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Chapter 3
Engineering mammalian cell for cancer

Abstract: The accuracy of products produced by mammalian cells can be explained by the fact that continuously stored environmental information leads to high-precision production capacity. Therefore, usage of mammalian cells in cancer might lead to progression of safe and efficient therapies. Synthetic biology has emerged as a transformative force in biomedical innovation, which aims to reprogram cells for precise diagnostics and therapeutics. Recent advances highlight the development of modular synthetic receptors, enabling customizable disease recognition and engineered mammalian cells exhibiting remarkable sensitivity and selectivity. Simultaneously, advances in mammalian synthetic biology facilitate the deliberate engineering of protein secretion, glycosylation, cellular metabolism, and cellular communication, unlocking new therapeutic possibilities that even lead to the construction of artificial tissues for innovative cancer therapies. This chapter focuses on the current techniques of mammalian cell engineering for cancer therapeutics including their drawbacks and future.

3.1 Introduction

Mammalian cells are eukaryotic cells. These are essential units of life in multicellular organisms, which play vital roles in various biological processes. The human body consists of trillions of individual cells in tissues and organs. These cells have a complex structure that enables them to adapt and specialize for specific functions. As a result, mammalian cells in the human body encompass a wide array of cell types carrying out various physiological functions. Therefore, understanding the mechanisms underlying the mammalian cell's various biological functions is essential [1].

Cancer can be defined as a complex and diverse group of diseases characterized by uncontrolled growth, survival, and, in some cases, the spread of abnormal cells in the body through metastasis [2]. Cancer is a genetic disease. Therefore, disruptions in mammalian cells' normal genetic regulatory mechanisms can lead to uncontrolled cell growth and proliferation, resulting in cancer progression [3]. As a result of the increased life span, the reoccurrence of cancer increases every year. By 2050, around 35 million people are estimated to have cancer annually, which indicates a 77% in-

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crease compared to the 2022 level. It encompasses various types and subtypes, each with distinct characteristics and behaviors that can be lethal. It is responsible for 16.8-22.8% of noncommunicable diseases in the twenty-first century. Based on the death records in 2022, the most lethal cancer type is lung cancer, followed by breast cancer. It is known that these numbers vary between genders and age ranges [4].

Evidence indicates that a series of genetic mutations in a single cell could lead to cancer [3]. Advancements in understanding the molecular genetics of cancer revealed the genetic basis of various types of cancer. For instance, mutations in critical genetic regulators of hematopoiesis have been associated with hematopoietic neoplasms, highlighting the link between genetic alterations and cancer development [5]. Furthermore, the dysregulation of transcription factors like RUNX1 (RUNX family transcription factor 1) has been implicated in leukemia, emphasizing the importance of proper gene expression regulation in preventing oncogenesis [6]. It is possible to tailor novel treatments based on mammalian cells by elucidating the relationship between genetic mutation and cancer development. In recent timelines, synthetic gene circuits in mammalian cells have shown promise in cancer therapy [7].

Synthetic biology is a multidisciplinary field that utilizes engineering principles to design systems for producing biological molecules, sensors, and systems. With synthetic biology, it is possible to create cell factories that provide the needs of industry or medicine from renewable sources. It allows the creation of highly specific and tunable cells for various functions. The use of these cells in different areas, such as agriculture, medicine, and industry, is increasing with the advancements in the field. For cancer, synthetic biology holds great promise since the optimized systems can be used for biomanufacturing, diagnosis, and therapy. In this chapter, the use of these systems for cancer will be given, and issues regarding engineering protein secretion, glycosylation, cellular communication, and metabolism will be discussed [1].

3.2 Engineering protein secretion

Proteins designated for secretion or residence in the plasma membrane pass through interconnected steps within the endoplasmic reticulum (ER) and Golgi apparatus. This protracted journey involves vesicle intermediates emanating from a donor compartment and fusing with an acceptor/target compartment. The adjustment of vesicle budding and cargo selection in the donor compartment involves coat protein complexes (COP) such as COPI (coat protein complex I), COPII (coat protein complex II), and clathrin. In contrast, vesicle fusion relies on the interplay between SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) and Sec1/Munc18 proteins. The secretory pathway, primarily unfolding in the ER, Golgi apparatus, and the endomembrane system, holds particular significance in biotechnology and the biopharmaceutical industry. Notably, the entry of proteins with *N*-terminal signal se-

quences into the ER represents the initial step in the secretory pathway, with preproteins gaining access either cotranslationally or posttranslationally. This complex process involves the signal sequence recognition particle (SRP). After synthesis on the ribosome-bound ER, secretory proteins undergo translocation into the ER lumen via a cotranslational mechanism. Transport vesicles facilitate the departure of secretory proteins from the ER, with vesicle budding mediated by COPs. Tethering and fusion of vesicles, crucial for cargo unloading, involve multicomponent heteromeric protein complexes (tethering factors) and evolutionarily conserved Rab GTPases (GTP-binding proteins). The secretion process is further characterized by two modes: constitutive, wherein proteins are secreted concurrently with synthesis, and regulated, where secretory proteins are stored in transport vesicles before release. Protein folding within the ER involves the formation of disulfide bonds, prolyl isomerization, and the sequestration of hydrophobic amino acids. The complexities of these processes underline the significance of comprehensive understanding and strategic engineering for improved protein secretion in mammalian cells. The secretory pathway encompasses complex processes predominantly occurring within the ER, Golgi apparatus, and the endomembrane system. Facilitating the proper targeting of newly synthesized proteins to their ultimate destinations is a crucial function of the secretory pathway, thereby upholding the cell structure and function. This pathway starts with entering proteins with *N*-terminal signal sequences into the ER, a process that can occur either cotranslationally or posttranslationally [8–10].

In biopharmaceutical manufacturing, the majority of products are secreted proteins. However, the limited secretion capacity of mammalian cells poses a significant challenge. Advancing our understanding of vesicle trafficking and developing engineering strategies to enhance secretion are crucial for maximizing production potential [11].

3.2.1 Signal peptides and optimization of signal peptides for enhanced secretion

Signal peptides, short sequences located at the *N*-terminal region of proteins, serve as directives for protein secretion. The initial phase of protein secretion relies on signal peptides, guiding the association of the translating ribosome with the SRP and facilitating translation arrest. While various signal peptide sequences show differing efficiencies in promoting the secretion of heterologous proteins, those from interleukins or immunoglobulins (Igs) are commonly used for directing recombinant protein secretion. However, challenges arise in selecting an appropriate signal sequence due to variations associated with host cells, secretion processes, and interactions with subsequent portions of the polypeptide [12, 13].

Bioengineering host cell lines can enhance the modification or secretion of heterologous proteins. A comprehensive understanding of these enhancements' molecular

mechanisms and their particular effects on diverse recombinant proteins requires systematic exploration [12, 13]. In this sense, literature following the methods developed in the literature is possible. In line with this, Kober et al. [14] focused on optimizing signal peptides to increase the secretory efficiency of recombinant biotherapeutic antibodies [14]. They identified signal peptides B and E, which they named in their study, as promising candidates for improving production rates suitable for commercial use. However, in addition to known signal peptides, a very large pool can be created to recognize signal peptides, and potential candidates can be selected and developed from these pools. Additionally, the research by Park et al. (2022) addressed the difficulties in developing an in vitro screening system for signal peptides in mammalian cells [15]. By combining degenerate codon-based oligonucleotide library construction, an Flp-In™ system, and a fluorescence-activated cell sorting-based cold capture assay, they created a screening system to characterize single clones transfected with signal peptides. Furthermore, studies have continued to support the statement of Bachhav et al. (2023), which stated that the importance of signal peptides in the secretion of therapeutic proteins and their recognition by SRP during translocation into the ER is crucial [16]. As a result, Cheng et al. [17] reported the effect of artificial signal peptides on the secretion activity of recombinant lysosomal enzymes in CHO (Chinese hamster ovarian) cell lines [17]. They engineered two recombinant human lysosomal enzymes, *N*-acetyl-α-glucosaminidase (rhNAGLU) and glucosamine (*N*-acetyl)-6-sulfatase (rhGNS), by replacing their native signal peptides with nine different signal peptides derived from highly secretory proteins in CHO K1 cells. When comparing native signal peptides, rhGNS has been noted to be secreted into media at higher levels than rhNAGLU. It is concluded that the secretion of rhNAGLU and rhGNS can be carefully controlled by changing the signal peptides. Finally, the existing computational tools for predicting unconventional protein secretion (UPS), such as SecretomeP and SPRED, are products of a hypothesis based on which all secretory proteins share common properties, independent of specific pathways. Focusing on this, it exploits classical secretory proteins by removing signal peptides. In another work, Zhao et al. (2019) introduced OutCyte, a new tool for UPS prediction that covers secretory pathways that do not contain a defined *N*-terminal signal peptide [18]. OutCyte-UPS outperformed existing tools in predicting UPS proteins, laying the foundation for improved UPS prediction in the future using experimentally validated data.

3.2.2 Cell line development to improve secretion efficiency

Cell line development is included in the drug development process, specifically at the stage following the identification of a potential drug as a protein. Cell line engineering is also included, especially in protein secretion enhancement studies, genetic circuit designs, and cleavage of ER-retention signal sequence studies. In pursuit of precise control over protein secretion, innovative platforms have been developed. Vlahos et al. [19] introduced RELEASE and RELEASE-NOT systems (Figure 3.1) to modulate in-

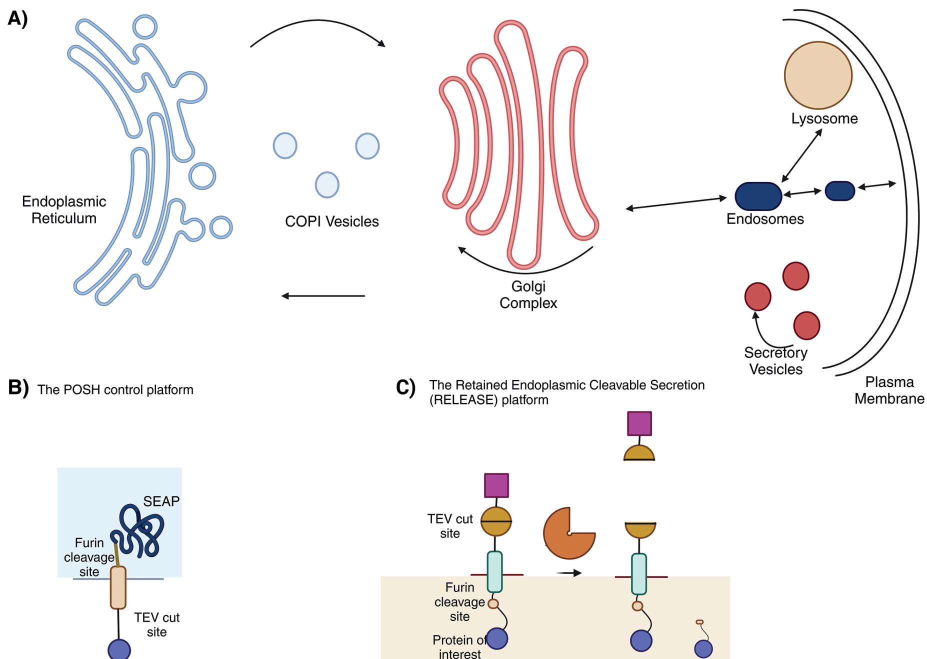

Figure 3.1: Schematic representation of (A) the engineerable protein trafficking network and secretion pathway in mammalian cells and (B) the POSH platform [20] that uses an inducible protease to remove the ER import signal from a synthetic reporter, allowing it to move to the trans-Golgi region, where it is cleaved by furin for secretion. (C) The RELEASE platform employs ER retention motifs to protect tagged proteins in the ER, which are then released through protease activation and transported via the conventional secretory pathway [19].

tercellular signaling through proteolytic removal of ER-retention motifs, paving the way for complex expression profiles. The RELEASE platform was modified to enable negative regulation of protein secretion (RELEASE-NOT) in response to protease activity. This advancement allows for tasks like logical processing and filtering of analog signals using just one protease input [19]. Combining these into compRELEASE, the authors achieved logic processing and signal filtering with a single protease input. In addition, Mansouri et al. [20] designed the programmable protease-mediated post-translational switch (POSH) platform, demonstrating efficient protein secretion with protease-driven protein circuits. This approach generates the desired protein in a chimeric form linked to an ER-retention signal (Lys-Lys-Tyr-Leu (KKYL)), keeping the protein within the ER under normal conditions. When triggered by an inducer, two fragmented cytoplasmic protease units merge to create an active protease. This activated protease removes the ER-retention signal, initiating the release and secretion of the protein stored in the ER [20]. Praznik et al. [21] utilized split potyviral proteases

for the controlled release of ER-lumen proteins, offering a faster alternative to transcription-based systems [21].

3.2.3 Manipulating vesicle transport for improved protein secretion

Intracellular vesicle trafficking is critical in maintaining organelle homeostasis in eukaryotic cells. Studies suggesting pharmacological manipulation of membrane fusion as a potential therapeutic approach are important [22]. In unusual protein secretion mechanisms that play an important role in releasing proteins outside the cell, this process occurs through extracellular vesicles (EVs), including microvesicles and exosomes. Exosomes are formed within endocytic vacuoles and are released by fusion with lysosomes or exocytosis. Ectosomes are released directly from the plasma membrane [23]. Interventions at the cellular level targeting the translocation machinery have proven effective in enhancing recombinant protein production in mammalian cells.

For instance, Le Fourn et al. [13] demonstrated increased translocation of proteins into the ER lumen by overexpressing SRP14 (signal recognition particle 14), resulting in a sixfold increase in cell-specific productivities for a low-yielding monoclonal antibody (mAb) [13]. Multitarget engineering approaches have been explored to address limitations in the secretory pathway of CHO cell lines. Cartwright et al. [24] overexpressed multiple ER proteins, identifying top-ranking effectors associated with increased titers and cell-specific productivities for an IgG1 (immunoglobulin G) LDH (lactate dehydrogenase) molecule [24]. For instance, Berger et al. [25] targeted the ER chaperone system, overexpressing PDI family members Erp27 and Erp57, leading to decreased accumulation of misfolded proteins, improved folding, and increased production of proteins such as IgG1, Fc-fusion protein, and interferon-β in CHO cells [25]. Another strategy is engineering the vehicle trafficking system to transport proteins from the ER to the cell surface. The production of secretory embryonic alkaline phosphatase was able to increase threefold when Peng et al. [26] overexpressed exocytic SNARE proteins such as SNAP-23 and VAMP8 [26]. Overexpressing ceramide transferase protein, involved in ER to Golgi transport, increased the output of tissue plasminogen activator, human serum albumin, and IgG in CHO cells [27].

3.2.4 Genetic engineering (knockdown or knockout strategies) of host cells for enhanced secretion

There are various activities of the discovered genes, and altering one gene may only have a little effect on the release of proteins. This highlights the need for multiple modifications, such as knockout, knockdown, or the coordinated overexpression of several

genes [28]. Host cell engineering strategies have significantly improved productivity in biopharmaceutical production, involving traditional recombinant DNA technology and RNA interference for stable gene suppression. Recent advancements in omics technologies have provided a nuanced understanding of cellular physiology, leading to identify gene targets specific to host cell lines. Focus on the secretory machinery has revealed the importance of ER chaperones and proteins in enhancing antibody and recombinant protein productivity. The overexpression of specific chaperones, foldases, and global regulators like XBP-1s (X-box binding protein 1) has demonstrated effectiveness [29–31]. Fierro et al. (2023) presented a DiCre-based knock-sideways tool, knockER, enabling the conditional fusion of KDEL (Lys-Asp-Glu-Leu) to secreted proteins, redirecting them to the ER, and offering new possibilities for studying secreted proteins [32]. Additionally, introducing Blimp1β into CHO cells has improved the secretory capacity by activating vital elements of the unfolded protein response and mechanistic target of rapamycin (mTOR) signaling [33]. mTOR, an important protein involved in protein synthesis, is known to improve various cellular processes, including cell growth, proliferation, and survival. The specific productivity of therapeutic immunoglobulins is significantly increased by overexpression of mTOR in CHO cells [34]. While Zhang et al. [35] emphasize the importance of genetic engineering to alleviate ER stress and increase productivity [35], He et al. [36] discuss the emerging role of circular RNAs (circRNAs) in posttranscriptional and translational gene regulation, highlighting their interactions with RNA-binding proteins. Their research reveals that circRNAs can modulate gene expression, ultimately affecting mRNA translation [36].

Enhancing the secretory capacity of mammalian production cells through genetic engineering offers a promising approach to increase productivity. Studies have shown that overexpression of various genes involved in the secretory pathway or manipulation of cytoskeleton dynamics can significantly improve recombinant protein production [37]. These diverse approaches highlight the continuous innovation in host cell engineering to optimize recombinant protein production.

3.3 Engineering glycosylation

Glycosylation, a frequent posttranslational modification, affects various physiological and pathological processes, including migration, cell growth, tumor invasion, host–pathogen interaction, differentiation, transmembrane signaling, and cell trafficking. There is a substantial history of modifying glycosylation in various organisms, including mammalian cells, plants, fungi (yeast), and bacteria, using genetic techniques, resulting in a repertoire of well-characterized glycosylation mutants. The glycosylation engineering aims to modify therapeutic glycoproteins' size, charge, and solubility, preventing swift elimination from the bloodstream. Additionally, glycoengineering has enhanced the existing therapeutic approaches or created novel modalities. Glycans can act as ligands for lectin recep-

tors, directing therapeutics to specific cells. Recent advancements, particularly in gene editing, offer precise control over glycosylation engineering, and the potential of the field appears boundless with expanding knowledge. The protein glycosylation can be divided into two main groups: *O*- and *N*-glycosylation. *O*-glycosylation is further divided into mucin-type and *O*-GlcNAcylation. The cotranslational and posttranslational processes involve glycosyltransferase enzymes, utilizing specific sugar nucleotide donors to attach carbohydrates to proteins, lipids, polypeptides, or polynucleotides [38–40].

Producing therapeutic enzymes for mammalian administration presents challenges due to specific glycosylation patterns crucial for ensuring correct biological activity, functionality, stability, and particularly low antigenicity. Over 50% of human proteins are recognized as potentially glycosylated, with many having multiple glycosylation sites. Protein glycosylation occurs at the carboxamide side chain of asparagines (Asns), glutamines (*N*-linked glycosylation), or the hydroxyl group of serine/threonine side chains (*O*-linked glycosylation) [41].

3.3.1 Types of glycosylation

3.3.1.1 *N*-linked glycosylation

N-glycosylation is a process initiated in the ER. Still, specific structural elements such as core fucosylation and branching are introduced later in the secretory pathway in the Golgi apparatus. *N*-glycans are modified by a series of sequentially acting glycosidases and glycosyltransferases that modify glycans in the Golgi apparatus and consequently dictate and ultimately determine the glycan profile of the whole cell. However, the structural modification of *N*-linked glycans is a complex process resulting in numerous glycan structures [42].

N-glycosylation plays a crucial role in the effector functions of IgG antibodies, and its clinical use includes therapeutic IgG antibodies with *N*-glycosylation tailored to enhance their cytotoxic properties [38]. *N*-Acetylglucosamine bound to asparagine (GlcNAcβ1-Asn) is the most common bond. The complexity and diversity of *N*-glycan structures and their implications in disease progression are still challenging. *N*-linked protein glycosylation begins with synthesizing the oligosaccharide precursor in the cytoplasm, which is then translocated to the ER lumen. The oligosaccharide precursors are transferred to the Asn residue of a newly formed protein when they undergo some modifications. Some trimming of the oligosaccharide chain is subsequently done in the ER, and the glycoprotein moves to the Golgi apparatus, where many additional sugar branches could be attached. *N*-glycosylation regulates the interaction of receptors and ligands with each other, coregulatory molecules, and distinct membrane domains in intact cells to alter signal transduction [38, 41].

Protein glycosylation encompasses the protein structure, secretory protein load, Golgi transport mechanism, enzyme protein levels, availability of monosaccharide nucleotides, and the organization of glycosylation enzymes within the Golgi apparatus [42].

3.3.1.2 *O*-linked glycosylation

O-glycosylation begins with the addition of *N*-acetylgalactosamine (GalNAc) to the hydroxyl group on certain serine or threonine residues. This process takes place in proteins traversing the Golgi compartment, facilitated by an enzyme known as *N*-acetyl galactosaminyltransferase (GalNAc-T), which transfers the GalNAc residue to the side chain of either serine or threonine. So far, it has been identified and characterized 15 unique members of the GalNAc-T family in mammals, potentially with up to 24 isozymes expressed in specific tissues. In *O*-linked protein glycosylation, posttranslational modifications start in the Golgi by attaching a GlcNAc molecule to the protein's serine or threonine residue. Next, multiple other sugars, such as sialic acid, can be added to the structure. *O*-glycosylation is crucial in various biological processes, such as pathogen interaction, cell adhesion, and proteolytic activities. Abnormal glycosylation has been linked to numerous diseases [43, 44].

O-glycosylation influences the protein structure and primarily affects surface-exposed regions. Mucin-type *O*-glycosylation, characterized by GalNac binding to serine/threonine residues, is predominantly found in mammalian-secreted and membrane-bound mucins. Bioinformatics tools such as NetOGlyc4.0 and ISOGlyP have been developed to estimate mucin-type *O*-glycosylation sites with approximately 75% and 70% accuracy, respectively [45, 46]. One of the primary challenges in *O*-glycobiology is still figuring out how the glycosyltransferases involved in *O*-glycan production in the secretory pathway are organized and vary dynamically [47].

3.3.2 Strategies for engineering glycosylation

Glycosylation is essential in many aspects of cancer development and progression, influencing tumor growth, evasion of immune surveillance, and metastasis, and also in determining the quality of recombinant glycoprotein therapeutics, particularly mAbs and protein stability [40, 48]. In cancer, glycosylation impacts critical processes like growth factor signaling, making it a target for biomarker identification. Glycosylation changes serve as indicators of stem-cell-like phenotypes within cancer and healthy tissues. Moreover, these alterations contribute to developing cancer-specific glycoproteins and can be exploited for therapeutic interventions. For instance, glycosylated biologics, such as otelixizumab and mogamulizumab, promise a therapeutic

potential in treating conditions like T-cell lymphoma and type 1 diabetes, demonstrating the diverse applications of glycosylation research in cancer therapeutics [40].

Genetic approaches can alter cellular glycosylation abilities: knockdown and overexpression are the most commonly used genetic methods. Knockdown is not successful in mammalian cell lines as in plants and *Drosophila*. The most important reasons for this are low efficiency and glycosyltransferase activity. Overexpression introduces desirable glycosyltransferase activities through glycogene transfection. However, it lacks control over genomic integration sites, expression levels, and gene copy numbers, disrupting glycosylation patterns and instability of introduced traits. Precision genome editing tools like nucleases ZFN (zinc finger nuclease), transcription activator-like effector nucleases, and CRISPR (clustered regularly interspaced palindromic repeats)/Cas9 have overcome challenges in knockout and knock-in strategies, enabling specific and efficient gene manipulation across all cell types. These tools extend beyond gene knockout or knock-in to activate silent genes, mimic disease mutations, and insert foreign genes at precise genomic locations [49].

3.3.2.1 *N*-glycosylation engineering

In the realm of glycoengineering, knockout strategies, such as targeting the MGAT1 (alpha-1,3-mannosyl-glycoprotein 2-beta-*N*-c2cosaminyltransferase) gene, have been pivotal in modulating glycosylation patterns for enhanced antibody-dependent cellular cytotoxicity through increased binding affinity to the Fcγ-IIIa receptor [50]. Significant strides have been made in *N*-glycosylation pattern alterations, notably the elimination of core α1-6-fucosylation in CHO cells, achieved through overexpression of bisecting GlcNAcT-III (MGAT3) or gene editing to knockout FUT8 alleles. In human HEK293-T cells (immortalized human embryonic kidney cells), GlycoDelete produced antibodies with truncated *N*-glycans, affecting Fcγ receptor (FcγR) affinity. Efforts to achieve homogeneous α2-6-sialylation faced challenges, but the GlycoDelete strategy and precise gene editing in CHO cells provided a framework for enhancing *N*-glycan homogeneity [49]. The GlycoDelete strategy further reduced *N*-glycan structural heterogeneity, promoting galactosylation and sialylation and showcasing advancements in modifying glycosylation patterns with applications in therapeutic protein production [51]. Moreover, MGAT1 disruption in CHO cell lines, facilitated by ZFN-mediated genome editing, results in consistent Man-5 (*N*-linked mannose-5) glycan glycosylation, offering specificity and efficiency for producing therapeutic proteins targeting the mannose receptor in cancer vaccine applications [52]. Sialylated IgGs, bearing nine mutations in the MGAT1 gene, exhibit reduced FcγRIIIA affinity, shedding light on the glycosylation enzyme's structural intricacies and influencing antibody function [53]. These glycoengineering approaches collectively contribute to the precision and versatility of manipulating glycosylation for various therapeutic applications.

Glycosylation of enzyme levels critically shape glycan biosynthesis, influencing the characteristics of therapeutic proteins [42]. Overexpressing α2-6-sialyltransferases in CHO cells enhances antibody production with α2-6-linked sialic acids, mirroring human blood glycosylation patterns [54]. The GlycoDelete glycoengineering method streamlines mammalian cell Golgi *N*-glycosylation, yielding therapeutic proteins with simplified and sialylated trisaccharide *N*-glycan structures, offering improved consistency and performance [51]. Natural and artificial miRNAs were harnessed to regulate glycosylation in CHO cells, demonstrating their versatility for fine-tuning mAb fucosylation and influencing various glycan features [55]. Genetic engineering in mammalian cells significantly advances the understanding of genetic and biosynthetic control over the cellular glycome, highlighting the vital roles of glycans in development, health, and disease [56]. Various strategies such as small interfering RNA (siRNA) and small hairpin RNA have regulated sialidase gene expression in CHO cells, impacting interferon-gamma sialylation [57]. Glycoengineering techniques targeting the fucosyltransferase enzyme, such as knocking down or knocking out the FUT8 gene, enable the production of antibodies without fucosylation, presenting strategies for modifying protein *N*-glycosylation pathways in mammalian cells [58]. Mammalian cell lines like CHO, BHK (baby hamster kidney fibroblasts), NS0 (recombinant murine cells), and C127 (murine mammary epithelial tissue-derived cells) are extensively utilized in pharmaceutical protein production. However, efforts to engineer these cell lines aim at achieving consistent and specialized glycosylation patterns, emphasizing the importance of overexpressing glycosyltransferases for improved glycan structures in recombinant proteins [59].

3.3.2.2 *O*-glycosylation engineering

Despite various *O*-glycosylation types performed by mammalian cells, there is limited interest in *O*-glycans for recombinant therapeutics. Some clinically used coagulation factors and drugs, like erythropoietin (EPO) and Enbrel, carry *O*-GalNAc, *O*-Fuc (*O*-fucosylation), and *O*-Glc glycans. However, engineering *O*-GalNAc glycans is complex due to the involvement of up to 20 polypeptide GalNAc transferases.

In the realm of *O*-glycosylation engineering, researchers focus on manipulating CHO and HEK293 cells by knocking out and knocking in GALNT (polypeptide *N*-acetylgalactosaminyltransferases) genes, providing insights into the control of *O*-GalNAc glycans [49].

Concurrently, efforts in synthetic glycobiology and cell surface display systems involve the presentation of glycan pathways on cell surfaces for research on glycosylation biosynthesis genes and exploring biological functions associated with specific glycosylation structures [60]. Gene editing technologies, particularly CRISPR/Cas9, facilitate precise modifications in glycosylation-related genes, exemplifying targeted ge-

nome editing for refined glycoengineering [61]. These advancements collectively contribute to the diverse and precise landscape of glycoengineering technologies.

Prabhu et al. [50] utilized CRISPR/Cas9 to modulate glycosylation in CHO cells, achieving independent control over galactosylation and fucosylation in the expressed IgG. By knocking out enzymes related to uridine diphosphate galactose and guanosine diphosphate fucose synthesis in CHO cells expressing IgG, the researchers achieved predictable and independent control over galactosylation and fucosylation [50]. Wong et al. [62] investigated intracellular glycosylation activities in CHO cells, highlighting the cell line's historical significance in glycoengineering [62]. Reinl et al. [63] demonstrated Golgi engineering in CHO cells to express novel Asn-linked oligosaccharide structures. Knocking out essential glycosyltransferase genes (FUT8 and β4GALT1) and integrating synthetic glycosyltransferase genes into the genome allowed fine-tuning of fucosylation and galactosylation levels. The system regulated by small-molecule inducers provided simultaneous and independent control, influencing antibody binding to cell surface receptors and impacting effector functions. This engineered platform offers a versatile tool for tailored glycosylation [63].

Yang et al. [64] aimed to achieve glycan remodeling of recombinant EPO for homogeneous glycoforms. Despite attempts to modify EPO expressed from HEK293T cells using endoglycosidase, success was limited [64]. Konstantinidi et al. [39] utilized a glycoengineered cell-based platform to study *O*-glycosylation in mucin tandem repeat domains. They discovered that potential Ser/Thr *O*-glycosides were fully occupied when expressed in human embryonic kidney 293 SimpleCells, offering insights into mucin *O*-glycosylation. The methods established in this study allow for a more comprehensive examination of native mucins and shed light on the *O*-glycosylation of mucins and mucin-like domains [39]. Prati et al. [65] demonstrated that simultaneous regulation of enzyme activities in CHO cells represents a significant advance in metabolic engineering. They reported the suppressing of CHO cell ST3Gal1 (ST3 beta-galactoside alpha-2,3-sialyltransferase 1) gene expression and overexpression of human C2GnT (Core2 β-1,6-*N*-acetylglucosaminyltransferase) gene to regulate the *O*-glycosylation pathway [65]. Moreover, Dinter et al. [66] developed tricistronic vectors for stable expression of two glycosyltransferases in CHO cells, showcasing efficient glycosylation and biosynthetic efficiency. Thus, additional glycosylation routes can be provided to CHO cells by multicistronic vectors expressing glycosyltransferases, enabling the in vivo glycosylation of target proteins with the appropriate glycan moiety [66]. In addition, Heffner et al. (2021) discussed strategies to control *O*-glycosylation by altering GnT (*N*-acetylglucosaminyltransferase) activity. Research indicated that elevating C2GnT levels in CHO DG44 cells might impact T-cell activation. Additionally, combining C2GnT overexpression with ST3Gal1 suppression could alter the *O*-glycosylation pathways. This study implies that cellular engineering can concurrently adjust the levels of competing glycosylation enzymes [67]. Collectively, these studies contribute to developing diverse and precise glycoengineering approaches with implications for therapeutic protein development.

3.4 Engineering cellular communication

Cellular communication has a significant role in the life cycle of a cell. It affects the signaling pathways that control cellular differentiation, proliferation, metabolism, and apoptosis. As cellular communication affects various pathways and causes numerous different responses, it can be controlled in many ways, resulting in a wide range of options for cancer treatment [68].

3.4.1 Extracellular vesicles

EVs are crucial for cancerous cellular communication, considering they provide signaling via packaging and delivery of bioactive constituents from one cell to another as well as they are an important target to optimize protein secretion as mentioned above [69]. The secretory pathway, a crucial facet of the vesicle trafficking system, provides the precise and regulated distribution of proteins, metabolites, and molecular cargo among and across cellular compartments [70]. Therefore, exosomes can be manipulated via engineering secretion frequency, packaging specificity, cytosolic merge ability, and targeting of exosomes to be used for natural carriers of various molecules [71]. For instance, Kojima et al. [72] created synthetic-biology-inspired EXOtic (EXOsomal transfer into cells) devices (Figure 3.2) that are created by manipulating all these aspects in a time of need. It consists of a system creating EVs that package expressed mRNA to release for targeting cells, producing packaged mRNA delivered and internalized through exosomes [69, 72]. Similarly, smart exosomes were developed [73] to target tumor-draining lymph nodes by engineering the exosome surface to exhibit L-selectin and OX40 ligand [73]. It is a comprehensive study since the cytosolic merge ability and targeting were achieved at the same time.

Moreover, immature dendritic cells (DCs) of mouse were engineered to generate exosomes with αv integrin-specific iRGD peptide (CRGDKGPDC) on the surface by genetically fusing the peptide and exosomal membrane protein (Lamp2b) for enhanced tumor targeting, and the loaded doxorubicin was delivered to the tumor site with enhanced specificity [74]. Therefore, EVs can be a promising tool to tune cellular communication as a more specific cancer therapy option.

3.4.2 Manipulating direct cell contact for diagnosis and therapy

Mammalian cells can communicate directly with molecules on the surface of other cells through proteins, fats, and carbohydrates, and their various complexes were displayed on their cell membranes. This causes similar or different signaling pathways to be affected in both cells and cellular responses [75]. Chimeric receptors can be used to stimulate directed cell migration, which can be a promising approach for reduce-

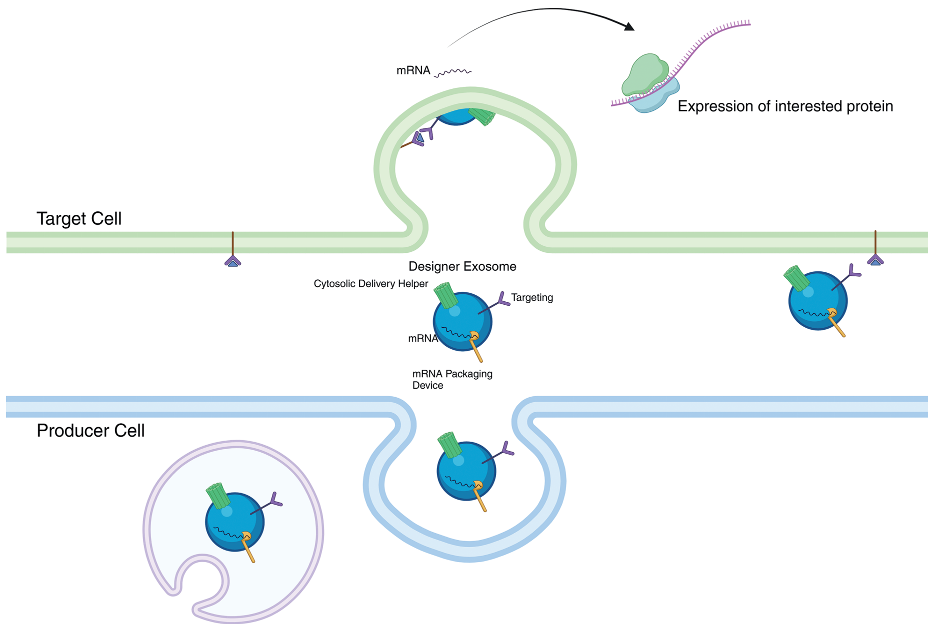

Figure 3.2: Schematic representation of the exotic device. It exhibits cytosolic delivery helper, targeting protein, and mRNA packaging devices on the surface of the designer exosome that packaged mRNA. Target cells internalize exosomes after recognition and cell membrane assembly of the exosome, and mRNA is translated to express the target protein in the target cell [69, 72].

ment of proximity between the therapeutic and the target cell. For example, fused IL-6R extracellular domain to vascular endothelial growth factor receptor intracellular domain on cells can be used for targeting IL-6-secreting senescent cells as a result of activated ros (Ras homolog family member A) GTPase activation [76]. Most of the direct cell contact-based therapies rely on the engineered cell therapies.

Mesenchymal stem cells (MSCs) are nonhematopoietic multipotent cells, which have the potential to differentiate into chondrogenic, osteogenic, and adipogenic cells. It is known that these cells display some specific antigens on their membranes and can migrate to tumor areas for the purpose of tissue repair and reconstruction. Therefore, in treatments where immune surveillance is maintained, the possible use of MSCs in targeting tumors and delivering potential cargo has brought to the fore their potential for treatment and diagnosis. For example, in thyroid cancer, MSCs engineered to express sodium iodide symporter (NIS) can be used to target iodide-concentrating thyroid glands, and this system can be used for imaging purposes with radioiodine labeling and also in the treatment of tumors in combination with radionuclide. There are even studies showing that imaging in other types of cancer can be done successfully in this way by providing ectopic NIS expression. A limiting factor for this relatively targeted system may be the damage experienced by off-target cells following MSC recruitment. To avoid

this situation, Schug et al. [78] developed a system that allows NIS expression to be selectively induced and is sensitive to TGFB1-inducible SMAD under hypoxia conditions, thus planning to increase the effect of targeting [77, 78]. Currently, Mayo Clinic conducted a treatment for patients with recurrent ovarian cancer in phase II as MSCs expressing NIS were infected with a genetically engineered measles virus [79]. Similarly, MSC-TRAIL (MSC-tumor necrosis factor-related apoptosis-inducing ligand) presents a targeted treatment of lung cancer through engineered MSCs to express TRAIL, which specifically promotes apoptosis in cancer cells in phase II [80].

DCs are immune cells that secrete antigens on their cell surfaces, stimulating the immune system in the presence of a possible pathogen or cancer cell, thereby creating an immune response. In addition, DCs have been studied for decades as an active immunotherapy in cancer treatments due to their ability to promote T-cell activation, apart from other antigen-presenting cells, and to be transported to lymph nodes, even if limited [81, 82]. In this context, Koch et al. [84] created a system including DCs transfected with IKKβ-activated RNA for T-cell and Natural killer (NK) cell activation to treat uveal melanoma, and this vaccine is in phase I trial [83, 84]. DC implantation can also be used as a supportive treatment. For example, it has been observed that when DCs are injected before nephrectomy in patients with metastatic renal carcinoma, they boost the immune response by inducing dense infiltration of T cells and may therefore increase the survival [81]. In addition, DCs are being investigated for use in the treatment of brain cancer because they can cross the barriers of the central nervous system. On this level, a phase III study supported by Oslo University Hospital to treat glioblastoma, which has a very high mortality rate, involves glioblastoma stem cells, mRNA transfected with DC vaccines injected together with the antiapoptotic peptide survivin, and human telomerase reverse transcriptase [85].

CAR-T (chimeric antigen receptor (CAR) T cells) are the most advanced immunotherapeutic treatment method among all chimeric antigen receptor-based treatments. Essentially, it involves activating T cells taken from the patient and reengineering them through CAR. Kymriah, Yescarta, and Abecma are among some CAR-T therapies approved by the FDA. Genetic engineering of CAR-T cells must be done very stringently to avoid toxicity in the body. One of the existing methods to do this is to control CAR expression through the inclusion of ON and OFF switches and molecules such as tetracycline and doxycycline. In addition, drug-inducible caspase-9 (iCasp9) is used as a kill switch and induces the apoptosis pathway. In addition, antigen escape and tumor antigen heterogeneity can be prevented by methods using improved circuits such as AND, OR, and NOT gates, which are used to increase the effectiveness and specificity of tumor targeting. Another approach involves genetic arrangements that enable CAR-T cells to interfere with immunologic factors in target cells. In fact, T cells obtained with this method are also known as Armored CAR-T cells. An example is an approach in which immune-checkpoint inhibitory molecules in cancer cells are inhibited by CAR-T cells via CRISPR-Cas9 or siRNA. Although the effectiveness of CAR-T has been proven, it has some limitations due to the costs and disruptions that may occur

during the processing of T cells taken from the patient. In addition, there is a need for more tuned and controllable systems to prevent tumor antigen escape. Interaction of CAR-T with normal and cancer cells can be intervened by genetic engineering through tuning of CAR expression and activation profile (Figure 3.3) [1, 86].

3.5 Engineering cellular metabolism

Mammalian cell metabolism encompasses producing and using energy necessary for cell growth, functionality, and survival. These processes involve transforming nutrients into energy and building blocks by cells through various chemical reactions [90]. The basic steps include glycolysis, pyruvate oxidation, tricarboxylic acid (TCA) cycle (Krebs cycle), oxidative phosphorylation, lipolysis, beta-oxidation, amino acid catabolism, pentose phosphate pathway, gluconeogenesis, and glycogenolysis [91]. Glycolysis is responsible for the production of energy and pyruvate, a valuable intermediate molecule transported to the mitochondria to be used in steps such as the Krebs cycle. Glycolysis is essential because it causes rapid energy provision and has a place in cancer metabolism [92]. During the oxidation of pyruvate followed by glycolysis, pyruvate is converted to acetyl-CoA, and carbon dioxide is released. Another fundamental step, the TCA cycle, plays a central role in energy production in the presence of oxygen. In the TCA cycle, NADH (reduced nicotinamide adenine dinucleotide) and FADH2 (flavin adenine dinucleotide) molecules are formed to be utilized for energy production during oxidative phosphorylation. In addition, the output of many intermediate products required for other metabolic pathways, such as gluconeogenesis, lipogenesis, and amino acid synthesis, is also achieved at this stage. Oxidative phosphorylation involves the oxidation of NADH and FADH2 molecules obtained in previous reactions in the cell during the electron transport chain (ETC) and the conversion of the proton gradient resulting from electron flow into ATP (adenosine triphosphate) by the ATP synthase enzyme. Water is released using oxygen during this highly energy-efficient phase [93]. At lipolysis and beta-oxidation, acetyl-CoA is produced as a result of the oxidation of fatty acids during fat lysis and is converted to ketogenic intermediates [94]. Amino acid catabolism is also a critical step, where carbon skeletons and ammonia are formed. In addition to contributing to energy production by entering the TCA cycle, carbon skeletons can also be used in protein synthesis [95]. The pentose phosphate pathway works parallel with glycolysis, producing ribose-5-phosphate necessary for nicotinamide adenine dinucleotide phosphate and nucleic acid synthesis. Finally, gluconeogenesis enables glucose production from various sources and glycogenolysis from glycogen, allowing cell metabolism to produce energy when required. When viewed this way, many intermediate molecules can be created in more than one stage and used in various stages, revealing the complexity of mammalian cell metabolism [96]. Mammalian cellular metabolism is an integrated

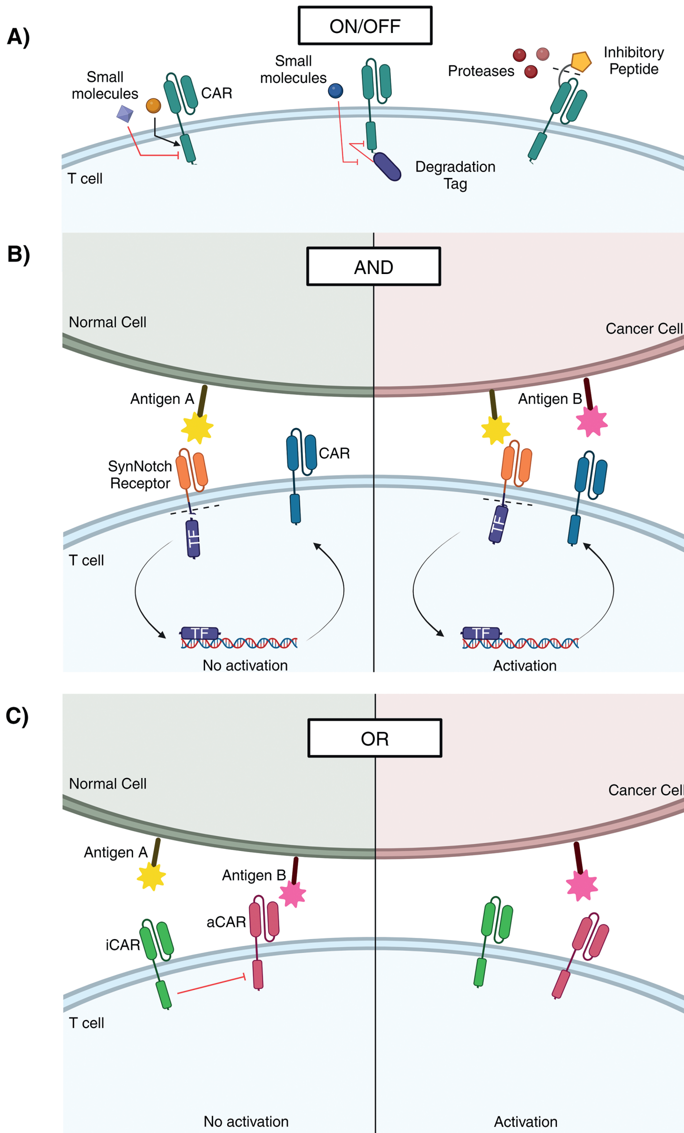

Figure 3.3: Schematic representation of CAR-T cell engineering methods to increase efficiency and specificity. (A) ON/OFF switches for controlling the expression and activity of CARs. Small molecules can be used for controlling the expression of CARs on the surface. Also, small molecules or low levels of O_2 can inhibit the degradation tags that CARs fused to activate the system with control, which is initially found in the OFF position. In addition, inhibitory peptides can be fused to the CARs where proteases are found in the tumor microenvironment to activate them on the tumor site. (B) An example of the use of AND gates in CAR-T technology. Upon recognition of antigen A with scFv extracellular domain, transcription factor (TF) on the synNotch receptor is cleaved and activates the transcription of CAR, yet this system only gets activated when antigen B is present since recognition of it with CAR is necessary for T-cell activation. (C)

system that ensures efficient energy production and use. Cells can regulate and adapt these pathways according to energy needs [97].

Cancer metabolism has become a studied and known phenomenon since Otto Warburg's breakthrough in the early twentieth century. The model developed by Warburg based on his observations on cancer cells was called the Warburg effect or aerobic glycolysis and was based on the fact that cancer cells, unlike normal cells, use glycolysis even in the presence of oxygen [98]. At that time, it was thought that cancer cells preferred glycolysis over mitochondrial respiration. It has been observed that oncogenes and pathways such as MYC, AKT, and mTOR increase glycolysis in the cancer scenario [99]. Also, due to hypoxia in cancer cells, the formation of ATP from substrates is directed to be carried out through glycolysis rather than the TCA cycle. The increased tendency to glycolysis is associated with negative feedback from accumulating irreducible NADH. However, this situation, which occurs due to inhibition of the TCA cycle due to hypoxia, should not be confused with NADH accumulation due to ETC error, as this may result in growth impairment and cell death [100]. In line with this, it is known today that mitochondrial respiration and the TCA cycle are still necessary for producing metabolites, such as oxaloacetate, which are essential for supporting tumor development [101]. This requirement is reinforced by the observation that nucleotide synthesis is also supported by glycolysis, one-carbon metabolism, and the TCA cycle in recent studies in pancreas and lung cancers with KRAS (Kirsten rat sarcoma virus gene) and p53 loss profiles [102]. This whole order is accompanied by reactive oxygen species (ROS), and cancer growth is promoted due to oxidative stress altering the metabolism [91]. Since cancer cells constantly develop, they require antioxidant supplements to increase survival through intensive nucleotide synthesis and removing toxic products [95]. Additionally, it is known that the cell exhibits a different metabolic profile during metastasis than that in a benign mass. This situation can be explained by the fact that the location of primary cancer cells, which try to dominate healthy cells in the secondary tumor area, is shaped by the metabolism according to the tissues' nutritional profile and the primary cells' capacity to adapt to this profile.

It is known that in some types of cancer, high expression of genes that interfere with the metabolic flow, such as MTC1, and genes that promote epithelial–mesenchymal transition and high production of ROS metabolites contribute to this profile [103]. In addition, the metabolic processes of cancer cells trying to adapt to the secondary tumor site can be shaped according to the nutritional content of this area. For example, when breast cancer cells invade the brain, their phosphoglycerate dehydrogenase expression increases, which enables their brain cells to make a metabolic adaptation regarding

Figure 3.3 (continued)
An example of the use of OR gates in CAR-T technology. Upon recognition of antigen A with iCar (inhibitory CAR), iCAR inhibits the aCAR (activating CAR), and this results in inactivation yet in the presence of antigen B only, iCAR cannot suppress the aCAR, and T-cell is activated. These two gate usages in CAR-T cells represent the tuning of CAR-T specificity toward cancer cells [87–89].

nutritional intake and nutrients in the environment, which increases their survival. Identifying targets that increase survival by causing such metabolic alteration creates targets for drugs that can be developed against metastatic cancers [104].

A good understanding of cancer metabolism is important for developing cancer cell lines and media optimization of these cell lines. For example, Akt or cMyc overexpression is found to be related to higher glucose uptake and lactate production. Therefore, manipulation of Akt or cMyc activations in cell lines such as CHO and NS0 can prevent lactate production, which burdens the cell, or alter the glucose flux [105]. Also, BHK and HEK293 cells were engineered to express cytosolic pyruvate carboxylase, resulting in increased viability and production. Moreover, partial knockout of LDH in hybridoma cells was found to be related to an increased titer and reduced lactate production [106].

Engineering metabolism may also have certain benefits in cell-based therapies. This may increase cell survival or effectiveness. For example, it has been observed that reprogramming the metabolism of CAR-T and CAR-NK cells with stimulants such as CD28 increases the viability of CAR-T. Similarly, knockdown of CAR-IL-15 and cytokine-inducible SH2-containing protein in NK cells was observed to promote the glycolytic pathway and lead to higher in vivo activity [107]. It is known that even anti-inflammatory cytokine activity can be inhibited with NK cells via alteration in their metabolism [108].

3.6 Conclusion

Glycosylation is crucial in cancer development and progression and determines the quality of recombinant glycoprotein therapeutics. The ability of cellular glycosylation can be modified and improved by genetic methods. Unique synthetic biology methods enable specific and effective gene manipulations. Glycosylation is often viewed as posttranslational modification. The methods focusing on different aspects are examined further. First, *N*-glycosylation engineering focuses on increasing the production of therapeutic proteins through methods such as targeting the MGAT1 gene and the GlycoDelete method. Second, although *O*-glycosylation engineering has generally received limited interest for recombinant therapeutics, some clinically useful coagulation factors and drugs carry *O*-GalNAc, *O*-Fuc, and *O*-Glc glycans. Glycosylation engineering has come to the fore in tailoring glycan structures to achieve optimal therapeutic protein properties. Gene editing techniques such as CRISPR/Cas9, ZFNs, and siRNAs help to holistically unravel the molecular mechanisms governing glycosylation. Engineering protein release and glycosylation patterns in mammalian cells have gained significant importance, especially in therapeutics.

The secretion of proteins requires several steps to proceed within the ER and Golgi apparatus. In this process, carrier vesicles pass from the donor compartment to

the target compartment, and in this transition, the interaction of COPI, COPII, clathrin, SNARE, and Sec1/Munc18 proteins is required for vesicle fusion. In this whole process, all proteins and pathways that play an active role can be directed to the desired purpose using synthetic biology techniques. In addition, secretion mechanisms can also be engineered directly. Optimization of signal peptides in CHO cells can increase the secretion of recombinant proteins, and this process involves optimization of signal peptides to increase the production efficiency of recombinant biotherapeutic antibodies. Manipulating vesicle transport plays a critical role in maintaining organelle homeostasis in eukaryotic cells and can increase protein secretion. These studies are frequently encountered in the literature as a result of the potential for knockdown, knockout, or overexpression of identified genes to significantly increase recombinant protein production. Thanks to these advances and genetic engineering methods that increase the production of recombinant proteins, optimize the secretion profiles of the produced proteins, and enable the developed proteins to emerge as high-quality products; cellular mechanisms have been better understood; and significant progress has been made to increase the efficiency.

Cellular communication is crucial for controlling differentiation, proliferation, and apoptosis. This complex communication system provides many options for possible cancer therapies. EVs, crucial for communication between cells, can be modified to transport active substances, offering precise cancer treatments. New creations such as synthetic biology-based EXOtic devices and intelligent exosomes demonstrate the possibilities of controlling vesicle trafficking for medical uses. Yet, there is a need for further development in the utilization of EVs since the actual concentration of vesicles that can reach the target points is generally low. Furthermore, the use of direct cell contact and chimeric receptors shows the potential for boosting therapeutic cell movement and performance in tumor settings. The difficulty of engineering cell-to-cell contact engages with the variations between patients, which creates a need for personalized medicine. Also, it rises the necessity for a precise tuning since any efficient application would require more than one circuit. Still, these developments highlight the potential of cell communication engineering in creating advanced and efficient cancer therapies in the future. Engineered cell-based therapies hold great promise. Yet, there are certain advantages and disadvantages of different cell types. MSCs can be off-target, and this may lead to severe side effects or misleading scanning results. The ability of MSCs toward targeting should be increased with tumor-specific engineering, and this requires an understanding regarding MSC tumor tropism. DCs are also important in terms of immunotherapy, especially for brain cancer types, since they can pass the barriers, yet their representation of predetermined antigens on the surface may lead to decreased efficiency in terms of recognition ability for further mutated cells. Yet, this can be turned into an advantage with the future advancements on sequencing and personalized medicine since low mutations will lead to higher responses on the individual this time. Also, restrictions of DCs to migrate to the lymph system might require conditioning of the cancer area to promote migration as

a previous study [109] demonstrated that preapplication of tetanus/diphtheria toxoid promotes DC migration in the cancer area [109]. Similarly, survival of glioblastoma patient was increased with a recall antigen injection including enhanced DC migration [81]. Therefore, DC vaccination stands as a promising way to treat early stage cancer patients despite these limitations. Cell-based therapies stand out due to their potential to enable safe and effective therapy beyond other methods, thanks to redirected cell functions through methods such as gene therapy. Gene therapy has some translational limits due to problems such as integration, localization, and frequency that occur during transgene delivery. Techniques developed to prevent this – for example, controlled transgene integration with engineered transposase – will contribute to eliminating the difficulties of cell-based cancer treatments.

Treatment techniques can be customized to disrupt specific metabolic pathways necessary for cancer cell survival and proliferation by identifying metabolic route dependencies in cancer cell lines and describing metabolic subtypes [110]. However, this poses some difficulties. First, this strategy may become particularly challenging as key elements of the immune system, such as macrophages and B cells, also undergo metabolic reprogramming with antigen recognition and stimulation. As a specific example of this situation, it has been observed that T cells' tendency to glycolysis increases as a result of antigen recognition and stimulation. Although this is promising for the treatment of autoimmune diseases, it may reduce the immune response and may cause metabolic engineering-related methods not to be preferred in cancer treatment [111]. In this area, changes in diet, in addition to treatments, have also shown potential in halting cancer progression. For example, high-fat diets may contribute to the development of fatty acid-preferring metastatic cells, which may complicate the patient's treatment. This situation, combined with the patient's genetic characteristics, may make treatment difficult for cancer metabolism. Therefore, advances in cancer metabolism are needed to develop a personalized medicine approach. In this way, methods that can reduce side effects in cancer treatment and can actually be quite effective are identified. In addition, the relationship between dormancy and metabolites, which can sometimes be seen in the case of metastasis, requires further investigation [112]. On the other hand, engineering cell metabolism can be a complicated task because healthy cells can also be quite sensitive to the targeted treatment approach. However, development in this field is used to increase the product yield and cell mass.

Engineering mammalian cells presents several challenges despite the advancements in genetic engineering technologies. This process is hindered by inexplicable failures, limiting the efficiency of circuit engineering in these cells. Additionally, the complexity of mammalian cell systems, including limited experimental data and complex regulatory mechanisms, presents obstacles in metabolic network analysis and engineering [113]. Furthermore, intercellular variation impacts therapeutic efficacy and safety, influencing allogeneic cell-based therapy development caused by risks like graft-versus-host disease. To overcome these difficulties, engineering mammalian cells for cancer needs to prioritize the versatility and adaptability of them while considering po-

tential trade-offs in therapeutic applications. For example, utilizing prokaryotic two-component regulatory systems as tools for creating orthogonal signaling pathways in mammalian cells shows the dose-dependent transduction of small-molecule ligands [114]. Utilization of mutually orthogonal aminoacyl-tRNA synthetase in mammalian cells demonstrated precise control over protein engineering [115]. These systems have prioritized enabling specific communication between engineered cells for various applications, but the trade-offs associated with insufficient orthogonal signaling pathways emphasized the limited implementation of these tools for therapeutic applications. In summary, trade-offs between engineering tools based on current advancement and unsolved potential challenges require crucial considerations and further prioritizations of some qualities in the design of mammalian cells for cancer treatment. The application of engineering tools based on synthetic biology promises potential solutions for current trade-offs and setbacks [116].

Overall, the conventional method of focusing on individual genes in cell manipulation is changing, as there is a growing understanding of the necessity to manipulate multiple genes in different cellular pathways. Understanding the interconnected cellular processes in mammalian cells helps to tackle the complexity of engineering mammalian cells. Yet, this complexity can also provide various alternatives for new approaches.

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