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厦门大学

博　士　学　位　论　文

CHO-K1 细胞基因编辑改造及其运用于抗体的重组表达

Engineered CHO-K1 by Gene Edition and Its Application to Recombinant Antibody Expression

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摘 要

抗体药物是近年来发展最快的一类生物技术药物，因其极强的靶向特异性，已成功运用于多种疾病的治疗；而目前大部分抗体药物均由中国仓鼠卵巢癌（Chinese hamster ovary, CHO）细胞进行生产。CHO有多个衍生品系，其中CHO-K1在工业中运用较广；CHO-K1主 要有两个品系CHO-K1-CCL-61和CHO-K1-CRL-9618，初始均以血清依赖的贴壁培养方式进行培养，工业生产中通常需要对其进行无血清悬浮适应，甚至对其基因组进行适当的改造；目前CHO-K1-CCL-61的运用和研究较为广泛；而关于CHO-K1-CRL-9618的运用和基础研究均较少。本研究以CHO-K1-CRL-9618为研究对象，对其开展无血清悬浮适应和基因编辑工作，力图开发新一代基于CHO-K1-9618无岩藻糖基化（Defucosylation）的谷氨酰胺合成酶（Glutamine synthetase, GS）表达系统。

本论文的第一部分将原始依赖血清且贴壁培养的 CHO-K1-9618(CHO-K1-A) 悬浮适应，获得具有大规模培养潜力的无血清悬浮培养的单克隆 CHO-9618s 细胞。利用逐步降血清法，将 CHO-K1-A 适应为无血清悬浮培养的细胞群，对细胞数为 1×10^{10} 个且变异度极高的悬浮细胞群进行单细胞克隆化，获得单细胞来源的亚克隆细胞株。通过连续、批次（Batch）、和流加（Fed-Batch）培养等评价，发现大部分亚克隆以 24h/代的速度进行增殖，支持长期连续培养，Fed-Batch 下最高活细胞密度（VCD, Viability cell density）可达 2×10^{7}c/ml，且关键营养代谢速率均正常。还验证了表现最好的一株亚克隆 CHO-9618s 能对外源蛋白进行表达，能在机械搅拌式生物反应器中 Fed-Batch 培养进行高细胞密度培养。

本研究的第二部分利用基因编辑技术构建 GS 和 Fut8 敲除的 CHO 细胞。内源的 GS 会干扰 GS 系统筛选的效率；而内源的岩藻糖基转移酶 8（Fucosyltransferase 8, Fut8）催化岩藻糖基（Fucose）转移至重组蛋白糖基化位点，会降低抗体依赖的细胞毒作用（Antibody-dependent cell cytotoxicity, ADCC）和吞噬效应（Antibody-dependent cell phagocytosis, ADCP）。本研究运用 CRISPR/Cas9 技术对 CHO-9618s 的基因组进行编辑，获得 GS-/Fut8-的单克隆细胞 CHO-NIDVD。运用 PCR, Surveryor 酶切、靶基因测序、谷氨酰胺（L- glutamine,

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LG）依赖性、Fitc 标记的凝集素细胞表面染色及 CHO-NIDVD 总蛋白 WB 检测，均证明了 CHO-NIDVD 已无内源 GS 和 Fut8 蛋白。

本研究的第三部分开展了 CHO-K1-9618 关键代谢特征、基因组和转录组学的初步研究。无血清悬浮适应后 CHO-9618s 的葡萄糖（Gluc）消耗速率和乳酸（Lac）合成速率下降；进一步基因编辑后 CHO-NIDVD 的 Gluc 消耗率和 Glu 合成速率显著增强。CHO-9618 的基因组有多个 SNPs（Single nucleotide polymorphism）和 Indels（Insertion-deletion）。在转录组方面，由贴壁转为悬浮培养后，与细胞增殖、细胞贴壁依赖和部分代谢相关的 18 条信号通路发生显著变化；与 CHO-9618s 相比，CHO-NIDVD 大部分显著差异表达的基因均与代谢相关。本部分还从基因组和转录组水平均验证了 CHO-NIDVD 基因组中 GS 和 Fut8 的成功编辑。

本研究的第四部分基于 CHO-NIDVD 建立了抗体的重组表达平台，分析其表达产物生物学功能上的变化，及其在构建高产稳定 cell pool 的效率、产能、表达的长期稳定性和产物质量上的特性。以 CHO-NIDVD 为宿主，显著增强了表达产物 Herceptin 的 ADCC 效应，以及乙肝治疗性抗体 162 对 HBsAg 的 ADCP 效应。本部分基于 CHO-NIDVD 初步构建了重组抗体的稳定表达平台，历时 65 天，获得的 cell pool 经 Fed-Batch，产量可达 1-2g/L，比生产率可达 50pg/cell/day；其表达产物的 Protein A 纯化特征、SDS-PAGE、CE-SDS 以及 HPLC-SEC 特征均与已上市抗体药物的质量属性基本相同；且这些 cell pool 能够稳定表达 63 代以上。

综上所述，本研究对 CHO-K1-CRL-9618 细胞开展无血清悬浮驯化及基因编辑工作，获得了能够进行无血清悬浮培养且 GS 和 Fut8 同时缺陷的 CHO-NIDVD；并开展了 CHO-9618 系列细胞的关键代谢、基因组和转录组研究；最后将 CHO-NIDVD 运用于抗体的表达，显著增强了抗体的 ADCC 和 ADCP，能够支持抗体高水平、质量可控且长期稳定的表达，具有运用于工业生产的潜能。总之，本论文的研究将为抗体药物表达生产提供新的表达系统，CHO 细胞基因组和转录组研究将为表达工艺的优化及开发下一代表达系统提供理论基础。本研究的应用不仅局限于抗体分子，也为其他单亚基或多亚基蛋白的表达提供新的参考。

关键词：CHO 细胞；无血清悬浮适应；基因编辑；基因组重测序；转录组测序；ADCC；ADCP；抗体表达

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Abstract

Antibody drugs have been one of the most fast-growing biotech drugs in recent years. Because of its high specificity, it has been successfully used in the treatment of many diseases. At present, most of the antibody drugs are produced by the Chinese hamster ovary cancer (CHO) cells. There are a number of CHO derivative lines in which CHO-K1 is widely used in industry. CHO-K1-CCL-61 and CHO-K1-CRL-9618 are the major strains of CHO-K1 . They were initially cultured adherently with serum. In industrial production, CHO-K1 cells are usually adapt to serum free suspension culture, and even engineered in the genome for optimizing the cell's performance. At present, the application and the basic research of CHO-K1-CCL-61 is extensive, while the use and research of CHO-K1-CRL-9618 is limited. In this study, serum free suspension adaption and gene editing was conducted on CHO-K1-CRL-9618, aiming to develop a new generation of defucosylation GS expression system based on CHO-K1-9618.

The first study aimed to adapt the serum dependent adherence CHO-K1-9618(CHO-K1-A) into serum free suspension culture (CHO-9618s). As the serum was decreased step by step, the adherent dependent CHO-K1-9618 was transformed into serum free suspension cell pools. The single colony was expanded and subjected to continuous culture, Batch and Fed-Batch. The doubling time of subclones is 24h in long time continuous culture stablising; the peak viable cell density (VCD) could reach 2×10^7c/ml in Fed-Batch culture. The key nutrient metabolic rate of CHO-9618s was normal. It was also verified that CHO-9618s, one of the subclones, could be used in expressing exogenous protein, and could be Fed-Batch cultured in mechanical stirred tank bioreactor with a high density culture.

The second part of this study aimed to develop a GS and Fut8 defective CHO with gene edition. The endogenous GS gene will interfere with GS screening system. Also, the endogenous fucosylation by Fut8 in CHO cells catalytic fucose transfers to the glycosylation site on recombinant protein will be reducing the Antibody-dependent cell cytotoxicity (ADCC) and Antibody-dependent cell phagocytosis (ADCP) effect. In this study, the GS and Fut8 was inactivated in CHO-9618s by CRSPRI/Cas9, a GS-/Fut8-construct CHO-NIDVD was obtained. The PCR, Surveryor enzyme digestion, DNA sequencing, LG dependence culture, F-LAC cell surface staining and total protein WB

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assay, verified that endogenous GS and Fut8 of CHO-NIDVD has been inactivated.

In the third part of this study, the key metabolic characteristics, the genomics and transcriptomics of CHO-K1-9618 was investigated. We found the glucose consumption rate and lactic acid synthesis rate was declined in post serum free suspension(CHO-9618s); the glucose consumption rate slightly increased and glutamate synthesis rate significantly enhanced in CHO-NIDVD compared to CHO-9618s. There are many SNPs and Indels in the genomes of CHO-NIDVD and CHO-9618s. At the transcriptomic level, compared to the serum-dependent adherent CHO-K1-A, 18 signal pathways related to the cell proliferation, cell adherence and partial metabolism were significantly changed in the CHO-9618s. Between CHO-9618s and CHO-NIDVD, the major DGE involved in metabolism. In this part, we confirmed the deficiency of GS and Fut8 in CHO-NIDVD at the genomic and transcriptomic level.

In the fourth part of this study, the CHO-NIDVD cells were applied to antibody expression. The changes of the antibody biological activity, the efficiency of stable cell pool construction, the yield, the stability and the product quality were considered in this study. CHO-NIDVD was used as the host cell to produce Herceptin and 162, a HBV therapeutic antibody. The ADCC of Herceptin and the ADCP of 162 has been enhanced significantly. A stable antibody expression platform had been developed in this study based on CHO-NIDVD. It lasted 65 days for cell pool construction based on this platform. The yield of cell pool could reach 1-2 g/L with Fed-batch process. And the Qab of cell pools could be up to 50pg/cell/day. The purification characteristics, SDS-PAGE, CE-SDS, and HPLC-SEC features of the products are basically the same as the commercial antibodies. And the cell pool could maintain stable expression ability at last 63 generations.

In summary, this study adapted the serum dependent adherence CHO-K1-9618(CHO-K1-A) into serum free suspension CHO-9618s, and engineered genes on CHO-9618s to obtain a GS and Fut8 defective CHO line (CHO-NIDVD), and further analyzed the genomic and transcriptomic features of CHO-9618; Finally, the CHO-NIDVD cells were used in the antibody

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expression, leading to the enhancements of ADCC and ADCP, and resulting in a high expression level, high quality and the long-term stable expression. The CHO-NIDVD and its derivative cell pools have the potential to be used in industrial production. In conclusion, the research of this thesis has provided a new expression system for antibody production, and the studies of CHO cell genomic and transcriptomic will provide the theoretical foundation for the expression process optimization for the development of next generation expression system. The application of this research is not only limited to the antibody, but also provides new reference for the expression of other proteins.

Keywords: CHO cells; serum free suspension adaptation; gene editing; genome sequencing; transcriptome sequencing; ADCC; ADCP; antibody expression

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