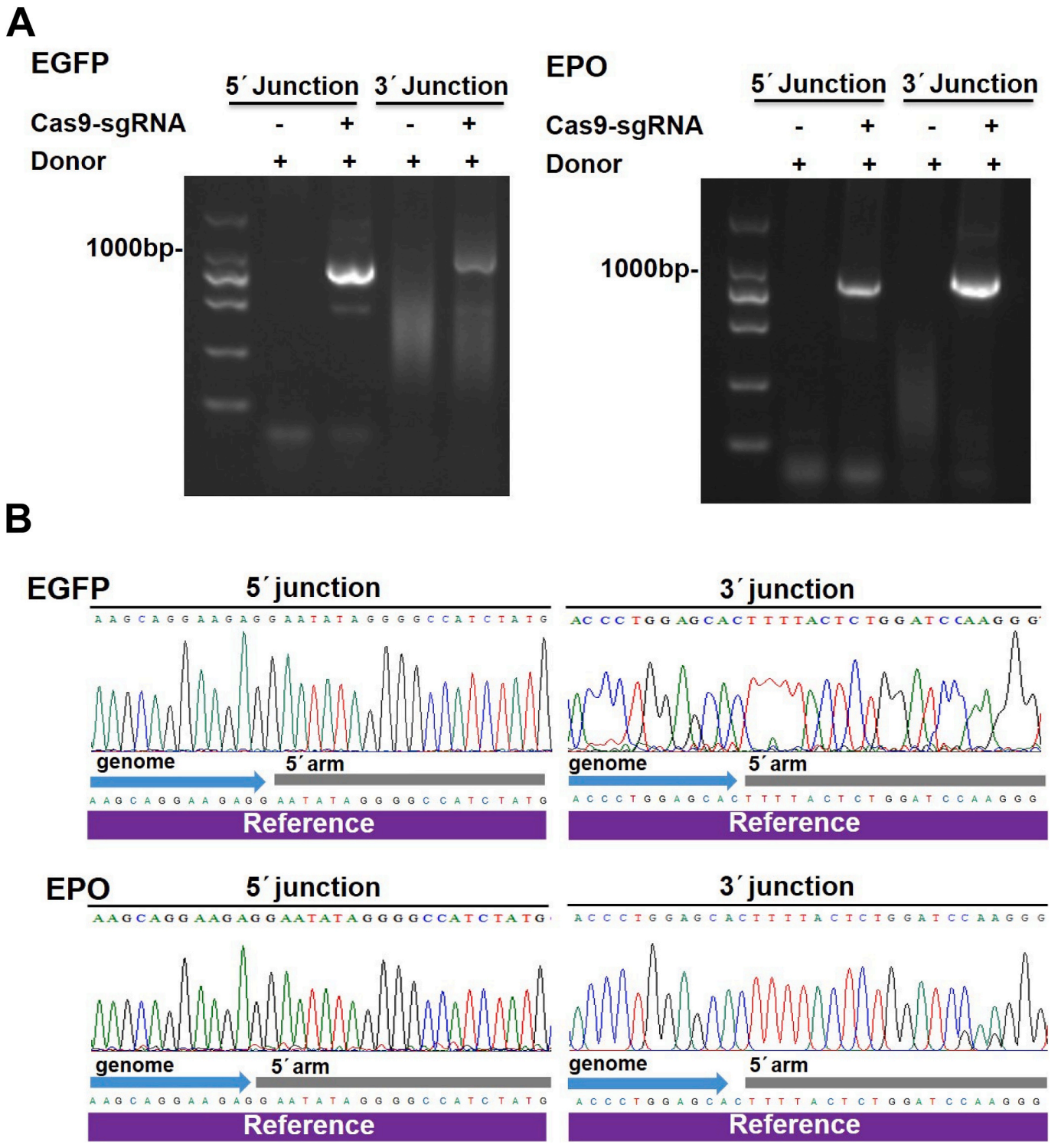

Fig. 2. Cell pool integration and identification. (A) Stable cells expressing EGFP and EPO were used to extract genomic DNA and amplification was performed using junction PCR. The PCR products were detected using agarose gel electrophoresis. (B) Sequence analysis.

expression stability and reduced heterogeneity.

3.6. Characterization of EPO glycosylation

EPO is a glycoprotein with one O-glycosylation site and three N-glycosylation sites. The glycosylation of EPO directly affects its function and half-life in serum [53]. Therefore, we focused on the glycosylation of EPO protein under different integration conditions. In this study, ultra-high-performance liquid chromatography (UPLC) was used to determine the quantity each EPO N-glucoside. We examined the differences in EPO glycosylation between the cell line EPO-7 with random integration and the cell line EPO-19 with site-specific integration. The samples were analyzed using UPLC, and the results showed that the expression levels were significantly lower in cells with CMAH site-specific integration than in those with random integration cell line EPO-7 (data not shown). Glycosylation characterization revealed that

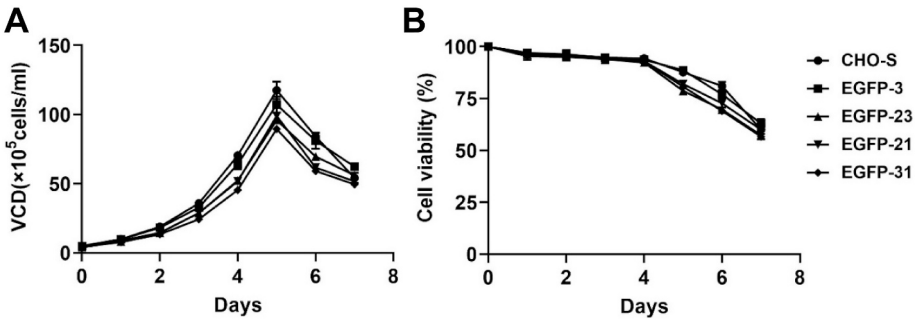

Fig. 3. Growth of CHO-S cells and integrated cell clones. (A) Viable cell density. (B) Cell viability analysis. The cells were seeded in six-well plates and suspended in CHO Pro Exp® serum-free medium at the same density for suspension expression. Samples were collected daily and a cell counter was used to analyze the viable cell density and cell viability.

the G0F-GN, G0, G0F, Man-5, G2FS1, G2FS2, FA3G3S3, F(6)M5A1G(4)1Ga(3)1, and FA4G4Lac4S4 levels of EPO-19 also changed significantly. The difference in the detection patterns and major glucosides of the EPO-19 cell line was that the proportion of EPO containingG0F-GN, G0, Man-5, G2FS1, G2FS2, and FA4G4Lac4S4 increased, while the proportion of EPO containing G0F, FA3G3S3, and F(6)M5A1G(4)1Ga(3)1 decreased, compared with the proportions in the cell line EPO-7 (Fig. 6A–C). These results suggest that integration at the CMAH site changed the glycosylation of EPO; however, the effects of N-glycosides on the serum half-life and *in vivo* biological activity need to be further studied.

3.7. Off-target analysis

CRISPR/Cas9 technology is a powerful knock-in and knock-out tool, but it may also introduce off-target mutations when editing genes [54,55]. We used the CCTOP CRISPR/Cas9 online prediction system (http://crispr.cos.uni-heidelberg.de) to analyze the potential off-target sites of the sgRNA used in this study and the results are shown in Table 5. Genomic DNA was extracted from the cell lines with site-specific integration (EGFP-3 and EGFP-23), and PCR amplification products were purified and sequenced to verify off-target effects. Analysis of the two cloned cell lines revealed that there was no off-target effects (Fig. 7A, B). These results demonstrated that CRISPR/Cas9-mediated site-specific integration at CMAH site is an efficient and reliable strategy for establishing CHO cell lines for industrial applications.

4. Discussion

Nearly 70 % of the approved RTPs are produced in CHO cell lines [2,15], and CHO cells have been the preferred hosts for production because of their increased productivity, ease of use, and expression stability [56]. Glycosylation is a key property of monoclonal antibodies and other RTPs that affects drug activity and half-life, and thus, it has been received considerable attention in the biopharmaceutical field [57–59]. A series of expressed glycosylated proteins have been identified in biologics produced using CHO cells through transgenes integrated into host cells [60]. However, the site-specific integration of transgenes and other specific requirements have been limited by low integration efficiency, low transcriptional activity, and high transgene silencing, which are not conducive to the construction of CHO cell lines [61–63]. The CRISPR/Cas9 system is a convenient and effective method for genome editing and has been widely used in CHO cells engineering to meet the demands of biopharmaceutical research [64–66]. Compared with other gene editing techniques, such as CRE-Lox, FLP-FRT, ZFNs, and TALENs, CRISPR/Cas9 technology has the advantages of high accuracy, user-friendly control and lower cost [67,68]. Neu5Gc4 produced by *CMAH* in CHO cells may induce an immune response and affect the activity of RTPs [8]. It is unknown whether the CMAH site plays a direct role in glycosylation and the long-term expression stability of transgenes. In this study, we selected the CMAH locus as the target site and integrated transgenes using CRISPR/Cas9 gene editing technology to establish a stable CHO recombinant cell line for applications in the biopharmaceutical industry.

We certificated the feasibility in this study using 5′/3′ junction PCR analysis of clonal CHO cells, and the site-directed integration efficiency was found to be 12.5 %–19.2 %, which was consistent with the previously reported site-specific integration efficiency of 7.4 %–27.8 % in CHO cells [61]. We successfully obtained 22 cell lines integrated at the CMAH site, and conducted a series of experiments. First, we performed an experiment on cell growth characteristics, and the growth curve was used to determine whether CMAH-site-specific integration of transgenes had a negative impact on CHO cells. Compared to the wild-type CHO-S cell lines, the growth characteristics of cell lines with site-specific integration (EGFP-3/EGFP-23) and random integration (EGFP-21/EGFP-31) did not change significantly. This result proved that the integration of transgenes at the CMAH site did not have a negative effect on the growth capacity of CHO cells.

Studies have shown that off-target effects caused by CRISPR/Cas9 are major obstacles to this site-specific integration approach. Base mismatches of sgRNAs may be the major reason for the off-target effects [69,70]. Target specificity can be evaluated by identifying potential non-targets for each sgRNA editing sequence [61]. We randomly chose a potential target (LOC100751614) and two clones expressing EGFP (EGFP-3 and EGFP-23) to verify the off-target effects. Our results showed that sgRNA1 used in this study did not induce off-target mutations, confirming the reliability of the CRISPR/Cas9 system for site-specific integration in CHO cells. Productivity and stability are critical factors for the stable development of cell lines [19]. According to EGFP fluorescence analysis by using flow cytometry, we confirmed that integration at the CMAH site showed higher protein expression stability than random integration for at least 25 generations.

In the past few years, several transcriptional hot-spots for site-specific integration have been identified in CHO cells, and their effects on protein expression have been verified. These hot-spots can effectively increase the number of transgene copies, improve transcriptional activity, and enhance expression stability [71–73]. Compared to the above hot-spots, CMAH site-specific integration mainly enhanced the long-term stability of recombinant protein expression, without significantly improving the transgenic copy numbers or protein expression levels. Many factors can affect the stability of protein expression, including changes in chromosome integrity, epigenetic modifications, and the culture environment (e.g., medium and supplements) [74,75]. CMAH-site-specific integration may have a location effect on chromosomes, which improves the stability of expression. However, the specific mechanism needs to be further explored.

Glycosylation is a critical quality attribute of RTPs and strategies for improving glycosylation in CHO cells has been widely reported [76].

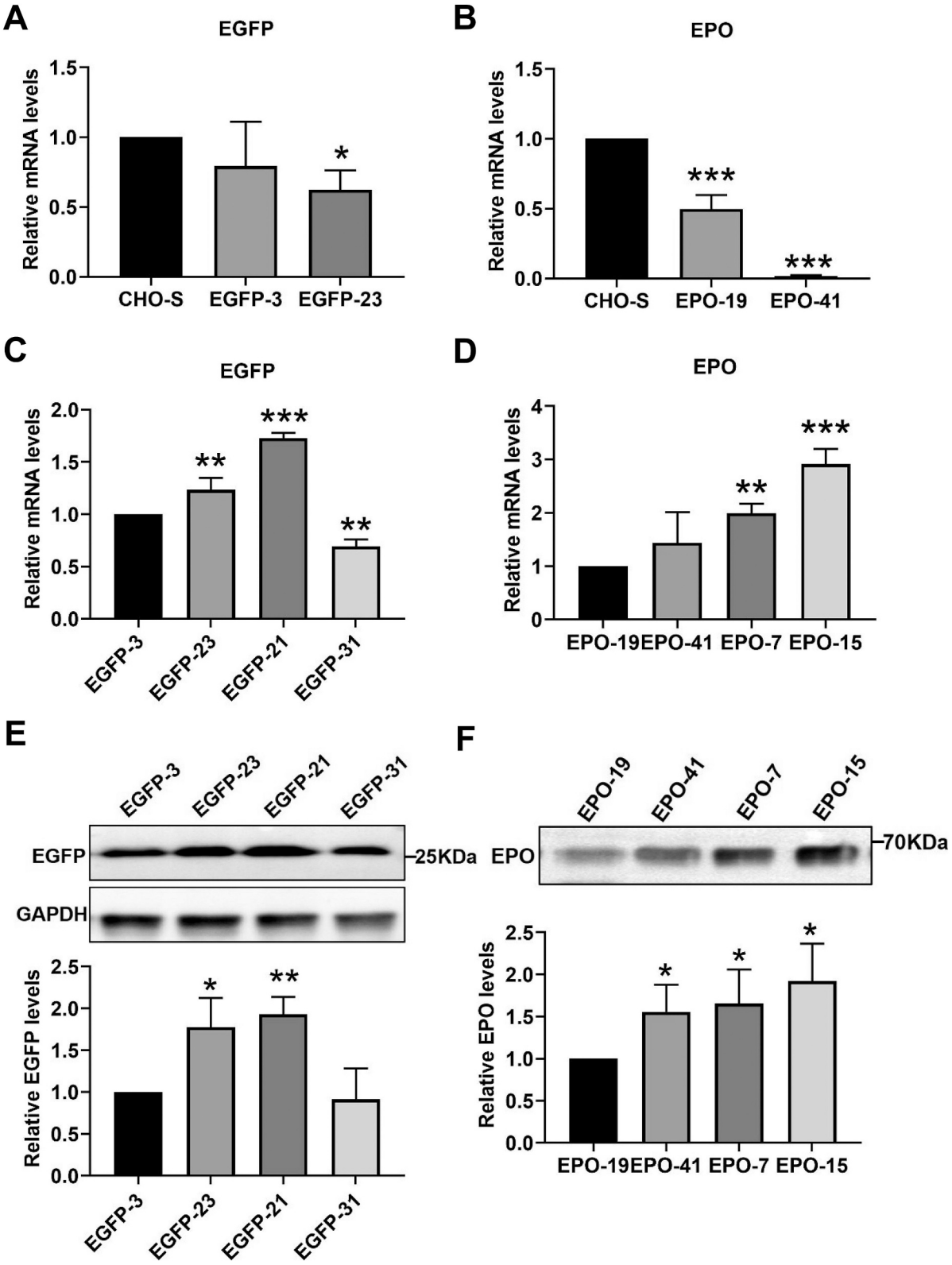

Fig. 4. mRNA expression levels of target genes. (A) CMAH mRNA expression levels in wild-type CHO-S cells and cell lines with site-specific integration (EGFP-3 and EGFP-23). (B) CMAH mRNA expression levels in wild-type CHO-S cells and cell lines EPO-19 and EPO-41 with site-specific integration. (C) EGFP mRNA expression levels in cell lines with site-specific integration (EGFP-3 and EGFP-23) and cell lines with random integration (EGFP-21 and EGFP-31). (D) EPO mRNA expression levels in cell lines with site-specific integration (EPO-19 and EPO-41) and random integration (EPO-7 and EPO-15). Total RNA was extracted and mRNA levels were detected by qPCR using *GAPDH* was used as an internal control. Relative protein levels of EGFP (E) and EPO (F) tested by Western blot in the CHO cells with random integration and site-specific integration. Proteins were extracted from cell supernatant or cells for Western blotting, and the bands were quantified using IMAGE J software (NIH, Bethesda, MD, USA). Each point represents the mean ± SD of three independent replicates (*$p < 0.05$; **$p < 0.01$).

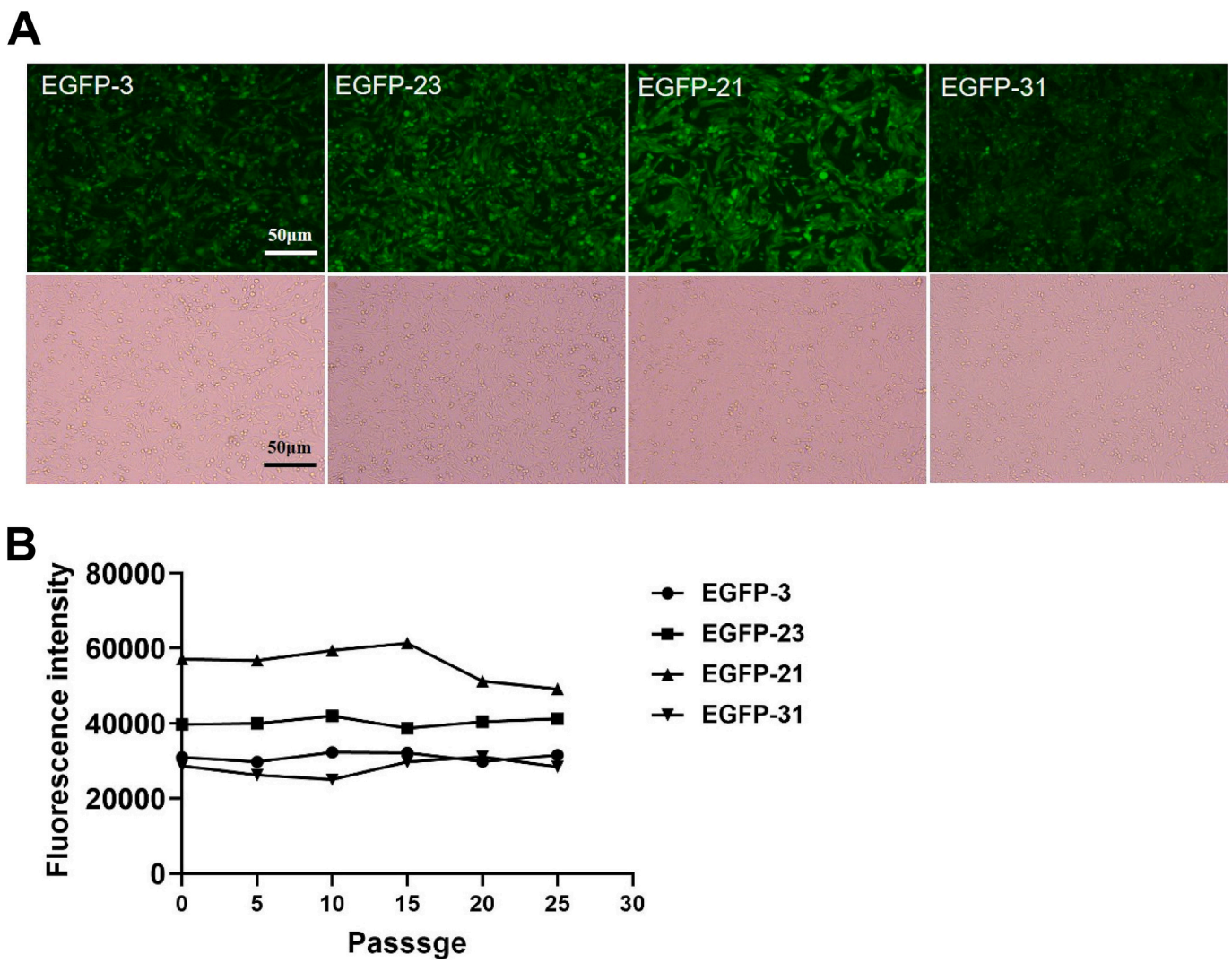

Fig. 5. EGFP fluorescence and protein expression stability analysis. (A) Fluorescence intensity of cell lines EGFP-3 and EGFP-23 with site-specific integration, and cell lines EGFP-21 and EGFP-31 with random integration, and corresponding bright-field images (below). (B) Stability of EGFP expression in cell lines with site-specific integration and random integration. EGFP-3 and EGFP-23 cells with site-specific integration and EGFP-21 and EGFP-31 cells with random integration were evaluated every 3 days. EGFP fluorescence intensity during cell passage was measured using flow cytometry every five generations, and a total of 25 generations were analyzed.

During upstream production, the forms of protein glycosylation can change depending on the cell line [77], process conditions [78], medium [79] feed formulations [80] and the genetic engineering approach applied [63]. However, the effect of CMAH site integration on glycosylation has not yet been reported. Therefore, we explored the glycosylation type of EPO integrated at the CMAH site using CRISPR/Cas9 technology. Clone EPO-7 with random integration and clone EPO-19 with site-specific integration were selected for analysis. Our results showed that the proportion of G0F-GN, G0, Man-5, G2FS1, G2FS2, and FA4G4Lac4S4 increased, whereas the proportion of G0F, FA3G3S3, and F(6)M5A1G(4)1Ga(3)1 decreased in clone EPO-19 compared to clone EPO-7. Fischer et al. found that the *CMAH* gene contains two putative binding sites for newly discovered *microRNA-111* and that CMAH mRNA levels exhibited a negative correlation with *microRNA-111* expression levels. The expression of *microRNA-111* depends on the transcription of sidekick cell adhesion molecule 1 (SDK1) and affects N-glycolylneuraminic acid sialylation levels [76]. However, the specific effects of the CMAH site integration on N-glycosides and the underlying mechanisms require further investigation.

We found that the mutated cell lines displayed different phenotypes in terms of stability and glycosylation forms compared to cell lines with random transgene integration, which provides insights into hot-spots in the CHO cell genome and theoretical support for related gene screening. The influence of CMAH knockout on the expression of other genes related to cell metabolism has not yet been investigated. One further research direction is to use omics studies, such as single-cell omics techniques, to identify new candidate genes and elucidate the underlying mechanisms promoting RTP production.

5. Conclusion

Our results confirmed that CRISPR/Cas9 is an effective gene-editing technology for site-specific integration of transgenes at the CMAH site in CHO cells. Compared to random integration, CMAH site-specific integration significantly improved the stability of transgenic expression and generated more desirable forms of glycosylation in the recombinant proteins. Our results revealed, for the first time, that the specific integration of transgenes at the CMAH site could improve the long-term stability of transgenic expression, showing great significance in the expression of therapeutic glycoproteins, and can provide a new approach for the construction of CHO cell lines.

Supplementary data to this article can be found online at https://doi.

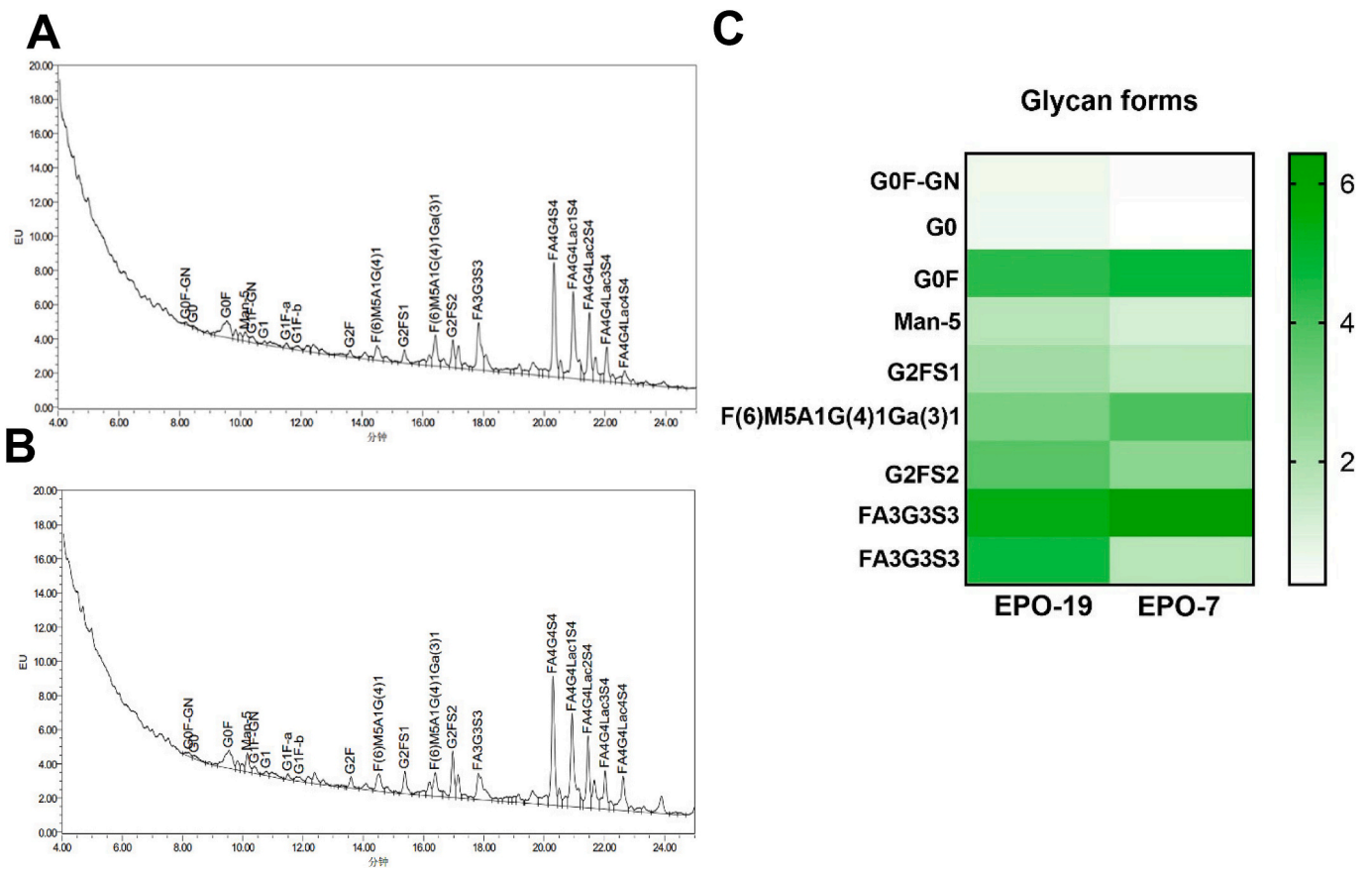

Fig. 6. EPO glycosylation. (A) UPLC analysis of the cell line EPO-7 with random integration. (B) UPLC diagram of the cell line EPO-19 with site-specific integration. (C) Glucoside analysis.

Table 5
Off-target analysis.

Clone	Potential target	Potential off-target sequence 5′-3′	Sequence result 5′-3′
EGFP-3	LOC100751614	**C**TGGA**G**TG[CA**G**C**T**GACCCAA] GGG	**C**TGGA**G**TGCA**G**C**T**GACCCAAGGG
EGFP-23	LOC100751614	**C**TGGA**G**TG[CA**G**C**T**GACCCAA] GGG	**C**TGGA**G**TGCA**G**C**T**GACCCAAGGG

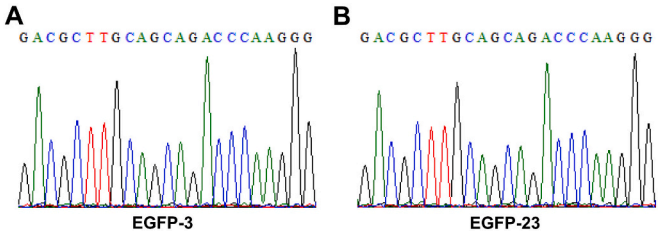

Fig. 7. Off-target sequencing analysis. Sequencing results of site-specific integration in (A) EGFP-3 cells and (B) EGFP-23 cells. DNA was extracted from EGFP-3 and EGFP-23 cells with stable expression and corresponding primers were designed for PCR amplification. The, products were purified for sequencing and off-target verification of the designed sgRNA.

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CRediT authorship contribution statement

Xiang-Xiang Cao: Writing – original draft, Methodology, Investigation, Conceptualization. **Jing-Jia Yuan:** Software, Methodology, Investigation. **Zhi-Yuan Bai:** Software, Methodology, Investigation. **Min Zhang:** Software, Methodology, Data curation. **Yi-Fei Yun:** Methodology, Data curation. **Xiao-Yin Wang:** Validation, Methodology, Investigation. **Chun-Liu Mi:** Validation, Software, Investigation. **Qiu-Li Sun:** Validation, Software, Methodology. **Shao-Lei Geng:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Tian-Yun Wang:** Writing – review & editing, Supervision, Project administration, Data curation.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability

The data that support the findings of this study are available from the

corresponding author upon reasonable request.

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作者	Cao, XX (Cao, Xiang-Xiang) [1], [2]; Yuan, JJ (Yuan, Jing-Jia) [1]; Bai, ZY (Bai, Zhi-Yuan) [1]; Zhang, M (Zhang, Min) [1]; Yun, YF (Yun, Yi-Fei) [3]; Wang, XY (Wang, Xiao-Yin) [1]; Mi, CL (Mi, Chun-Liu) [1]; Sun, QL (Sun, Qiu-Li) [1]; Geng, SL (Geng, Shao-Lei) [1], [3]; Wang, TY (Wang, Tian-Yun) [1], [3]
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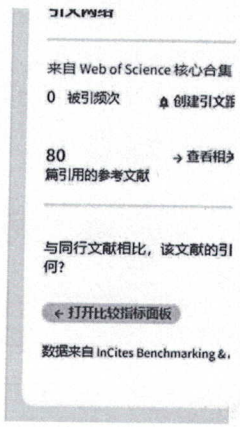

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作者

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摘要

Chinese hamster ovary (CHO) cells are the most widely used platform for recombinant therapeutic protein (RTP) production. Traditionally, the development of CHO cell lines has mainly depended on random integration of transgenes into the genome, which is not conducive to stable long-term expression. Cytidine monophosphate N- acetylneuraminic acid hydroxylase (CMAH) is expressed in CHO cells and produces N-hydroxyacetylneuraminic acid, which may cause a human immune response. However, the effects of transgene integration at the CMAH site on RTP expression in CHO cells remain unclear. In this study, we selected CMAH gene, which is lacking in humans, as the target site to construct recombinant CHO cell line using the CRISPR/Cas9 technique. Erythropoietin (EPO) and EGFP integration at the CMAH site resulted in more stable expression levels and lower heterogeneity than random integration. In addition, the proportion of N-glycosylation levels in the EPO glycoside of CMAH integration site also changed. In conclusion, CMAH site integration improved the stability of RTP expression in CHO cells.

关键词

作者关键词: CHO cells; Targeted integration; CRISPR/Cas9; Glycosylation
Keywords Plus: SITE-SPECIFIC INTEGRATION; PSEUDO ATTP SITES; TRANSGENE EXPRESSION; GENOME; KNOCKOUT; ANTIBODY; IDENTIFICATION; CRISPR/CAS9; CASSETTE; PRECISE

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

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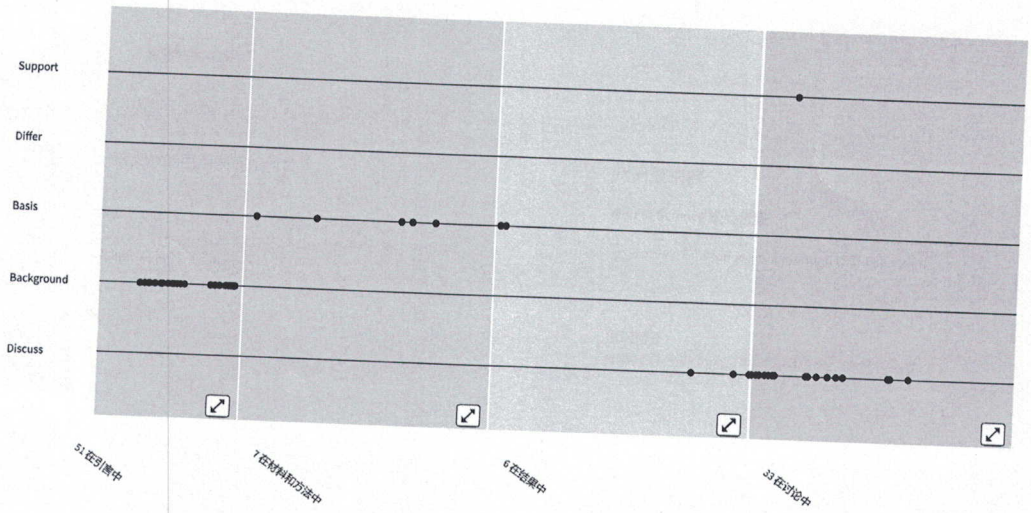

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