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# Increasing Production of Therapeutic mAbs in CHO Cells through Genetic Engineering

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# INCREASING PRODUCTION OF THERAPEUTIC MABS IN CHO CELLS THROUGH GENETIC ENGINEERING

by

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ABSTRACT

Increasing Production of Therapeutic mAbs in CHO Cells through Genetic Engineering

by

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Large scale production of therapeutic monoclonal antibodies (mAbs) has become a continuously relevant topic in healthcare. As the primary producer of mAbs, Chinese Hamster Ovary (CHO) cells play a vital role in manufacturing these molecules. Despite improved practices in scale-up and cell culture media optimization, cell line development remains the most effective route to increase mAb productivity.

In CHO cells, the gene *C12orf35* has been identified as a candidate for consideration to improve productivity. Knockout of this gene has led to increased productivity and altered cell morphology. Additionally, this site has been shown to have a high transcription rate. These characteristics make *C12orf35* a desirable candidate for both knockout and knock-in gene editing using the CRISPR/Cas9 system. This research demonstrated increased viable cell density (VCD) and IgG titer in pooled CHO-M cells expressing a plasmid designed to knockout *C12orf35*.

Since *C12orf35* also represents a potentially valuable knock-in site, mammalian target of rapamycin (mTOR) was chosen as a candidate for testing. When expressed transiently in CHO cells, mTOR increases mAb productivity, VCD, and dramatically alters cell morphology. This implies that a stable clone expressing mTOR at *C12orf35* may have a drastically altered productivity profile due to the combined effects of both methods. An additional plasmid was designed to allow for mTOR knock-in at the *C12orf35* cut site.

(135 pages)

# PUBLIC ABSTRACT

## Increasing Production of Therapeutic mAbs in CHO Cells through Genetic Engineering

Charles Barentine

Between 2014 and 2018, the global market for therapeutic monoclonal antibodies (mAbs) rose from $60 billion to $115.2 billion with a projected value of $300 billion by 2025. These molecules are used to effectively treat some of the most challenging illnesses from auto-immune diseases to cancer. While mAbs are highly valuable with potent applications, their production at scale remains an outstanding challenge. These molecules are largely produced in Chinese Hamster Ovary (CHO) cells that require highly specific conditions to produce a useful product.

Genetic engineering presents one solution to overcome productivity limits. With the advent of CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated proteins) systems, engineering the CHO genome has never been easier. CRISPR/Cas9 allows for site-specific editing and gene integration. Within the CHO genome, a variety of sites have been identified that warrant further investigation for editing. Among these sites is the gene *C12orf35*. The deletion of *C12orf35* has been shown to lead to increased productivity in CHO cells. Additionally, *C12orf35* has been identified as a site with a high transcription rate, implying that genes at this site are likely to be expressed more frequently.

The gene coding for mammalian target of rapamycin (mTOR) has been demonstrated to alter CHO cell phenotype characteristics such as cell size, viable cell density, and antibody productivity when expressed transiently. This study aims to

evaluate the potential synergism of deleting the gene *C12orf35* by editing the gene coding for mTOR between a cut site made in *C12orf35*. Splicing the gene coding for mTOR at this site has the potential combined benefit of disrupting *C12orf35* while simultaneously stably expressing the mTOR gene at a highly transcribed site in the CHO genome.

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# Chapter 1

# Introduction

## Overview

Therapeutic monoclonal antibodies (mAbs) are highly specific antibodies utilized for significant therapeutic applications for diseases such as cancer, Crohn's disease, rheumatoid arthritis, ulcerative colitis, and many more (Shen, 2016). The popularity of mAbs is largely due to their high specificity and wide array of applications for disease treatment. Producing these antibodies on a large scale is becoming an increasingly lucrative business as the industry has been steadily growing over the past several decades to match ever-increasing demand. From 2008 to 2014, yearly sales of full-length mAbs produced in mammalian cell culture rose to over $60 billion (Ecker et al., 2014). In 2018, the global therapeutic monoclonal antibody market was valued at $115.2 billion. This value is projected to increase to $300 billion as early as 2025 (Lu et al., 2020). Addressing this demand requires investigation into manufacturing factors such as cell line choice, cell line development, process optimization, and quality assurance.

## Significance

Disease treatment has evolved quickly over recent decades. As new methods continue to be developed to combat some of the most challenging diseases, it is important to keep these treatments as accessible options with manageable costs. Even after a molecule has been developed, the significant barriers of productivity and

manufacturing scale remain. To keep healthcare accessible, it is important to start optimization as far upstream as possible. Incorporating a cell line specifically engineered for maximum productivity in the early stages of development provides an opportunity to attack this issue at its source. Higher productivity at the cell level means more affordable healthcare for the patient far downstream. While it is important to consider current market values and projections, the ultimate consideration should remain as patient impact, and how we as researchers can help keep the world healthy.

**Research Hypotheses**

This research will focus on the impacts of deleting a gene in the telomeric region of chromosome 8 in CHO cells and inserting a gene at this site for the purpose of increasing mAb productivity.

1. A Cas9 expressing plasmid can be successfully transfected into in-house mAb producing CHO line (CHO-M)
2. Knocking out the gene *C12orf35* will impact the productivity of CHO-M cells.
3. Knocking out the gene *C12orf35* will impact the viable cell density of CHO-M cells
4. An additional plasmid can be constructed to add mTOR gene to *C12orf35* site

**Objectives**

This study's overall objective is to determine how the deletion of genes in the telomeric region of chromosome 8 in CHO cells will impact their performance as a mAb manufacturing platform. We further aim to use this site as a location for insertion of genes known to positively impact productivity. This research aims to disrupt this region using CRISPR/Cas9 and simultaneously integrate a gene known to positively impact cell

performance when overexpressed. Furthermore, impacts on productivity, stability, and protein quality are considered. This can be broken down into five main objectives:

1. Obtain baseline culture information for the targeted cell lines. This includes historical stability and productivity.
2. Design and generate a system for deleting *C12orf35*
3. Test the impacts of deletion on productivity and stability with a growth study using pooled cultures and isolated clones.
4. Design and generate a repair template for insertion of mTOR gene at cut site.
5. Generate stable cell lines with improved productivity.

# Chapter 2

# Literature Review

## Brief History of Antibodies

Antibodies are a key component in immune systems that allow for recognition of foreign substances in the blood stream. These proteins are produced in B lymphocytes and function in recruitment of cytotoxic effector cells to a targeted antigen (Meyer et al., 2014). Since their discovery in 1890 as a substance that seemed to neutralize infection by the bacteria causing diphtheria (Behring, 1890), antibodies have held great promise for disease treatment. Early administration of antibody treatment took the form of serum therapy, which utilized the serum of immunized animals to directly treat humans (Winau & Winau, 2002). This method was used to treat a wide variety of viral diseases in the early 20th century (Alexander & Leidy, 1946; Hammon et al., 1953; Janeway, 1945; Luke et al., 2006) and developed into the field of passive immunization (Graham & Ambrosino, 2015). While effective, this form of treatment proved risky in humans with severe side effects resulting from an immune response to the animal serum (Casadevall & Scharff, 1994). While it was clear that this field held value, the risks needed to be mitigated for effective treatment to be possible. The field continued to develop towards an understanding of how antibodies are produced and how we may utilize this means of production for therapeutic purposes. In 1973, Dick Cotton and César Milstein demonstrated the successful fusion of two myeloma cell lines (Cotton & Milstein, 1973). This research was a milestone in antibody development because it

demonstrated that two separate cell lines, each individually producing distinct antibodies, could be fused to form a hybrid producing antibodies from both parental cell lines.

## Antibody Structure and Function

### *Immunoglobulin Classes*

Immunoglobulins are Y-shaped molecules consisting of two heavy chains and two light chains joined by disulfide bonds (Charles A Janeway et al., 2001). Within this structure are constant regions and variable regions with the variable regions providing the antibody functionality. Human immunoglobulins (Ig) are grouped into five different classes: IgM, IgG, IgA, IgD, and IgE. These classes are defined by the isotypes of heavy chains (μ, α, δ, γ, and ε) present in each class (Bengtén et al., 2000). These categories are based on the structure of the antibody, with IgG being the most used antibody for therapeutic applications (Woof & Burton, 2004).

When an immune response is initiated, IgM is the first antibody to be constructed and is an effective neutralizing agent during the early stages of a disease (Boes, 2000). This is also the first antibody to appear during neonatal development (Bengtén et al., 2000). IgM is the most common antibody isotype and its unique structure, along with its natural presence, allows it to respond quickly to infection. The IgM class is typically composed of 5 monomeric subunits. Each subunit consists of two light chains and two heavy chains, which are bound together into a pentamer by

disulfide bonds and a joining J (cysteine-rich polypeptide) chain. Human serum Ig content is approximately 10% IgM.

IgA is the most abundantly produced isotype in humans largely due to its strong presence in secretions, which require continuous replacement (Herich, 2017; Monteiro, 2010). In these secretions IgA functions to guard mucosa from bacterial infection and maintain a balanced microbiome (Fagarasan, 2008; Kerr, 1990). This antibody accounts for approximately 15% of total serum Ig in humans and exists both as a monomer and dimer. Monomeric IgA consists of two heavy and two light chains. While this form is the most common, dimeric IgA may also be formed as a dimer linked by disulfide bonds and a J chain (Kerr, 1990). Two subclasses, IgA1 and IgA2, exist in humans that differ in structure, function, and typical concentration. The primary structural difference between these subclasses is the length of the hinge region that connects the Fab arms and the Fc region (Woof & Kerr, 2004).

IgD is a monomeric immunoglobulin found at very low total Ig levels (0.2%) in human serum. IgD also consists of two heavy and two light chains with a hinge region that is particularly sensitive to proteolysis (Schroeder & Cavacini, 2010). The function of this antibody is poorly understood, but it is possible that this molecule is involved in homeostasis and B-cell fate (Geisberger et al., 2006). IgD is thought to be recently evolved as it has only been identified in primates and rodents (Bengtén et al., 2000).

IgE is present at the lowest total Ig concentration (0.002%) in human serum and was the last of the classes to be discovered (Sutton et al., 2019). Thus far, IgE has only been identified in mammals (Bengtén et al., 2000). This class also consists of two heavy

and two light chains connected by a hinge region. IgE possesses a high affinity to the FcεRI receptor found on mast cells and basophils. As such, bound IgE can help increase mast cell resistance to apoptosis. This indicates that IgE may play an important role in immune response to parasites and allergies (Kawakami & Galli, 2002).

IgG is by far the most abundant isotype in human serum at approximately 75% of total Ig content and has the longest serum half-life of all isotypes. Four subclasses, IgG1, IgG2, IgG3, and IgG4 have been identified. These subclasses exhibit variability in the constant regions of the heavy chains and express varied levels of functional activity despite a more than 90% similarity in amino acid sequence (Vidarsson et al., 2014). IgG1 is the most abundant (60% of IgG subclasses) subclass and typically responds to proteins, polysaccharides, and allergens. IgG2 is the second most abundant (32%) subclass and responds well to polysaccharides. IgG3 and IgG4 both constitute about 4% each of IgG subclasses. IgG3 responds to proteins and is associated with a strong inflammatory response. IgG4 responds mostly to proteins and allergens following repeated exposure (Vidarsson et al., 2014).

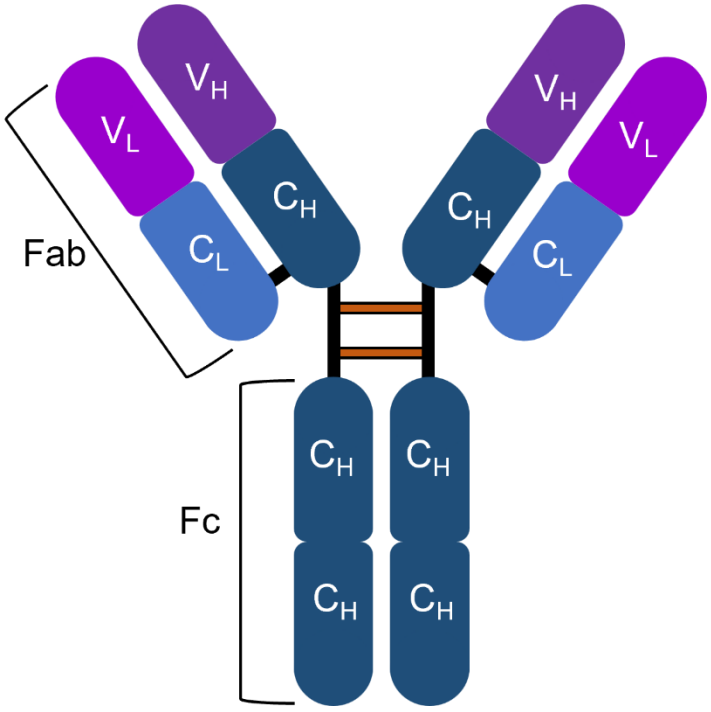
### *IgG Structure and Function*

The arms of IgG molecules, called fragment antigen-binding (Fab) fragments, are made up of two light chains ($V_L$, $C_L$) and two heavy chains ($V_H$, $C_H$), which are approximately 25 kDa and 50 kDa respectively (Carrara et al., 2021). The Fab fragments provide the variable portion of the molecule that is responsible for antigen binding. The lower portion of the molecule, known as the Fc region, is the constant region responsible for effector cell recruitment and molecule interactions (Figure 2.1). The Fc

region is capable of binding to Fc gamma receptors (FcγRs) expressed on the surface of cytotoxic effector cells, thereby recruiting these cells to a bound antigen (Meyer et al., 2014).

**Figure 2.1**.

*IgG Structure*




Targeted cell death may be achieved through effector cell recruitment and activation or delivery of conjugated cytotoxic agents. Another function of mAbs is to reduce pathway activity by blocking target receptors or ligands (Awwad & Angkawinitwong, 2018). The versatility and high specificity of mAbs make them an ideal molecule for use in targeted thereapeutic applications.

### *Polyclonal versus Monoclonal Antibodies*

Initial research surrounding treatment with antibodies utilized polyclonal antibodies (pAbs), which are derived from many different B cell lineages (yielding the 'polyclonal' nature of pAbs) and target the whole antigen as the result of an immune response. The multi-epitope binding action of pAbs provide increased sensitivity for a variety of applications and robustness against epitope variability(Ascoli & Aggeler, 2018). pAbs also benefit from their rapid generation times and stability but suffer from decreased consistency and concentration(Lipman et al., 2005). While extremely useful for some applications, the low productivity of pAbs and heterogenicity limit their application for pharmaceutical purposes. Monoclonal antibodies (mAbs), however, target a single epitope, are homogenous, and consistent compared to pAbs. mAbs may also be produced in immortalized cell lines, whereas pAbs are typically harvested from immunized animal blood(Leenaars & Hendriksen, 2005). This allows for relatively on-demand production in higher quantities than what would be reasonably supplied from pAb production.

## Antibody development

### *Hybridoma Technology*

The traditional pathway for antibody development uses a method known as hybridoma technology. Hybridoma technology was first developed by César Milstein and Georges Köhler in 1975, which earned them a shared Nobel Prize in 1984 in physiology

and medicine (Köhler & Milstein, 1975). Antibodies are manufactured solely by B cells, which can be harvested from the spleen (Alberts et al., 2002). This technology generates antibodies by injecting mice with a desired antigen, prompting an immune response. Mouse spleen cells are then isolated and fused with a myeloma cell line. These fused cells are cultured in a hypoxanthine-aminopterin-thymidine (HAT) selection medium. Aminopterin blocks the function of dihydrofolate reductase, which in turn inhibits *de novo* DNA synthesis. This forces cells to utilize salvage pathways that require media containing thymidine and hypoxanthine. Myeloma cells used for generation of hybridomas are genetically modified to have a non-functional thymidine kinase (TK) and/or hypoxanthine-guanine phosphoribosyltransferase (HGPRT), which are both required for the salvage pathways to function. Therefore, the myeloma cells will die off by themselves in HAT medium. The mouse splenocytes, while possessing functional TK and HGPRT, will eventually die off by themselves since their replication number is limited due to the nature of the cells (myeloma cells, however, are an immortalized cell line). With these conditions, the only surviving cells will be a fusion of myeloma and splenocytes (Parray et al., 2020). Surviving clones are then screened via an enzyme-linked immunosorbent assay (ELISA) for expression of the antibody of interest. Limiting dilutions are then performed followed by expansion of antibody expressing clones to produce the desired monoclonal antibodies.

### *Phage Display*

More recently, phage display technology has gained attention as a viable way to isolate antibodies against a specific antigen. For this discovery, the Nobel Prize in

Chemistry was awarded to George P. Smith and Sir Gregory P. Winter in 2018 (*The Nobel Prize in Chemistry 2018*, n.d.). This technology utilizes characteristics found in viruses that infect bacteria known as phages and made its first appearance in 1985 (G. P. Smith, 1985). Early studies showed that a foreign DNA sequence could be expressed on the protein coat of phage particles. By splicing a foreign sequence between the amino terminal half and the carboxyl terminal half of phage gene III, a fusion protein is formed. Phage gene III codes for pIII, which is a minor coat protein at the tip of the filamentous phage. This process embeds the foreign peptide into the coat protein while maintaining functionality of pIII, which plays an important role during infection. Of particular importance is the intact immunological activity of the foreign peptide. This was demonstrated by expressing an *Eco*RI gene on the surface of phage. Infectivity remained active after this modification but was blocked when the phage was exposed to anti-*Eco*RI antibody.

While an individual phage expressing a single peptide at its surface may not be of great interest for antibody production, the true value of this technology lies within the generation of entire peptide-displaying libraries. These libraries can be generated to display a vast array of random peptides of many varieties (Clackson et al., 1991; Parmley & Smith, 1988). This principle can be further applied to potentially eliminate the need for hybridoma technology. Genes coding for the light and heavy variable chains of antibody-binding fragments may be isolated from spleen cells of immunized mice through PCR. These genes may then be combined randomly using a linker region of DNA coding for a short peptide that, when expressed, will act as a hinge connecting the

fragments (Clackson et al., 1991). This process generates a library of coding sequences that express randomly combined single-chain Fv (scFV) antibody fragments. These sequences may then be incorporated directly into the phage genome or provided as phagemid vector which will compete with the wild-type coat protein expression (Ledsgaard et al., 2018).

Once a library of antibody-expressing phage has been created, these viral particles may now be screened to isolate high affinity variations. This process, known as biopanning, utilizes affinity selection to isolate high affinity clones (Christensen et al., 2001). For this process there are 5 basic steps: 1) binding of target molecule, 2) phage binding, 3) washing, 4) phage elution, and 5) amplification. In the first step the target molecule is immobilized on a surface such as a microtiter plate. Next, the phage library is applied to the immobilized molecule followed by a washing step to remove any unbound phage. The target molecule is then denatured allowing for the elution of isolated phage. Isolated phage may now be used to infect bacteria for amplification. This process may be repeated for additional cycles to ensure a high affinity molecule is produced.

Antibodies may be isolated with hybridoma technology or phage display. Both methods require immunized animals to produce target specific antibodies. Hybridoma technology seeks to isolate a single clone producing the desired antibody. Alternatively, phage display allows for the generation of entire libraries of antibodies with varying affinity for a target molecule. Both methods possess unique advantages and disadvantages for the discovery of therapeutic antibodies.

***Antibody Humanization***

Early antibody constructs were developed with traditional hybridoma technology and thus were of murine origin. High immunogenicity from murine antibodies resulting from human anti-murine antibody (HAMA) response, prompted the development of humanized antibodies (Schroff et al., 1985; Shawler et al., 1985). HAMA response dramatically reduced therapeutic efficacy and resulted in rapid removal of the mAb from patient systems (Hwang & Foote, 2005). Antibody humanization may be accomplished through several methods such as complementary determining regions grafting (CDR), germline humanization, genetic engineering, and antibody resurfacing (O'Mahony & Bishop, 2006; Safdari et al., 2013). Chimeric antibodies may be generated by modifying human constant antibody domains with xenogeneic variable domains to reduce immunogenicity (Billetta & Lobuglio, 1993). Replacing the mouse constant region of a murine-derived mAb with a human constant region through CDR reduces immunogenicity and provides for better drug retention times (Steinitz, 2014).

Somatic hypermutation is a process employed by B-cells to accumulate mutations in the variable regions in antibodies (Martin et al., 2015). This process allows for diverse pool of antibodies to be generated but is also another potential source for immunogenicity to develop during antibody production. One potential method for mitigating this source of variability is to utilize germline frameworks, which do not manifest as much somatic hypermutation, instead of IgG frameworks (Safdari et al., 2013).

### *Antibody Biosimilars*

Biosimilars are rapidly entering the market as the fastest growing class of products for therapeutic treatments (Center for Drug Evaluation and Research., 2017). Patents on the first generation of biotechnology drugs developed in the 1980s have been expiring over the past decade (Udpa & Million, 2015). This opens the market up for development of drugs that replicate the activity of original therapeutics (Genazzani, 2007). Biosimilars are alternatives to original biological products that offer similar treatment with lower costs. These drugs cannot be marketed simply as "generics" as their classification and composition are too complex. Biosimilars must mimic the original molecule according to rigorous approval standards set by the Food and Drug Administration (FDA). Similarity is determined through purity, chemical identity, and bioactivity. As of May 26$^{th}$, 2022, 35 biosimilars have been approved for use by the FDA (Table 2.1) (*Biosimilar Product Information*, 2022). The rise in biosimilar production has generated an increase in demand for therapeutic antibodies produced in large quantities.

**Table 2.1.**

*List of FDA Approved Biosimilars*

| Biosimilar Name | Approval Date | Reference Product |
|---|---|---|
| Fylnetra (pegfilgrastim-pbbk) | May 2022 | Neulasta (pegfilgrastim) |
| Alymsys (bevacizumab-maly) | April 2022 | Avastin (bevacizumab) |
| Releuko (filgrastim-ayow) | February 2022 | Neupogen (filgrastim) |
| Yusimry (adalimumab-aqvh) | December 2021 | Humira (adalimumab) |
| Rezvoglar (insulin glargine-aglr) | December 2021 | Lantus (insulin glargine) |
| Byooviz (ranibizumab-nuna) | September 2021 | Lucentis (ranibizumab) |
| Semglee (Insulin glargine-yfgn) | July 2021 | Lantus (Insulin glargine) |
| Riabni (rituximab-arrx) | December 2020 | Rituxan (rituximab) |
| Hulio (adalimumab-fkjp) | July 2020 | Humira (adalimumab) |
| Nyvepria (pegfilgrastim-apgf) | June 2020 | Neulasta (pegfilgrastim) |
| Avsola (infliximab-axxq) | December 2019 | Remicade (infliximab) |
| Abrilada (adalimumab-afzb) | November 2019 | Humira (adalimumab) |
| Ziextenzo (pegfilgrastim-bmez) | November 2019 | Neulasta (pegfilgrastim) |
| Hadlima (adalimumab-bwwd) | July 2019 | Humira (adalimumab) |
| Ruxience (rituximab-pvvr) | July 2019 | Rituxan (rituximab) |
| Zirabev (bevacizumab-bvzr) | June 2019 | Avastin (bevacizumab) |
| Kanjinti (trastuzumab-anns) | June 2019 | Herceptin (trastuzumab) |
| Eticovo (etanercept-ykro) | April 2019 | Enbrel (etanercept) |
| Trazimera (trastuzumab-qyyp) | March 2019 | Herceptin (trastuzumab) |
| Ontruzant (trastuzumab-dttb) | January 2019 | Herceptin (trastuzumab) |
| Herzuma (trastuzumab-pkrb) | December 2018 | Herceptin (trastuzumab) |
| Truxima (rituximab-abbs) | November 2018 | Rituxan (rituximab) |
| Udenyca (pegfilgrastim-cbqv) | November 2018 | Neulasta (pegfilgrastim) |
| Hyrimoz (adalimumab-adaz) | October 2018 | Humira (adalimumab) |
| Nivestym (filgrastim-aafi) | July 2018 | Neupogen (filgrastim) |
| Fulphila (pegfilgrastim-jmdb) | June 2018 | Neluasta (pegfilgrastim) |
| Retacrit (epoetin alfa-epbx) | May 2018 | Epogen (epoetin-alfa) |
| Ixifi (infliximab-qbtx) | December 2017 | Remicade (infliximab) |
| Ogivri (trastuzumab-dkst) | December 2017 | Herceptin (trastuzumab) |
| Mvasi (Bevacizumab-awwb) | September 2017 | Avastin (bevacizumab) |
| Cyltezo (Adalimumab-adbm) | August 2017 | Humira (adalimumab) |
| Renflexis (Infliximab-abda) | May 2017 | Remicade (infliximab) |
| Amjevita (Adalimumab -atto) | September 2016 | Humira (adalimumab) |
| Erelzi (Etanercept-szzs) | August 2016 | Enbrel (etanercept) |
| Inflectra (Infliximab-dyyb) | April 2016 | Remicade (infliximab) |
| Zarxio (Filgrastim-sndz) | March 2015 | Neupogen (filgrastim) |

*Note.* Adapted from *Biosimilar Product Information* by U.S. Food and Drug Administration, 2022 (https://www.fda.gov/drugs/biosimilars/biosimilar-product-information). In the public domain.

### *Recombinant antibodies*

Recombinant antibodies allow for production to be carried out in more suitable platforms, such as CHO cells. To produce a recombinant antibody, the gene coding for the antibody must be isolated. One method in which this is accomplished is by first isolating mRNA coding for the antibody. Antibody-producing cells such as hybridomas, spleen cells, or B lymphocytes are used as a source for mRNA, which may be purified through affinity chromatography (Karu et al., 1995). mRNA contains a poly(A) tail, which allows for purification via affinity chromatography. Columns or beads containing poly(T) are often used for purification of mRNA. Following mRNA purification, reverse transcriptase, which generates DNA from single-stranded RNA, is used to produce a coding sequence for the antibody.

## Production Cell Line

With increasing demand for antibody therapeutic drugs comes an increase in the demand for production. As such, one of the most critical decisions during drug development is the production cell line. A mAb-producing cell line must be capable of high cell densities, high titer, and cater well to fed-batch processes. Ideally, a manufacturing cell line is adapted to suspension cell culture, which allows for high densities and scale-up. While many cell lines exist that can produce mAbs and biosimilars, none are quite as suited to the task as Chinese Hamster Ovary (CHO) cells. CHO cells are the most prevalent cells used in the production of therapeutic proteins (Li et al., 2010; Wurm, 2004). These now invaluable cells were first isolated by Theodore T. Puck and his team in 1958 (Puck et al., 1958). These cells were originally of interest due

to their low chromosome number and applications in genetic studies, but soon proved to be a viable cell line suitable for long-term culture.

Further developments in CHO cells led to the commonly used cell lines we see in industry today. These include CHO-S, CHO K1, and CHO-DG44 (Dahodwala & Lee, 2019). The development of dihydrofolate reductase (DHFR) deficient CHO cells in 1980 proved particularly valuable (Urlaub & Chasin, 1980). This enzyme is responsible for the synthesis of several amino acid precursors. Without this enzyme, CHO cells become dependent on hypoxanthine, thymidine, and glycine (GHT) in the culture medium. This allows for selection pressure to be implemented by transfection of DNA plasmids that contain the DHFR gene and the gene of interest followed by culture in medium lacking GHT.

CHO cells are a robust cell line that cater well to production of mAbs and biosimilars on a large scale, with nearly 70% of all recombinant therapeutic proteins being produced in these cells (J. Y. Kim et al., 2012). CHO cells have proven to be regulatory friendly, amenable to genetic modification, and effective at post-translational protein modification, which is essential for therapeutic protein production. CHO cells are also capable of being cultured in adherent culture or suspension culture depending on the adaptation applied. Suspension cell culture has proven to be the most effective method at culturing mammalian cells on a large scale and CHO cells have overcome many of the barriers associated with this process (Chu & Robinson, 2001).

## Bioreactor Manufacturing

Bioreactors are a major component in large-scale mAb production, as they are the only current method capable of delivering oxygen and nutrients at the levels required. Bioreactors also allow for the scaling of cell cultures to large scale production volumes. Cell lines producing mAbs originate from a single cell and are scaled up over time to production levels upwards of 25,000 L (Kelley, 2009). During scale-up of mAb production, many factors must be considered including raw material availability, time frame, risk factors, purification, and cost. While productivity has increased significantly in the past decade, mAbs can still only regularly be expressed at around 5 g/L in fed-batch culture (Shukla et al., 2017).

### *Single-Use Technology*

Regulatory concerns, batch consistency, and ease-of-use have led to the quick integration of single-use technologies (SUT) in biopharmaceutical manufacturing (Allison & Richards, 2014; Langer & Rader, 2014). SUT has evolved to replace traditional stainless-steel manufacturing as the biopharmaceutical industry gradually comes to a fuller realization. Traditional manufacturing methods require cleaning validations and high-quality systems to support regular cleaning and turnover. SUT circumvents the need for the same level of cleaning but requires characterization of the films used in these systems to reduce the potential for leachables and extractables. Additionally, SUT requires disposal of large, unwieldy bags that often will include their own mixing systems (Eibl & Eibl, 2010; Shukla & Gottschalk, 2013). As antibody technology develops and demand continues to increase, it becomes clear that a viable, long-term solution for

antibody production is needed. Further research into this area is certainly required to manufacture these products sustainably.

**Cell Culture Media Optimization**

At the heart of every successful production run lies a reliable, well-characterized cell culture medium. Classical cell culture media are largely based on the early research of Harry Eagle, who developed the combination of amino acids, sugars, vitamins, and salts now known as Eagle's Minimal Essential Medium (EMEM) (Eagle, 1955, 1959). This led to the development of an optimized version known as Dulbecco's Modified Eagle's medium (Dulbecco & Freeman, 1959). While these media supported cell growth and high densities, they required the addition of serum. Serum supports cell growth quite well, but requires careful sourcing and carries with it the risk of adventitious agents such as viruses, bovine spongiform encephalopathy, and transmissible spongiform encephalopathy (BSE/TSE) (Erickson et al., 1991; Siegel & Foster, 2013). Since serum is a biological product, it is highly subject to lot variability, which can be affected by uncontrolled factors such as seasonal changes and disease.

The desire for a chemically defined medium led to the development of Ham's nutrient mixture F12 (Ham's F12). Ham's F12 still suffered from an inability to promote high cell densities. These challenges promoted further investigation into cell culture media development and the commercial interest in suitable formulations. Currently, several companies offer off-the-shelf proprietary media that are typically tailored to specific types. Companies also offer media development services, which use modern software and Design of Experiments (DOE) to develop chemically defined media custom

tailored to specific cell lines(Mandenius & Brundin, 2008). The high variability in nutritional need and response even between clones makes development of platform media a unique challenge.

**Genetic engineering to increase productivity in CHO cells**

While process optimization significantly impacts performance and productivity of cell lines during mAb production, it is not entirely sufficient to maximize these parameters. Continued cell line development plays a key role in creating a sustainable, profitable product. CHO cells have proven themselves to be hardy, dependable producers that are amenable to a variety of engineering formats (Omasa et al., 2010). In order to turn these cells into production workhorses, methods exist to down-regulate undesirable genes, overexpress desirable genes, eliminate a gene's function, or add an entirely new function to the genome (Fischer et al., 2015). Genes that are often impacted by these modifications include those that influence productivity, growth, cell metabolism, apoptosis, and glycosylation. Finding the correct synergy in these edits to create an industry-friendly organism is the basis for mammalian cell line development.

In developing cell lines intended to produce antibodies for use as therapeutic drugs, several considerations must be made including specificity, immunogenicity, and production viability (Starr & Tessier, 2019; Tiller & Tessier, 2015). Of particular concern and relevance to mAb production is the glycosylation of proteins produced in mammalian cells. Glycosylation is a post-translational modification that leads to the processing of carbohydrate moieties on the protein backbone of antibodies (Jefferis, 2005; Narula, 2016). These modifications have profound consequences for therapeutic

applications. Changes in media composition, cell culture age, cell culture conditions, and genetic factors all play a role in antibody glycosylation (Andersen, 2000; Goochee & Monica, 1990). The glycan profile, which is produced by enzymatically releasing the *N*-linked oligosaccharides from a mAb, is an indicator of product quality and batch consistency (Shang, 2014). Glycosylation also impacts protein stability and bioactivity (Zhang et al., 2015).

CHO cellular engineering can exist in many different forms and techniques. For gene knockouts, the most common, current methods are through zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS), or Clustered Regularly Interspaced Palindromic Repeats (CRISPR/Cas9) system (Fischer et al., 2015).

***Zinc-Finger Nucleases***

ZFNs offer a method for inducing double-stranded breaks in DNA by combining zinc-finger proteins (ZFPs) with the nuclease domain of the *Fok*I restriction enzyme (Urnov et al., 2010). ZFPs contain $Cys_2His_2$ fingers capable of recognizing a 3 base pair sequence and binding to DNA via the α-helical portion of each finger (Wolfe et al., 2000). For this to be useful in site-specific recognition, several ZFPs must be assembled in a specific order. This necessitates a library of ZFPs that allow for standardized, modular assembly (Bhakta & Segal, 2010; Gonzalez et al., 2010; Wright et al., 2006). Drawing from these now publicly available protein libraries, a zinc finger array may be assembled that recognizes a specific locus. Recognition and binding are further complicated by context-dependent effects, so-called to illustrate the lack of independence between adjacent ZFPs (Cathomen & Keith Joung, 2008). While methods

exist to design arrays around these effects, these interactions remain problematic for binding efficiency (Sander et al., 2011). Additionally, advanced methods for array design require a high level of understanding and expertise.

The activity of *Fok*I is dependent on its dimerization. Therefore, when designing ZFNs, the arrays must flank the cut site with the *Fok*I cleavage domain between them. This results in two monomer subunits that dimerize the *Fok*I cleavage domain across the cut site, resulting in a double-stranded break. This break may be resolved through non-homologous end joining (NHEJ) or homology-directed repair. NHEJ is the most common path and can result in mutations that disrupt the gene. HDR is the less common outcome and requires a supplied repair template to insert a gene at the cut site (Urnov et al., 2010).

### *Transcription Activator-Like Effector Nucleases*

TALENs, like ZFNs, utilize engineered nucleases to perform DNA alterations. Much like ZFNs, TALENs also utilize the *Fok*I nuclease domain and a customizable DNA binding domain (Joung & Sander, 2013). This DNA binding domain consists of transcription activator-like effectors (TALEs), which are found in the pathogenic bacteria *Xanthomonas*. These bacteria utilize TALEs to alter gene transcription by injecting them into host plant cells. TALEs are then able to bind to host DNA and promote bacterial colonization (Boch & Bonas, 2010).

TALEs are highly conserved repeats of 33-35 amino acids that each recognize a single base pair, which allows them to be constructed into modular arrays. The customizability of these arrays allows for flexible gene recognition and cutting (Gaj et

al., 2013). TALE-nuclease chimeras can be formed by linking a *Fok*I nuclease domain to the C-terminal of TALE arrays (Miller et al., 2011). Like ZFNs, TALENs require recognition sites flanking the cut site. Once recognition occurs at the site, a *Fok*I dimer at the C-terminal of each array forms and initiates a double-stranded break (Sun & Zhao, 2013). The DNA may then be repaired through either NHEJ or HDR and a repair template may be provided. Entire libraries of TALENs have been developed, even spanning the human genome (Y. Kim et al., 2013).

While ZFNs and TALENs dominated genetic engineering methods for several years, their required specialty knowledge and barrier to entry made genetic engineering of eukaryotes a continually difficult endeavor. Genetic engineering was still in its infancy during this time with the development of CRISPR/Cas systems just on the horizon.

***CRISPR/Cas***

In 2020, two scientists, Jennifer Doudna and Emmanuelle Charpentier, were awarded the Nobel Prize in Chemistry “for the development of a method for genome editing” (*The Nobel Prize in Chemistry 2020*, 2022). This understated prize was a result of their contributions to the development of CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated proteins) systems for the purpose of precise gene editing. Their landmark research recognized that adaptive bacterial immunity systems may be used to cleave target DNA (Jinek, 2012).

CRISPR systems are bacterial and archaeal immune defense mechanisms that have been adapted for use as a genetic engineering platform. CRISPRs are characterized by hypervariable spacer sequences that are acquired from foreign invading DNA

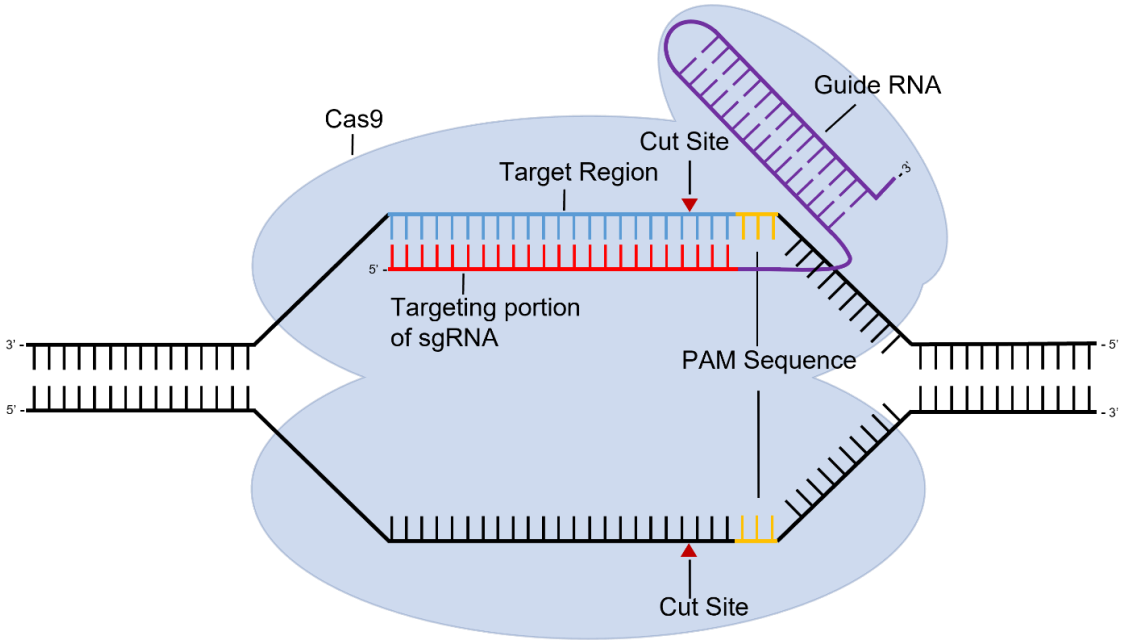
interspaced between repeat sequences (Bhaya et al., 2011). These sequences are transcribed to produce pre-CRISPR RNA (pre-crRNA), which is then cleaved to isolate individual spacers and a partial repeat. The crRNA is then able to recognize and bind to matching invading DNA (termed the protospacer) to initiate cleavage via recruited Cas proteins.

CRISPR/Cas9 made its debut into mammalian cells in 2013 with the promise of providing a system for mammalian genetic engineering with several advantages over more traditional ZFN or TALENS methods (Jinek, 2012; Mali, 2013). These systems function by guiding an endonuclease to invasive DNA and cleaving the foreign genetic material (Wiedenheft et al., 2012). Cleavage occurs 3 base pairs upstream of the protospacer adjacent motif (PAM) sequence that must be present for identification of a cleavage site (Jiang, 2016). The Cas9 protein is guided to the target site with the help of two RNAs. The first RNA, called CRISPR RNA (crRNA) is the genetic material associated with bacterial immunity that is used to identify and cleave previously encountered foreign DNA (Bhaya et al., 2011). The second RNA component necessary for the system to function is known as trans-activating crRNA (tracrRNA). When mature crRNA and tracrRNA are provided to the Cas9 enzyme the result is targeted DNA cleavage at the site complementary to the crRNA sequence (Jinek, 2012). This targeting process can be simplified by fusing the crRNA with the tracrRNA and incorporating a protospacer adjacent motif (PAM) sequence. The PAM sequence (typically $^{5'}$NGG$^{3'}$) is vital to recognition of the cleavage site as its presence initiates the interrogation process (Sternberg, 2014). The resulting molecule containing a fusion of the PAM, crRNA, and

tracrRNA, referred to as single guide RNA (sgRNA), can be transfected alongside the Cas9 enzyme for a simple, rapid method of genome editing (Mali, 2013). In practical applications, the sgRNA and Cas9 may be transfected directly into the cell or provided as a plasmid to be produced within the cell. More recently, new CRISPR/Cas systems have been introduced with Cas12 and Cas13 coming under investigation (Rusk, 2019; Yan et al., 2019).

**Figure 2.2.**

*Cas9 Mechanism*

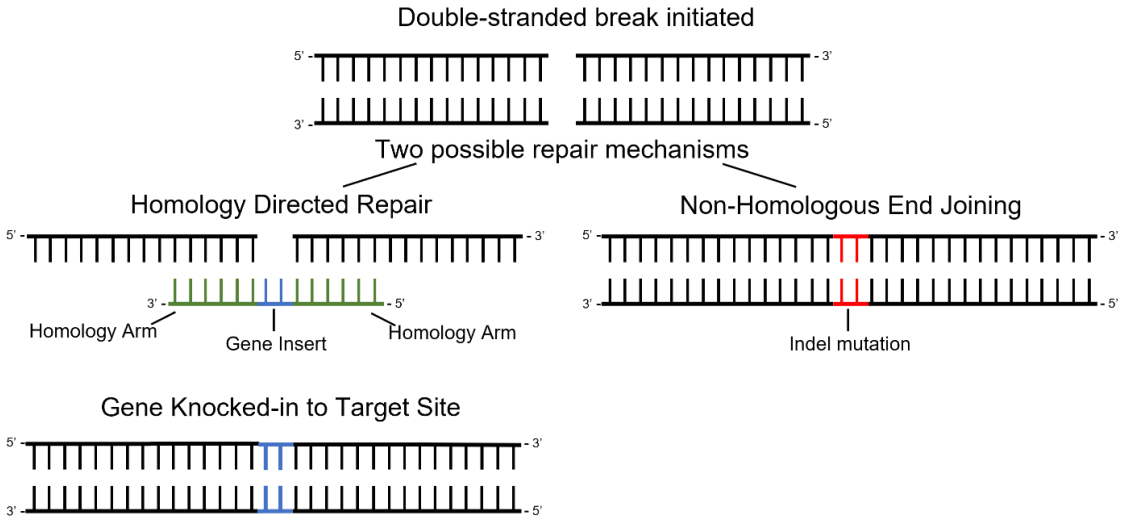



*Note.* Cas9 (blue region) interrogates the PAM sequence and initiates a double-stranded break 3 bp upstream. This activity is directed by the sgRNA with a targeting region homologous to the desired cut site.

Cas9 endonuclease induces a double-stranded break upstream of the PAM sequence. The repair mechanism for this break can take one of two different paths. The first, less likely path is through homology-directed repair (HDR) and the second, more likely path is through non-homologous end joining (NHEJ) (Cong, 2013). HDR relies on a template DNA sequence for precise repair and is utilized for insertion of genes through the CRISPR/Cas9 system. NHEJ is an error-prone repair mechanism that often results in single nucleotide insertions or deletions and the formation of indels in the absence of a repair template (Gratz, 2014). These indel mutations can manifest themselves as gene knockouts and provide a reliable method for gene disruption (Figure 2.3).

**Figure 2.3.**

*Schematic of Possible DNA Repair Mechanisms*



## Genes of interest

### *C12orf35*

*C12orf35*, also known as retroelement silencing factor 1, *Resf1* (RefSeq NW_003615627.1), is a gene located in the telomeric region of chromosome 8 in *Cricetulus griseus*. In humans, this gene has shown involvement in gene regulation, specifically through regulation of repressive epigenetic and chromatin modifications (Fukuda et al., 2018). Retroelements are transposable genetic elements that are extremely common in mammals. The majority of transposable elements, which constitute over 40% of the human genome, consist of retroelements (Deininger & Batzer, 2002). These so-called "selfish" genetic elements are thought to be regulated largely through chromatin modifications such as methylation (Molaro & Malik, 2016). It follows that deletion of a gene involved in down-regulating gene expression may impact overall transcription rates and productivity.

In previous studies, the gene *C12orf35* has been shown to impact CHO-K1 cell productivity (Ritter, 2016b, 2016a). Disruption of *C12orf35* and surrounding genes leads to increased mAb productivity and cell line stability (Ritter, 2017). Previous disruption of this region has been performed using siRNA-mediated repression or knockout with TALENs. *C12orf35* has also proven to be a valuable location for site-specific gene integration (Zhao, 2018). Integration at this site has proven to be sustainable and stable with minimal impact on protein quality.

### *Mammalian Target of Rapamycin (mTOR)*

Mammalian target of rapamycin (mTOR) pathway is a serine/threonine protein kinase that presents an exciting opportunity for cellular engineering. Mitochondrial oxygen consumption has been shown to be regulated by mTOR, and inhibition of this pathway negatively impacts cell functionality (Schieke, 2006). This pathway is vital for regulating cellular metabolism in response to nutrient availability and has been directly linked to cell mass accumulation and viability. Dysregulation of mTOR signaling has been linked to many diseases in humans including obesity, cancer, diabetes, tuberous sclerosis, cardiac hypertrophy, inflammatory diseases, and more (Hall, 2008). Rapamycin is used as an inhibitor of mTOR and a treatment for many of these conditions. Previously, mTOR has been transiently expressed in CHO cells with a resulting increase in cell productivity, cell size, cell proliferation, and a reduction in cell death (Dreesen & Fussenegger, 2011). MTOR directly controls the phosphorylation of at least 335 proteins in response to environmental health (Robitaille, 2013). With this in mind, it stands to reason that combining a viable integration site with known productivity benefits upon disruption (*C12orf35*) with the knock-in of a gene related to increased cell metabolism (mTOR) will likely generate a highly productive CHO cell line.

***Fam60A***

The gene *Fam60A* in CHO cells has been show to play a role in the stability of isolated clones where a stable clone is defined as losing no more than 25% productivity over a 12 week time frame (Ritter, 2017). Human family with sequence similarity 60A (Fam60A) has been characterized as a subunit of the Sin3 histone deacetylase complex (Muñoz et al., 2012; K. T. Smith et al., 2012, p. 60). Highly acetylated chromatin is

associated with higher transcription rates in mammalian cells. Negating the charges in histone tails through acetylation interferes with the winding of the nucleosomal array. This unwound form of DNA is more readily available for transcription than DNA that is tightly wound around the histone proteins. Histone acetyltransferase and deacetylase enzymes regulate this process. Histone deacetylases (HDACs) are a class of enzymes that play a crucial role in the acetylation of DNA. HDACs are one of two families of deacetylases that have been identified along with the silent information regulator-like family. Of the HDACs, the best characterized are HDAC1 and HDAC2 which are found in three protein complexes: Sin3, Nurd, and CoREST (Grozinger & Schreiber, 2002). The Sin3 complex is thought to be coded for by the telomeric region in chromosome 8 of CHO cells (Ritter, 2017).

*Fam60A* remains of interest due to its potential for impacting clone stability. Previous studies have identified this gene as a relevant player in cell line stability. In designing systems for increased productivity, stability should remain a consideration so that a cell line may continue to produce viable, high-quality protein. While investigation of the synergistic effect of *Fam60A* deletion, *C12orf35* deletion, and mTOR gene insertion remained outside the scope of this study, it is worth considering *Fam60A* as another tool for effective CHO engineering.

# Chapter 3

## Baseline Establishment

### Background

Since full-length monoclonal antibodies (mAbs) undergo post-translational modifications such as glycosylation, these proteins are primarily manufactured with mammalian cell lines capable of this process (Hossler et al., 2009). Chinese hamster ovary (CHO) cells are the most widely used cells for this purpose, owing to their demonstrated safety, ease of engineering, and adaptability to serum-free media (J. Y. Kim et al., 2012). Many different CHO cell lines exist with widely varying productivity (Kang et al., 2014).

Choosing a suitable cell line for modification requires the determination of original parameters. CHO-K1 cells expressing immunoglobulin G are frequently used as model organisms for overexpression of proteins. To this end, a growth promotion study was performed on two IgG-producing CHO-K1 cell lines, Mab7 and CHO-M, to determine suitability for knockout and anticipated results. This study measured industry relevant growth and productivity parameters to determine cell line suitability for gene knockout.

### Materials and Methods

#### *Media Preparation*

HyClone™ ActiPro™ cell culture media (catalog number SH31039) was supplemented with 100 µM methionine sulfoximine (MSX). MSX inhibits endogenous glutamine synthesis and acts as a selection pressure for mAb producing clones.

### *Cell Culture and Sampling*

Two proprietary CHO-K1 cell lines – CHO-M and Mab7 – both producing IgG were cultured in triplicate for this study. Cells were cultured in suspension 125 mL Erlenmeyer shaker flasks with 30 mL cell culture media and incubated at 37°C in shaking incubators with 5% $CO_2$ and 95% humidity. 1 mL samples were taken from each culture and placed in 1.5 mL microfuge tubes. 300 µL of this sample was used for viable cell density (VCD) measurements, while the rest was centrifuged at 100xg for 5 minutes. Since IgG is secreted, the supernatant was then collected for titer measurement.

Cultures were sampled at Day 0 and daily after Day 2 for 7 days in batch mode. VCD and viability measurements were taken with the Beckman Coulter Vi-CELL™ XR Cell Viability Analyzer (Beckman Coulter Life Sciences, Indianapolis, IN), which utilizes the Trypan Blue Exclusion method for analysis.

Spent media analysis was performed with the Roche Cedex Bio HT analyzer (Roche Diagnostics Corporation, Indianapolis, IN). Samples were measured for IgG titer. This instrument can measure a wide range of industry relevant spent media parameters including total protein, glucose, osmolality, IgG, lactate, and many others.

### **Growth Studies**

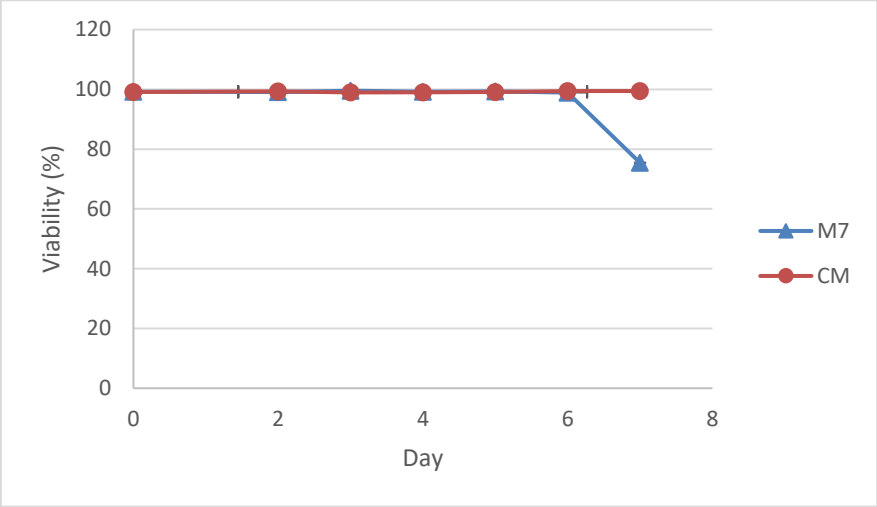
Three performance indicators – viability, VCD, and IgG titer – were analyzed for both cell lines. Viability, measured in percent viable cells, is a measure of the percentage of live cells in a sample (Figure 3.1). VCD measures viable cells per mL through a trypan blue exclusion assay (Figure 3.2). Titer, measured in grams of IgG per liter (Figure 3.3), is

often considered the most important factor as it represents productivity of the culture. All cultures were performed in triplicate.

These data are representative of a typical growth curve for mammalian cells with the Mab-7 cell line reaching a peak average VCD of $14.81 \times 10^6$ viable cells/mL on day 6 and the CHO-M cell line reaching a peak average VCD of $17.69 \times 10^6$ viable cells/mL on day 7. Mab-7 showed a drop in viability on day 7, an event that precedes the culture crashing, while CHO-M maintained consistent viability for the duration of the study. CHO-M cells reached a peak average titer of 0.68 g IgG/L at the end of the study while Mab-7 cells only reached a peak average titer of 0.10 g IgG/L on day 7. From these data it becomes clear that CHO-M is a higher producing cell line with greater culture longevity.

**Figure 3.1.**

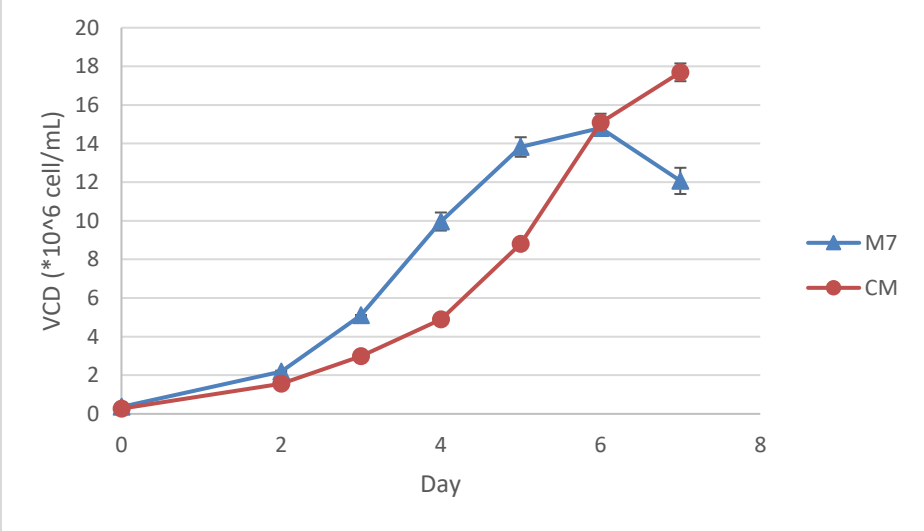
*Average Viability (%) vs. Day for Two mAb Producing CHO Cell Lines*




*Note.* Viability was averaged for triplicate samples of Mab-7 (M7) and CHO-M (CM).

**Figure 3.2.**

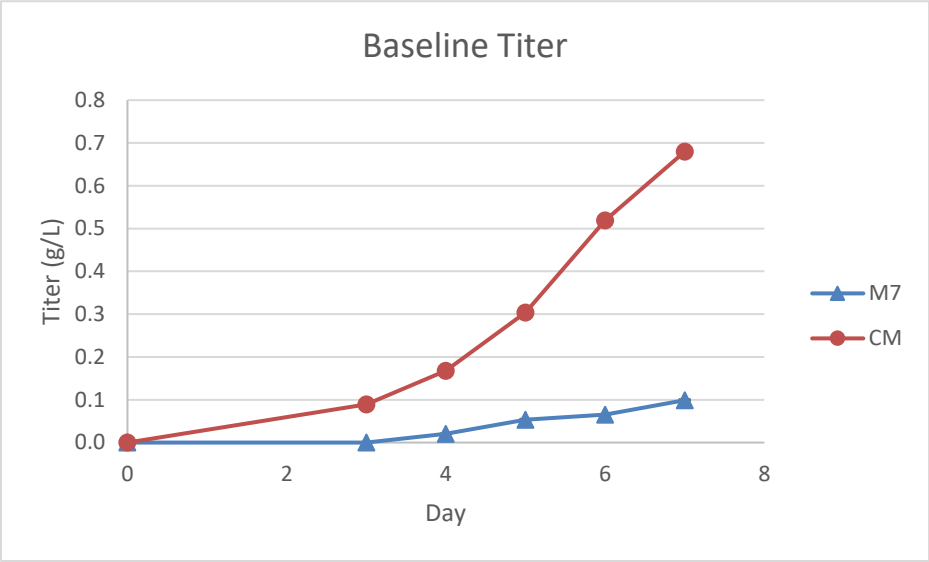
*Average VCD vs. Day for Two mAb Producing CHO Cell Lines*




*Note.* Viable cell density (VCD) was averaged for triplicate samples of Mab-7 (M7) and CHO-M (CM).

**Figure 3.3.**

*Average IgG Titer (g/L) vs. Day for Two mAb Producing CHO Cell Lines*




*Note.* IgG titer was averaged for triplicate samples of Mab-7 (M7) and CHO-M (CM).

**Results**

CHO-M cells demonstrated dramatically higher productivity and an increased culture lifetime. When the cultures were terminated on day 7 of the experiment, CHO-M cells displayed a 31.7% higher average VCD, 24.01% higher average viability, and 85.3% higher average titer. While cell viability (Figure 3.1) was comparable between the two cell lines, VCD and titer were dramatically different with CHO-M vastly outperforming the Mab-7 cell line. To optimize its performance further, CHO-M was chosen as the candidate for further research to push the limits of CHO cell productivity.

## Conclusions

CHO-M proved to be the heartier cell line in batch culture and easily outperformed Mab7 in IgG productivity. Combined with a longer viable culture length, these characteristics make for a desirable model that is easy to manage and robust in its cell culture profile.

# Chapter 4

# C12orf35 Knockout

## Background

The gene *C12orf35* in Chinese hamster ovary (CHO) cell lines has been shown to play a role in monoclonal antibody (mAb) production. Disruption of this gene has led to increased mAb productivity in CHO cells (Ritter, 2016b, 2016a). Additionally, siRNA-mediated repression of the gene *Fam60a* led to increased stability in CHO cells. High-producing, stable cell lines are an extremely desirable resource in therapeutic mAb production. A simplified system for generating commercially relevant cell lines using CRISPR/Cas9 would be a valuable addition to upstream mAb development.

*C12orf35*, also known as retroelement silencing factor 1, Resf1 (RefSeq NW_003615627.1) is a gene located in the telomeric region of chromosome 8 in the Chinese hamster *Cricetulus griseus* genome. In humans, this gene plays a role in DNA regulation. Resf1 is thought to regulate repressive epigenetic and chromatin modifications (Fukuda et al., 2018). Thus, this gene may have significance in upregulation of protein expression in mammalian cells.

## Materials and Methods

### *Screening*

CHO-M cells were screened for the positive presence of *C12orf35* and *Fam60a*. Three potential target sequences for each gene knockout were generated as indicated in Table 4.1. Target sequences for *C12orf35* were generated using the web tool CHOPCHOP

v2 (Labun et al., 2016, 2019; Montague et al., 2014). CHOPCHOP is a powerful tool used for designing sgRNAs that aids in optimizing target selection. This software takes into consideration a wide variety of criteria for selecting ideal target sites including GC content, cutting efficiency, and off-target binding.

Table 4.2 summarizes the results of target optimization for *C12orf35* where MM0, MM1, MM2, and MM3 represent 0, 1, 2, or 3 (respectively) base pair mismatch off-targets found for each site. Efficiency for each site is scored between 0 and 1 by taking several factors and scoring criteria into consideration.

**Table 4.1.**

*Generated Target Sequences for Deletion*

| Target ID | Target Sequence | Source |
|---|---|---|
| C12orf35-1 | ATCAAGAACTAGTACAACAGCGG | Kornel Labun, et al (2016); Tessa G. Montague et al (2014) |
| C12orf35-2 | AGAACTAGTACAACAGCGGTTGG | |
| C12orf35-3 | GATGAGTATGTTGAAGCAAACGG | |
| Fam60a-1 | GATAGAGTTTTCACTTTCTTTGG | Ronda C, Pedersen et al (2014) |
| Fam60a-2 | GTGTGCTGCTTGTGAAAAGATGG | |
| Fam60a-3 | GTCCGTGAACCGAGAGCTGGAGG | |

**Table 4.2.**

*Summary of CHOPCHOP Results for C12orf35*

| Target | Rank | Location | GC (%) | MM0 | MM1 | MM2 | MM3 | Efficiency |
|---|---|---|---|---|---|---|---|---|
| C12orf35-1 | 1 | NC_048601.1:370719 | 43.5 | 0 | 0 | 0 | 6 | 0.68 |
| C12orf35-2 | 2 | NC_048601.1:370715 | 47.8 | 0 | 0 | 0 | 3 | 0.59 |
| C12orf35-3 | 3 | NC_048601.1:369908 | 43.5 | 0 | 0 | 0 | 23 | 0.54 |

Verification of the target regions was performed through PCR and gel electrophoresis. C12orf35-1 and C12orf35-2 contained overlapping sequences; therefore, verification was only performed on C12orf35-1 and C12orf35-3. Primers were designed flanking the target sites (Table 4.3). These primers were designed using NCBI reference sequence NW_003615627.1 with the intent to overlap targets C12orf35-1 and C12orf35-2. The Eurofins Genomics PCR Primer Design Tool (https://eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/) was used to design ideal forward and reverse primers. Primers were chosen based on position and melting temperature ($T_m$) similarity.

**Table 4.3.**

*Primer Design for Target Site Verification.*

| Gene | Forward Primers | Reverse Primers | Product Length |
|---|---|---|---|
| C12orf35-1 | ACCAACCTCCAAAAACCAC | TCCTTTTCCAACAGCTACATC | 236 |
| C12orf35-3 | CAAAATGAGCCCCAGAAACC | TCTCCGTTTGCTTCAACATAC | 212 |

Target sequences for *Fam60a* were generated using CRISPy, which is another web-based bioinformatics tool used to aid in sgRNA selection (Ronda et al., 2014). NCBI reference sequence NW_003614152.1 was used for design. CRISPy is used to scan the CHO-K1 genome for the gene of interest and provides a selection of potential targets. This software also searches for and displays the results of potential off target matches

scanned against the CHO-K1 genome. Targets were chosen based on exon uniqueness while also attempting to minimize the potential number of off-target effects. Table 4.4 summarizes the data output from the CRISPy software.

**Table 4.4.**

*Summary of CRISPy Results for Fam60a*

| Target | GC Content (%) | MM0 | MM1 | MM2 |
|---|---|---|---|---|
| Fam60A-1 | 34.8 | 27 | 835 | 11537 |
| Fam60A-2 | 47.8 | 8 | 276 | 4369 |
| Fam60A-3 | 65.2 | 4 | 105 | 2476 |

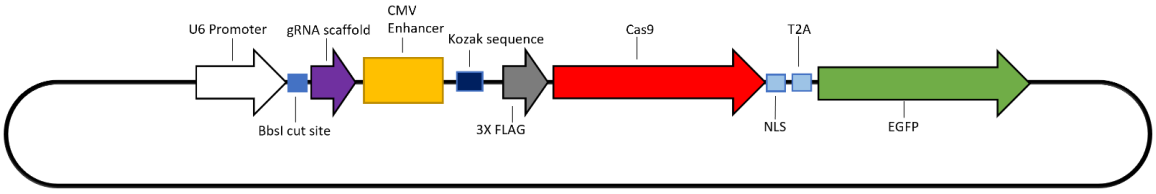
***Plasmid Design***

A targeting plasmid as outlined in Ran et al. 2013 was designed using C12orf35-1 as the guiding sequence. The plasmid pSpCas9(BB)-2A-GFP (PX458) (Addgene 48138) was chosen to generate the targeting plasmid. This plasmid contains an RNA polymerase III promoter for human U6 small nuclear RNA, gRNA scaffold, Cas9 gene, ampicillin resistance gene, and EGFP gene for expression verification (Figure 4.1). The Cas9 gene is derived from the *Streptococcus pyogenes* and initiates a double-stranded break when directed by bound sgRNA. This targeting plasmid also contains a Bbsl restriction enzyme cut site, which allows for addition of a custom guiding sequence. Upstream of the Cas9 sequence is a human cytomegalovirus (CMV) immediate early enhancer followed by a

vertebrate consensus (Kozak) sequence to aid in strong translation initiation. A 3X FLAG epitope tag is expressed as a fusion protein to Cas9 to allow for affinity chromatography if desired. A nucleoplasmin nuclear localization signal (NLS) is added to the C-terminus of Cas9 to aid in transport of the protein into the nucleus. Ribosomal skipping is initiated by the self-cleaving T2A peptide before translation of EGFP, thereby allowing Cas9 and EGFP to function as separate proteins. Cells taken from an agar stab swab were cultured in LB media containing 100 µg/mL ampicillin. Plasmid was purified using ZymoPURE Plasmid Miniprep Kit (catalog number D4210).

**Figure 4.1.**

*Plasmid pSpCas9(BB)-2A-GFP (PX458)*



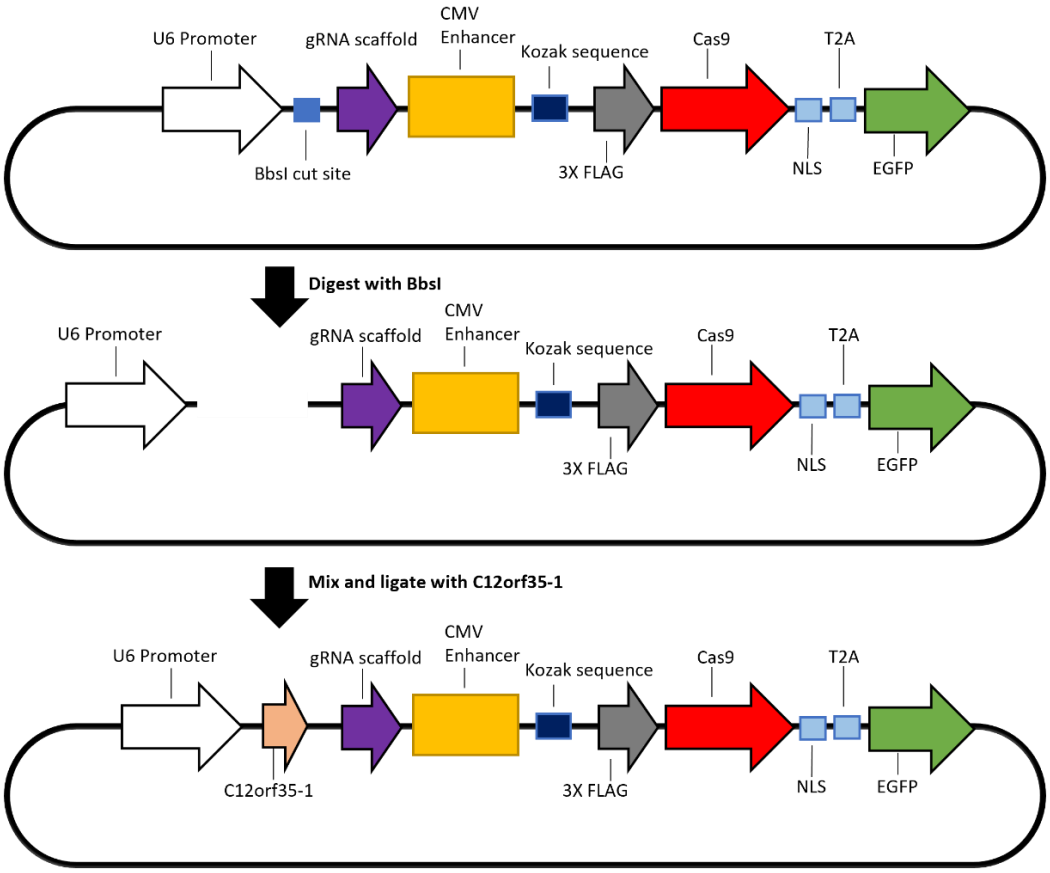

***Targeting Plasmid Construction***

The restriction enzyme BpiI (BbsI) (Thermo ER1011) was used for digestion to cut the plasmid between the U6 promoter site and gRNA scaffold site. Oligonucleotides were designed to contain C12orf35-1 as a guide sequence with sticky ends complementary to the BbsI restriction site. These were annealed by mixing equal molar ratios of oligonucleotides, heating the samples to 94°C for 2 minutes in a thermal cycler

and allowing to cool at room temperature. Annealed oligos were added to the digested plasmid and ligated with T7 DNA ligase (New England BioLabs M0318S). This generates a construct that expresses the targeting sequence and gRNA scaffold to create a functional sgRNA for Cas9 guidance (Figure 4.2).

**Figure 4.2.**

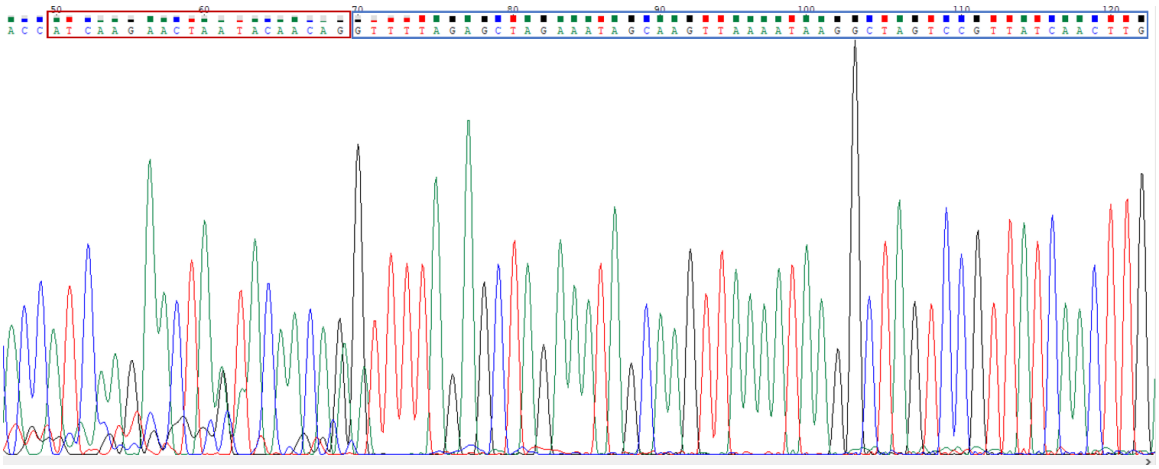
*Targeting Plasmid Construction*



The resulting plasmid was then transformed into competent DH5alpha cells. Competent cells were thawed on ice immediately followed by the addition of 5 µL of purified pSpCas9(BB)-2A-GFP plasmid. The mixture was incubated on ice for 30 minutes followed by heat shock at 42°C for 30 seconds in a water bath. Cells were then immediately incubated on ice for 5 minutes. 200 µL sterile LB media was then added to the mixture and placed in a shaking incubator at 37°C for 1 hour. Cells were then plated on LB agar plates containing 100 µL/mL ampicillin and incubated in a stationary incubator at 37°C overnight. Single colonies were picked into liquid LB media containing 100 µL/mL ampicillin and incubated overnight. Plasmid was then purified using ZymoPURE Plasmid Miniprep Kit (catalog number D4210). Purified plasmid was submitted for DNA sequencing to confirm the successful addition of the targeting sequence to the gRNA scaffold (Figure 4.3).

**Figure 4.3.**

*Sequencing Results for Targeting Plasmid*



*Note.* The C12orf35-1 target sequence is contained in the red box followed by the sgRNA sequence in the blue box.

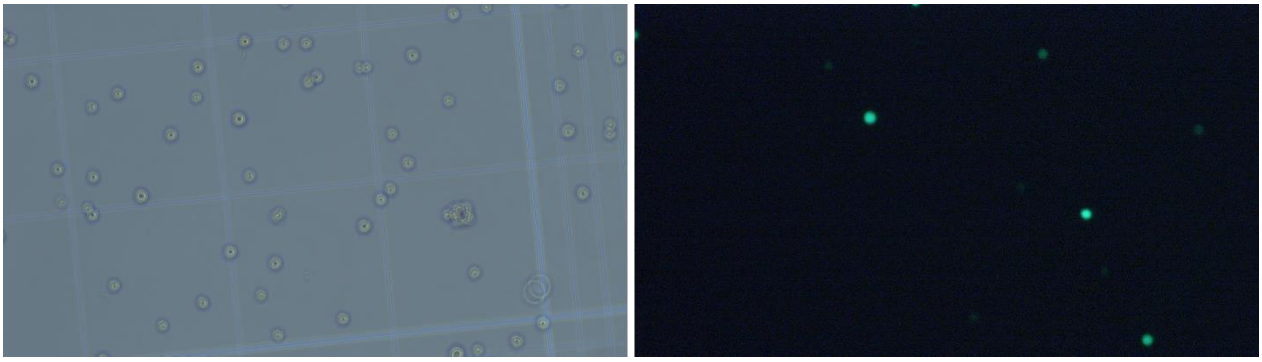
***Targeting Plasmid Transfection***

Targeting plasmid was transfected into log phase CHO-M cells using the SF Cell Line 4D-Nucleofector™ Kit L (Lonza V4XC-2024). 400 µL of HyClone™ ActiPro™ was added to 5 wells in a 24-well ultra-low attachment plate (Corning CLS3527) and placed in a stationary incubator at 37°C. CHO-M cells were centrifuged at 100g for 5 minutes to pellet 1.0E+06 cells per transfection. Cells were then resuspended with 100 µL nucleofector solution per transfection and aliquoted into electroporation cuvettes. Complete targeting plasmid was added to each cuvette. 10µL of PBS was added to one cuvette as a negative control, while 2 µg of EGFP vector was added to one cuvette as a positive control. Following transfections, samples were placed in a fresh 24-well ultra-low attachment plate and placed in a stationary 37°C incubator. Cells were verified for

fluorescence after 48 hours using a fluorescent microscope. Fluorescence indicated that transfections were successful.

**Figure 4.4.**

*GFP Fluorescence of CHO-M Cells Transfected with Targeting Plasmid*



***Pooled Growth Study***

Samples with positive fluorescence, thereby indicating sgRNA and Cas9 expression, were transferred from 24-well plates into 125 mL Erlenmeyer flasks containing 30 mL of ActiPro™ cell culture medium with 100 µM MSX. Cultures were allowed to reach 3.0E6 cells/mL before passage. Transfected CHO-M cells (CHO-MTF) were then cryopreserved at 10E6 cells/mL in ActiPro + MSX + 10% DMSO. To assess any differences between pooled transfected CHO-M cells and unmodified cells, a growth study was performed comparing the two cell populations. Both conditions were seeded in triplicate at 0.3E6 cells/mL in 30 mL of ActiPro™ + MSX in 125 mL Erlenmeyer shaker flasks. Cells were cultured in shaker incubators at 37°C and 5% $CO_2$. Flasks were sampled

at days 0, 3, 5, 6, 7, 10, and 11. Cell counts and viability were measured using a Vi-CELL™ XR Cell Viability Analyzer. IgG titer was measured using a Roche Cedex Bio HT analyzer.

***Single Cell Culture***

Without selection pressure, isolation of a single clone in a pool of transfected CHO-M cells is a necessary step in identifying the effects of C12orf35 knockout. To accomplish this, several methods were attempted.

***Deep Well Study***

The first isolation method utilized single-cell dilution of CHO-M TF cells into a 1.5 mL 96-deep well plate. CHO-M TF cell stocks were cultured to a concentration of 1.68E+06 cells/mL in growth medium (ActiPro™ + 100 µM MSX). This cell stock was diluted to a target concentration of 5 cells/mL with growth medium. A 96-deep well plate was prepared by adding 400 µL growth medium and 400 µL conditioned growth medium. Diluted cell stock was poured into a trough and 100 µL was quickly transferred to the deep well plate. A concentration of 0.5 cells/well was targeted to ensure a maximum of one cell per well was added. The plate was covered and placed in a shaking incubator at 37°C, 5% $CO_2$, and 95% humidity. Cultures were allowed to grow for 20 days. On day 20 all wells were fed with 95 µL conditioned growth medium to combat evaporation. On day 21 VCD measurements were taken for each well using the Sartorius iQue® 3 flow cytometer and analyzed with Forecyt® software. A control CHO-M TF culture of known concentration was used to properly gate events. No events were detected from 96-deep well plates. Without any apparent growth in any wells the experiment was terminated on day 21.

***Stationary Incubation***

Without growth present in the deep well plate format, stationary incubation was attempted using 96-well clear bottom plates. A stock solution was made according to the previous protocol to target 0.5 cells/well. This solution was then divided into a single row of a 96-deep well plate. This plate was used as the source for seeding a 96-clear bottom plate using a BioTek Precision Microplate Pipetting System (PRC384U). A program was developed specifically for this application.

This plate was then covered and incubated at 37°C, 5% CO2, and 95% humidity. Growth was monitored daily microscopically for each well. After 16 days of culture each well was fed with 100 µL conditioned growth medium. On day 30, wells that exhibited growth were transferred by pipette to a 24-well plate format containing 150 µL of conditioned media and returned to the incubator. Inspection of the 96-well plate following transfer revealed that the majority of cells remained adhered to the plate and were not transferred to the larger format. On day 5, 24-well cultures were fed with 100 µL conditioned growth medium. On day 12, cultures were fed with 200 µL unconditioned medium. Cultures were monitored daily and regularly fed with unconditioned growth medium however, significant growth was not observed in any of the 24-well plates.

To overcome cell adhesion to the plate surface, ultra-low attachment 96-well plates (Corning 3474) were utilized. These plates incorporate a covalently bonded hydrogel that inhibits cell adhesion and protein binding. Plates were seeded with single cell limiting dilutions and monitored for growth according to the previous protocol.

Wells that exhibited growth were transferred to an ultra-low binding 24-well plate format. These plates were then placed on a rocker platform inside of a stationary incubator to mimic suspension conditions while limiting exposure to shear forces. Evaporation became a concern in this format as the slow doubling time led to long incubation periods that reduced culture volume. Osmolality was monitored using a freeze-point osmometer for the most promising wells that appeared to exhibit growth. Sterile-filtered DI water was added to wells with high osmolality to maintain this value within an acceptable range. Despite these efforts, growth in all wells ceased before confluency and the cultures were unable to be scaled-up.

**Results and Discussion**

***Pooled Growth Study***

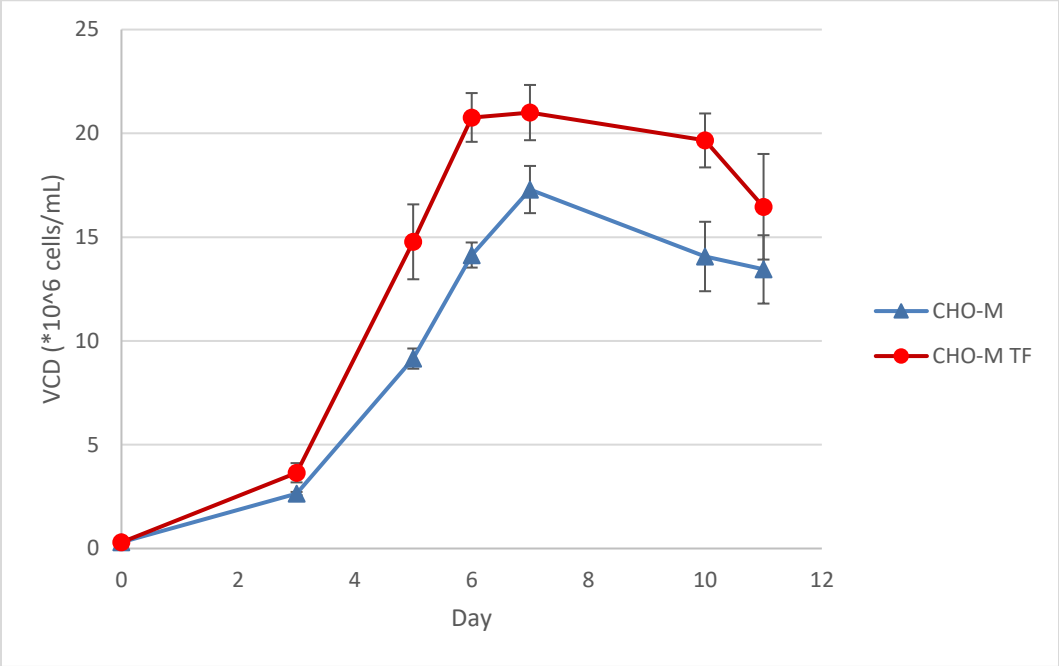
CHO-M cells were transfected with a plasmid intended for expression of Cas9 in tandem with single-guide RNA (sgRNA) targeting the C12orf35-1 cut site. This transfected cell population is denoted as CHO-M TF. Following transfection, a growth study was performed comparing CHO-M TF to the unaltered CHO-M control. All growth studies were carried out in triplicate. Muted differences between cell populations were observed, with CHO-M TF exhibiting higher VCD and IgG titer. A one-tailed one sample t-test was performed to compare CHO-M TF attributes against the CHO-M control. For both populations, VCD peaked on Day 7, so this day was chosen to compare all outputs. For VCD, the mean value of CHO-M TF on day 7 (M = 21.00*10^6 cells/mL, SD = 1.33*10^6 cells/mL) was significantly higher than the control CHO-M mean (M = 17.47*10^6 cells/mL, SD = 1.14*10^6 cells/mL) with $t(2) = -3.298$, $P(T \leq t) = 0.040$ (Figure

4.5). Mean IgG titer was also significantly higher in CHO-M TF on day 7 (M = 0.827 g/L SD = 0.011 g/L) than the control CHO-M (M = 0.731 g/L SD = 0.022 g/L) with t(2) = -4.729, P(T≤t) = 0.021 (Figure 4.7). There was no significant difference in mean viability between the two populations on day 7 with CHO-M TF (M = 99.15 percent viable, SD = 0.13 percent) and the control CHO-M (M = 99.32 percent viable, SD = 0.18 percent) both exhibiting close to 100 % viability and t(2) = 1.105, P(T≤t) = 0.192 (Figure 4.6).

Logarithmic growth was observed between days 3 and 5. As such, this range was used to calculate the mean doubling times of CHO-M TF and CHO-M using VCD data. Doubling time is calculated as DoublingTime = (duration*log(2))/(log(FinalConcentration) – log(InitialConcentration)). The CHO-M cell line had an average VCD of 2.65*10^6 cells/mL on day 3 and 9.15*10^6 cells/mL on day 5. CHO-M TF had an average VCD of 3.65*10^6 cells/mL on day 3 and 14.77*10^6 cells/mL on day 5. Approximately 46 hours passed between samples, giving a doubling time of approximately 26 hours for CHO-M cells and 23 hours for CHO-M TF cells.

**Figure 4.5.**

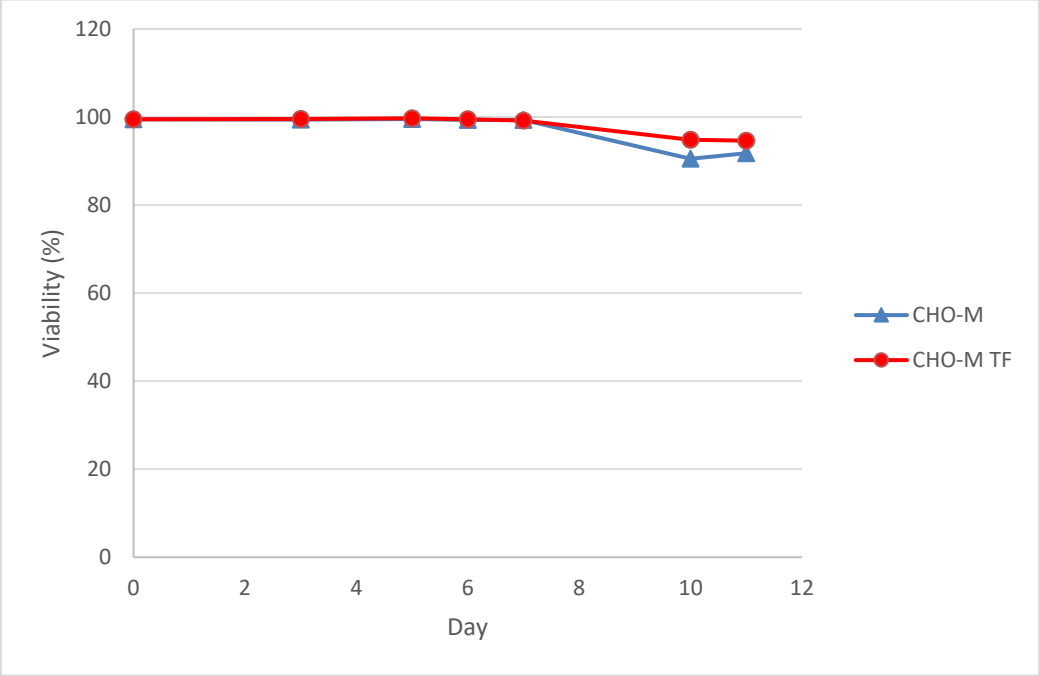
*VCD Comparison for CHO-M and CHO-M TF Cell Populations*




*Note.* VCD was averaged for triplicate samples of control CHO-M and transfected pool CHO-M TF with P(T≤t) = 0.040 for a one-tailed t-test on Day 7.

**Figure 4.6.**

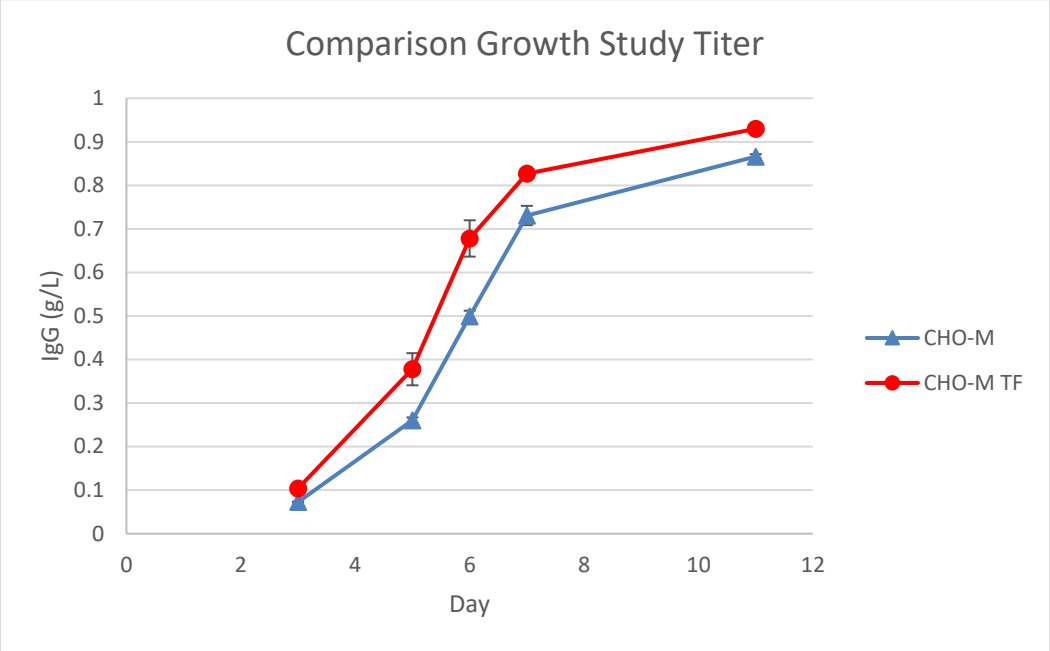
*Viability Comparison for CHO-M and CHO-M TF Cell Populations*




*Note.* Viability was averaged for triplicate samples of control CHO-M and transfected pool CHO-M TF with P(T≤t) = 0.192 for a one-tailed t-test on Day 7.

**Figure 4.7.**

*Titer Comparison for CHO-M and CHO-M TF Cell Populations*




*Note.* IgG titer was averaged for triplicate samples of control CHO-M and transfected pool CHO-M TF with P(T≤t) = 0.0.021 for a one-tailed t-test on Day 7.

Despite a low transfection rate and a mixed population of transfected CHO-M cells, statistically significant differences were observed on Day 7 of culture. It is reasonable to expect that an isolated clone may then have much higher productivity and peak VCD than the pooled populations.

***Single Cell Culture***

Efforts to isolate a single clone from transfected CHO-M cells were met with mixed success. While it appeared that the limiting dilution effectively placed individual cells in many of the wells, growth past this point was extremely limited and never

successfully entered a logarithmic growth phase. Low growth factor concentrations may have contributed to stunted growth, however, supplementation with growth factors or conditioned media did not increase growth rate adequately. Due to the extended culture duration, osmolality became a major concern that was not initially anticipated. While mitigation was attempted with media feeds, a more appropriate solution may be to supplement the culture with a low osmolality alternative based on evaporative loss modeling. Single-cell culture may also cater better to classical media and adherent conditions, but this has yet to be tested with this cell line.

# Chapter 5

## MTOR Knock-In

### Background

Scale-up of single-cell cultures from transfected CHO-M cells proved ineffective. This conclusion led to the need for a selection pressure to better isolate clones that were altered because of CRISPR/Cas9 modification. A selection pressure will ensure that surviving populations are modified and will reveal a better understanding of the overall impact of modification prior to isolation of a single clone. To accomplish this, knock-in must be performed that contains a suitable resistance gene. This also allows for a new gene of interest to be added at the knock-in site which has the potential to increase productivity even further since *C12orf35* has been shown to be a highly expressed region of the CHO genome. The gene expressing mTOR was chosen for this purpose. If a successful knock-in of mTOR is performed with this method, a gene known to increase CHO productivity will be expressed at a highly transcribed region along with puromycin resistance.

### Materials and Methods

#### *mTOR Addition to pBABE-puro*

Adding a selection pressure to CHO-M cells requires a repair template plasmid with adequate cloning sites, promoter, and selection pressure. The vector pBABE-puro (addgene 1764) is an empty backbone for mammalian gene expression that contains restriction sites flanking a puromycin resistance gene (PuroR) with an SV40 promoter

and ampicillin resistance. Between the SV40 promoter and PuroR lies a HindII restriction site, which allows for the addition of a gene of interest.

The target site C12orf35-1 was chosen as the insertion site for mTOR due to its viability as a cut site for Cas9. The gene chosen for stable integration of mTOR into *C12orf35* was taken from pcDNA3-Au1-mTOR-Wild type (Addgene 26036). This plasmid contains the upstream restriction site HindIII and the downstream restriction site NotI. To allow for addition of mTOR downstream of the SV40 promoter in pBABE-puro, oligonucleotides with sticky ends complimentary to NotI were designed. The plasmid pcDNA3-Au1-mTOR-Wild type was digested with HindIII and NotI followed by gel purification. The annealed oligonucleotides were then ligated to the purified mTOR followed by HindIII digestion and gel purification. This process generated an isolated mTOR gene with both upstream and downstream HindIII sticky ends.

The vector pBABE-puro was digested with HindIII and treated with calf intestinal alkaline phosphatase to prevent self-ligation. Isolated mTOR was ligated into the digested pBABE-puro plasmid (Figure 5.1). The resulting construct was transformed into DH5alpha cells and plated onto LB agar plates with 100 mg/mL ampicillin. This culture was incubated at 37°C overnight. Isolated colonies were selected the next day and used to seed 5 mL liquid LB cultures with 100 mg/mL ampicillin. These cultures were incubated overnight at 37°C followed by plasmid purification. Purified plasmid was digested with HindIII to separate the plasmid into two distinct bands. Gel electrophoresis confirmed bands of the expected size. A primer was designed to initiate sequencing approximately 50 base pairs upstream of the HindII restriction site on the

pBABE-puro vector. DNA sequencing confirmed that the mTOR gene was oriented correctly (Figure 5.2). Since the restriction site is identical on both sides of the gene, the potential exists for incorrect orientation following ligation. Sequencing results showed the sequence for pBABE-puro immediately upstream of the HindIII restriction site as expected. Following the HindIII site, the sequence did not align with the NotI restriction site or the pBABE-puro sequence immediately following the original HindIII site. Were the NotI site present directly downstream of the HindIII restriction site, this would indicate that the insert was oriented backwards.

**Figure 5.1**

*Workflow for Construction of MTPB-8*

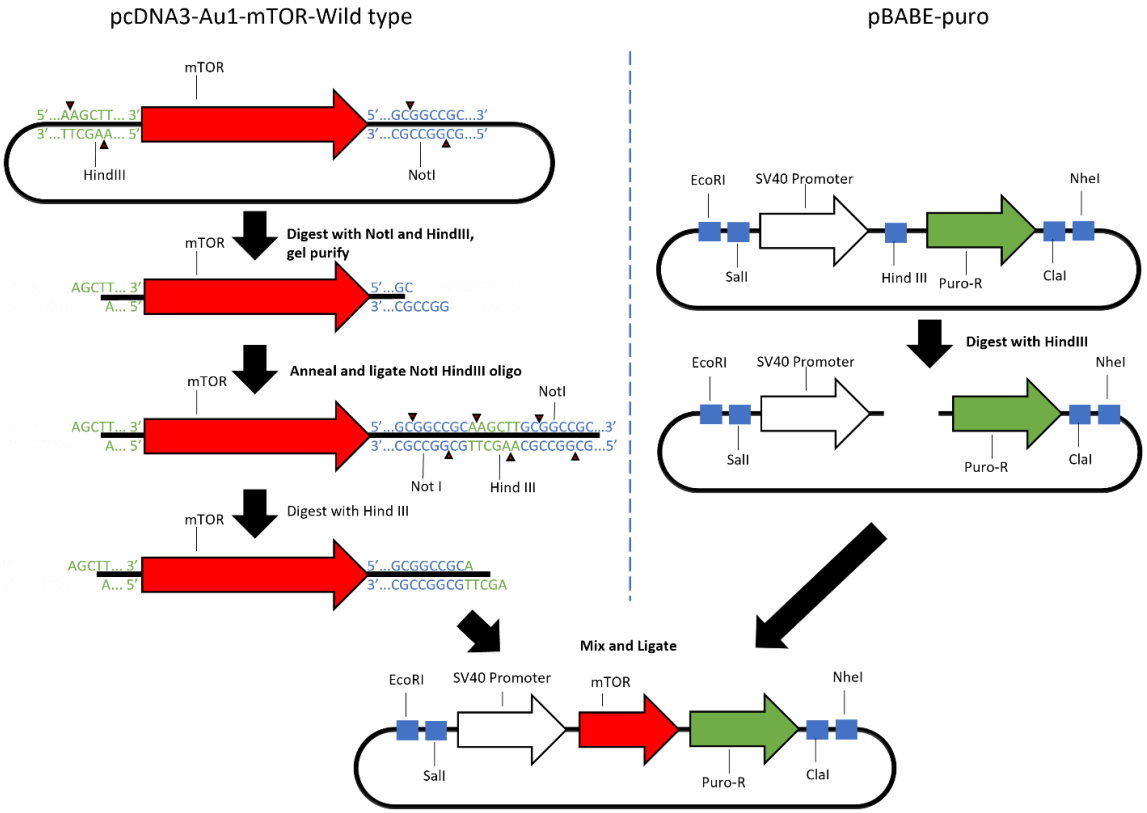
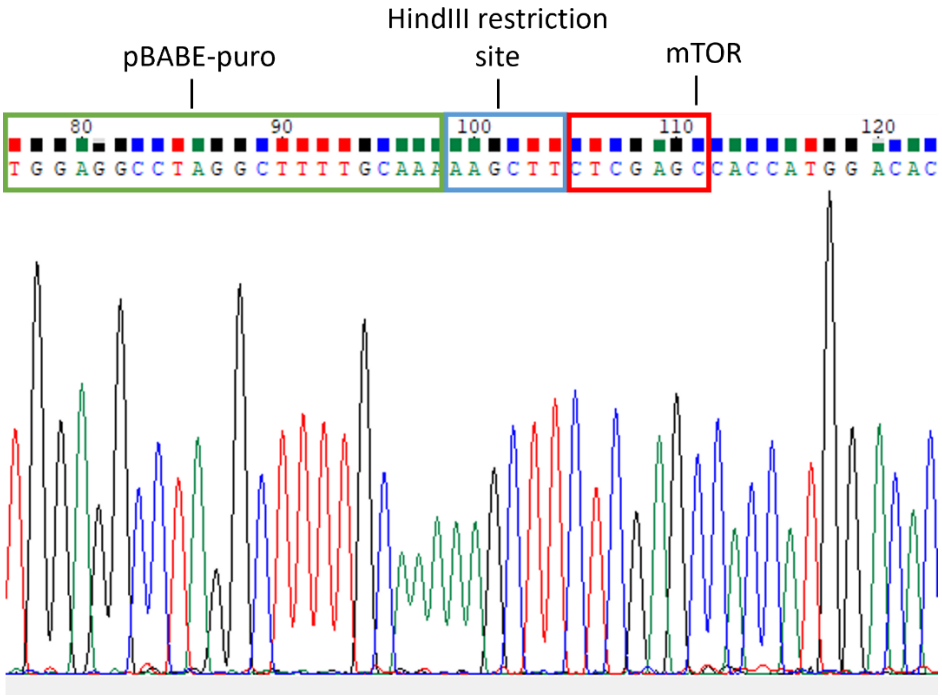


**Figure 5.2.**

*Sequencing Results from MTPB-8 Showing Correct mTOR Orientation*




***Homology Arms***

Homology arms are a key component to adding a gene through homology directed repair. These arms are typically approximately 800 bp in length and should be designed to directly flank the Cas9 cut site, which lies 3 bp upstream of the protospacer adjacent motif (PAM) sequence. Primer pairs were designed to amplify homology arms from the CHO-M genome. The PAM sequence was modified on the forward primer for Homology Arm 2 to prevent repeated recognition and cutting by Cas9. Homology arm 1 was modified to contain the restriction site EcoRI at the 5’ end and SalI at the 3’ end.

Homology arm 2 was modified to contain the restriction site ClaI at the 5’ end and NheI at the 3’ end.

**Figure 5.3.**

*Schematic Showing Homology Arm Amplification with Addition of PAM Sequence and Restriction Site*

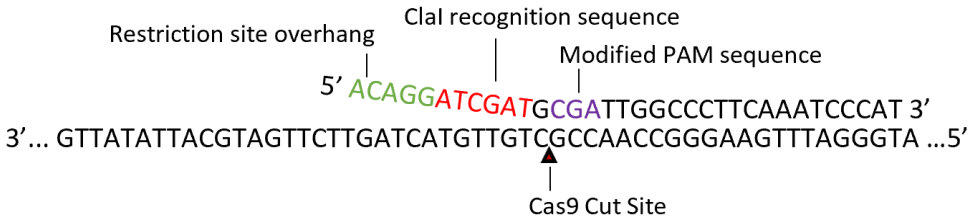



**Table 5.1.**

*Primer Pairs for Homology Arms*

| | |
|---|---|
| Homology Arm 1 | |
| Forward Primer | 5' TAAGCAGAATTCCCTTATTTACCACAAAGCTTTGTGC 3' |
| Reverse Primer | 5' CCTGGTCGACGTACTAGTTCTTGATGC 3' |
| Homology Arm 2 | |
| Forward Primer | 5' ACAGGATCGATGCGATTGGCCCTTCAAATCC 3' |
| Reverse Primer | 5' ATAAGCTAGCGCTTCAACATACTCATCAGATGTTG 3' |

These primers were used to amplify homology arms to be used in the repair template during mTOR knock-in. Genomic DNA isolated from CHO-M cells was used for homology arm PCR. 0.5 mL of CHO-M cell culture was centrifuged at 1000 rpm for 5

minutes. The supernatant was then removed, and the cells were resuspended in 20 µL TE buffer. Resuspended cells were heated in a thermal cycler at 100°C for 10 minutes followed by 2 minutes chilling on ice. The sample was spun at 100xg and the supernatant was collected to be used as the PCR template. Distal homology arm amplification was performed with the protocol outlined in Table 5.2. Gel electrophoresis was used to verify homology arms of the proper size were generated. This protocol proved ineffective for amplification of the proximal homology arm.

**Table 5.2.**

*Distal PCR Protocol*

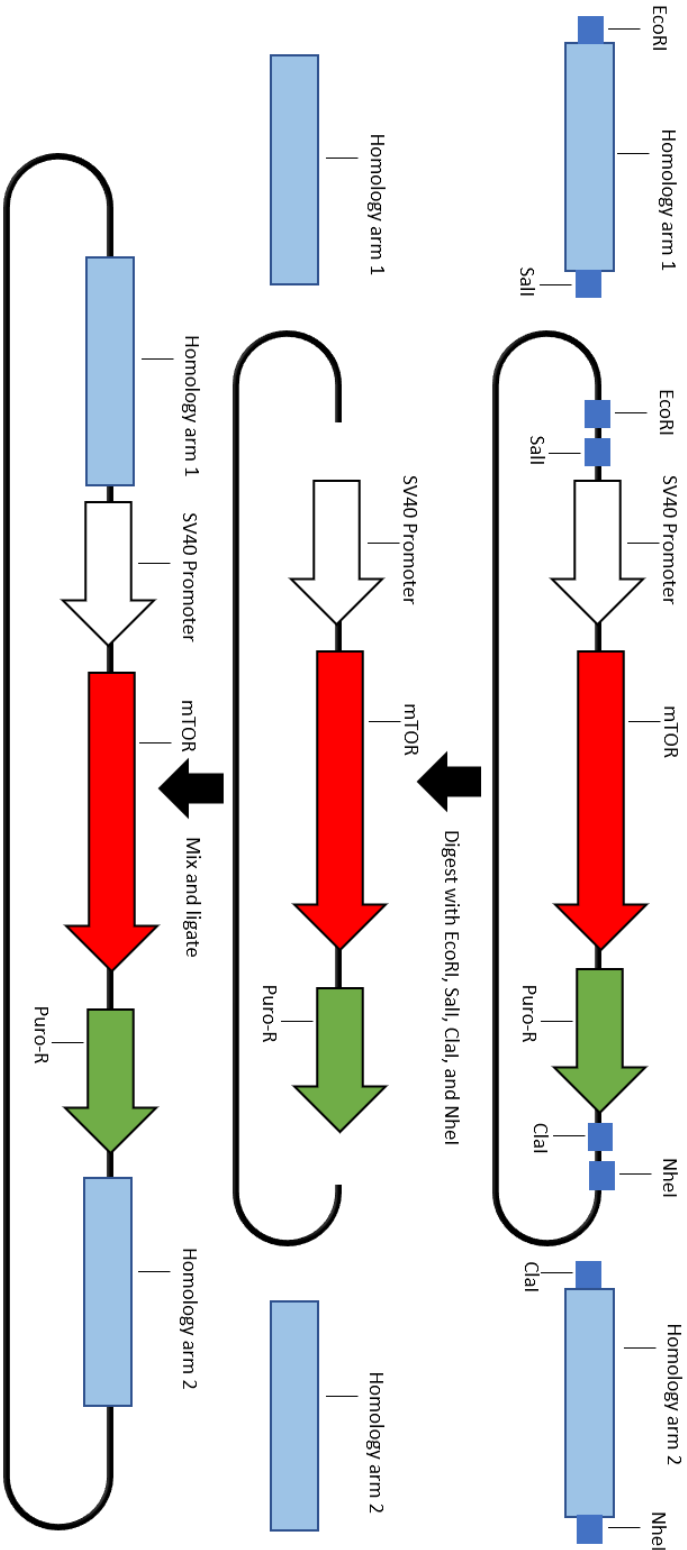
| Step | Setting | Duration |
|---|---|---|
| 1: Initial Denaturation | 95°C | 10 min |
| 2: Denaturation | 95°C | 1 min |
| 3: Annealing | 57°C | 2 min |
| 4: Extension | 72°C | 3 min |
| 5: Repeat | Goto 2 | 40x |
| 6: Final Extension | 72°C | 7 min |
| 7: Hold | 4°C | Hold |

The PCR protocol was optimized to amplify the proximal homology arm. Touchdown PCR was performed to optimize annealing temperatures but did not generate the expected bands. Since touchdown PCR was still unable to product bands of the appropriate size, a PCR gradient spanning annealing temperatures from 50°C to 37°C was used. This method generated proximal homology arms of the expected size.

A complete repair template will be generated by adding the homology arms to the mTOR Puro-R construct MTPB-8 (Figure 5.4). This template will be transfected along with the previously generated targeting plasmid.

**Figure 5.1.**

*Addition of Homology Arms to MTPB-8 Plasmid Containing mTOR and Puromycin Resistance*



**Results and Discussion**

The gene coding for mTOR was successfully ligated into the pBABE-puro vector and its orientation confirmed with sequencing. Homology arms flanking the cut site for C12orf35-1 are to be added to this vector, but so far ligation has not yielded a successful construct.

# Chapter 6

## Summary

As disease treatment evolves and the needs of the biopharmaceutical industry continue to change, we must first look upstream for solutions to meet demand. Engineered cell lines offer an undeniable opportunity to establish a strong basis for manufacturing on the outset of drug development. With the advent of CRISPR/Cas technology, genetic engineering of mammalian cell lines has never been easier. A myriad of opportunities presents itself to experiment with methods that push biology well past its previously established limits. This open-ended outlook must be tempered with caution regarding how engineered cell lines may deleteriously affect molecule functionality and immunogenicity.

This research demonstrated the successful design, construction, and transfection of a plasmid targeting *C12orf35*. This plasmid allows for gene recognition by Cas9, thereby inducing a double-stranded break and potential gene knockout via non-homologous end joining. Significant differences were observed between transfected cells and control cells even in pooled populations not derived from a single clone. With this plasmid, a simple knockout to increase productivity may be performed in any CHO cell line following screening for *C12orf35* with the designed primers. With successful clone isolation, it is reasonable to assume that high-producing CHO clones may be generated with this technique. This provides another tool for creating cell lines that are efficient producers of extremely valuable therapeutics.

To further optimize a CHO cell line, overexpressing genes that are known to positively impact productivity can go a long way toward creating a high-producing clone. An important consideration for adequate gene expression is the transcription rate of the targeted site. Since *C12orf35* is in a highly transcribed region of the CHO genome, it makes logical sense to attempt stable expression of a known, beneficial gene at this site. To this end, a donor template designed to knock-in the gene coding for mTOR was designed to target *C12orf35*. The mTOR gene was successfully cloned into the plasmid pBABE-puro, but ligation of the homology arms to this site has not yet proven effective.

Future research should focus on two outcomes: 1) generating a stable cell line from a single clone transfected with the *C12orf35* targeting plasmid and 2) completing the repair template allowing for knock-in of the gene coding for mTOR.

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# Appendices

**Appendix A: Manuscript submitted to Biologicals and currently under review.**

# Modern Development of Monoclonal Antibodies

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**Abstract**

To effectively manufacture monoclonal antibodies (mAbs), multidisciplinary considerations must be made to ensure acceptable levels of productivity and product safety. From molecule discovery to large-scale manufacturing, careful planning and intent are needed to make a viable product. A variety of technologies exist and are still in development to push the limits of what is possible in mAb manufacturing. mAb development involves immunization, genetic engineering, process optimization, and regulatory concerns. This article provides an overview of widely used practices along with developing fields currently shaping the future of biological therapeutics.

## 1. Introduction

Therapeutic monoclonal antibodies (mAbs) are molecules that are used to provide treatment for some of the most challenging diseases including Crohn's disease,

rheumatoid arthritis, cancer, and more recently, COVID-19 [1–4]. Manufacturing these molecules on a large scale presents challenges and opportunities for development that help to build a better future for medicine. Researchers must decide the appropriate paths for molecule discovery, cell line development, and manufacturing conditions prior to engaging in costly studies. This article aims to provide a starting point for researchers to make important decisions early on in the development process.

## 2. Brief History of Antibodies

Antibodies are a key component in immune systems that allow for recognition of foreign substances in the blood stream. These proteins are produced in B lymphocytes [5] and function in recruitment of cytotoxic effector cells to a targeted antigen [6]. Since their discovery in 1890 as a substance that seemed to neutralize infection by the bacteria causing diphtheria [7], antibodies have held great promise for disease treatment. Early administration of antibody treatment took the form of serum therapy, which utilized the serum of immunized animals to directly treat humans [8]. This method was used to treat a wide variety of viral diseases in the early 20$^{th}$ century [9–12] and developed into the field of passive immunization [13]. While effective, this form of treatment proved risky in humans with severe side effects resulting from an immune response to the animal serum [14]. While it was clear that this field held value, the risks needed to be mitigated for effective treatment to be possible. The field continued to develop towards an understanding of how antibodies are produced and how we may

utilize this means of production for therapeutic purposes. In 1973, Dick Cotton and César Milstein demonstrated the successful fusion of two myeloma cell lines [15]. This work was a milestone in antibody development because it demonstrated that two separate cell lines, each individually producing distinct antibodies, could be fused to form a hybrid producing antibodies from both parental cell lines.

## 2.1 Polyclonal versus Monoclonal Antibodies

Initial research surrounding treatment with antibodies utilized polyclonal antibodies (pAbs), which are derived from many different B cell lineages (yielding the 'polyclonal' nature of pAbs) and target the whole antigen as the result of an immune response. The multi-epitope binding action of pAbs provide increased sensitivity for a variety of applications and robustness against epitope variability [16]. pAbs also benefit from their rapid generation times and stability but suffer from decreased consistency and concentration [17]. While extremely useful for some applications, the low productivity of pAbs and heterogenicity limit their application for pharmaceutical purposes. Monoclonal antibodies (mAbs), however, target a single epitope, are homogenous, and consistent compared to pAbs. mAbs may also be produced in immortalized cell lines, whereas pAbs are typically harvested from immunized animal blood [18]. This allows for relatively on-demand production in higher quantities than what would be reasonably supplied from pAb production.

## 2.2 mAb Market Value

Manufacturing antibodies at a large scale is becoming an increasingly lucrative business as the industry has been steadily growing over the past several decades to match ever-increasing demand. From 2008 to 2014, yearly sales of full-length mAbs produced in mammalian cell culture rose to over $60 billion [19]. In 2018, the global therapeutic monoclonal antibody market was valued at $115.2 billion. This value is projected to increase to $300 billion as early as 2025 [20]. As patents for some of the most successful mAbs expire, opportunities arise for biosimilars to enter the market. Biosimilars face many difficulties when trying to copy an approved mAb, including demonstrating comparable clinical quality. Factors such as immunogenicity can have unpredictable effects when attempting to replace such a complex product [21]. The introduction of biosimilars is a developing field requiring complex navigation and a large pool of experience to draw from [22]. Despite these challenges, the number of Food and Drug Administration (FDA)-approved biosimilars has more than doubled in the last three years from 16 (2015-2018) to 35 approved products (2019-2022) [23]. With no sign of slowing down, there is clear value in developing methods to safely generate these products in large quantities.

# 3. Antibody Production Techniques

The first step in generating antibodies against a specific antigen is the discovery of a suitable molecule. The most common and traditional method uses hybridoma cell lines derived from antibody-producing B-cells found in the spleen of an immunized animal. Surface display technologies provide an alternative method for antibody discovery and effectively mimic the *in vivo* immune response [24].

## 3.1 Hybridoma Technology

A major hurdle present in antibody production was the short lifespan of B cells, which are required for antibody production. To address this, Georges Köhler and César Milstein, building off of previous work, developed a fusion cell line of immunized mouse spleen cells with the long-lived myeloma cell line [25]. The technique became known as hybridoma technology and is capable of producing a stable cell line manufacturing antibodies against a specified antigen [26]. For this technique, an animal subject is first immunized with the target antigen. Sera from immunized animals is screened for affinity against the antigen. Spleen cells from high affinity subjects are isolated from the animal and fused with myeloma cells such as Sp2/0-Ag14 [27]. These fused cells are cultured in a hypoxanthine-aminopterin-thymidine medium (HAT) selection medium. Aminopterin blocks the function of dihydrofolate reductase (DHFR), which in turn inhibits *de novo* DNA synthesis. This forces cells to utilize salvage pathways that require media containing thymidine and hypoxanthine. Myeloma cells used for generation of hybridomas are genetically modified to have a non-functional thymidine kinase (TK)

and/or hypoxanthine-guanine phosphoribosyltransferase (HGPRT), which are both required for the salvage pathways to function. Therefore, the myeloma cells will die off by themselves in HAT medium. The mouse splenocytes, while possessing functional TK and HGPRT, will eventually die off by themselves since their replication number is limited due to the nature of the cells (myeloma cells, however, are an immortalized cell line). With these conditions, the only surviving cells will be a fusion of myeloma and splenocytes [26]. Limiting dilution may then be used to isolate a single clone for further investigation [28].

Early antibody constructs were developed with traditional hybridoma technology and thus were of murine origin. High immunogenicity from murine antibodies resulting from human anti-murine antibody (HAMA) response, prompted the development of humanized antibodies [29,30]. HAMA response dramatically reduced therapeutic efficacy and resulted in rapid removal of the mAb from patient systems. While there are still many questions surrounding the effectiveness of antibody humanization to reduce immunogenicity, the practice continues to be popular for mAb development [31–33].

## 3.2 Phage Display

More recently, phage display technology has gained attention as a viable way to isolate antibodies against a specific antigen. For this discovery, the Nobel Prize in Chemistry was awarded to George P. Smith and Sir Gregory P. Winter in 2018 [34]. This

technology utilizes characteristics found in viruses that infect bacteria known as phages and made its first appearance in 1985 [35]. Early work showed that a foreign DNA sequence could be expressed on the protein coat of phage particles. By splicing a foreign sequence between the amino terminal half and the carboxyl terminal half of phage gene III, a fusion protein is formed. Phage gene III codes for pIII, which is a minor coat protein at the tip of the filamentous phage. This process embeds the foreign peptide into the coat protein while maintaining functionality of pIII, which plays an important role during infection. Of particular importance is the intact immunological activity of the foreign peptide. This was demonstrated by expressing an EcoRI gene on the surface of phage. Infectivity remained active after this modification but was blocked when the phage was exposed to anti-EcoRI antibody.

While an individual phage expressing a single peptide at its surface may not be of great interest for antibody production, the true value of this technology lies within the generation of entire peptide-displaying libraries. These libraries can be generated to display a vast array of random peptides of many varieties [36,37]. This principle can be further applied to potentially eliminate the need for hybridoma technology. Genes coding for the light and heavy variable chains of antibody-binding fragments may be isolated from spleen cells of immunized mice through PCR. These genes may then be combined randomly using a linker region of DNA coding for a short peptide that, when expressed, will act as a hinge connecting the fragments [36]. This process generates a library of coding sequences that express randomly combined single-chain Fv (scFV) antibody fragments. These sequences may then be incorporated directly into the phage

genome or provided as phagemid vector which will compete with the wild-type coat protein expression [38].

Once a library of antibody-expressing phage has been created, these viral particles may now be screened to isolate high affinity variations. This process, known as biopanning, utilizes affinity selection to isolate high affinity clones [39]. For this process there are 5 basic steps: 1) binding of target molecule, 2) phage binding, 3) washing, 4) phage elution, and 5) amplification. In the first step the target molecule is immobilized on a surface such as a microtiter plate. Next, the phage library is applied to the immobilized molecule followed by a washing step to remove any unbound phage. The target molecule is then denatured allowing for the elution of isolated phage. Isolated phage may now be used to infect bacteria for amplification. This process may be repeated for additional cycles to ensure a high affinity molecule is produced.

Antibodies may be isolated with hybridoma technology or phage display. Both methods require immunized animals to produce target specific antibodies. Hybridoma technology seeks to isolate a single clone producing the desired antibody. Alternatively, phage display allows for the generation of entire libraries of antibodies with varying affinity for a target molecule. Both methods possess unique advantages and disadvantages for the discovery of therapeutic antibodies.

## 3.3 Other Surface Display Technologies

Yeast, a well-characterized and established organism for research, may also be used as a platform for surface display. This method uses the fusion of antibody fragment genes to cell wall genes, thereby displaying the protein on the cell surface [40–42]. Similar to the biopanning method used to screen antibody candidates in phage display, magnetic activated cell sorting (MACS) may be used to narrow the selection pool followed by fluorescence-activated cell sorting (FACS) [43,44].

*Escherichia coli* cells have a long history of use in protein manufacturing and genetic engineering. These cells seem an obvious choice for display technology development, however, these cells struggle to display complex molecules such as full-length mAbs at the cell surface. This issue was addressed in 2008 using bacterial periplasmic display in “E-clonal” mAbs [45]. This technique stabilized the antibodies and allowed for FACS screening.

Mammalian expression systems offer the benefit of post-translational modifications necessary for clinical applications. These systems are capable of generating display libraries similar to other display technologies with the added potential benefit of greater control over immunogenicity [46]. This technology was largely limited to generating libraries against a specific antigen; however, the process has recently been adapted to create more broadly applicable libraries. Mammalian surface display has been developed in a variety of cell lines including HEK293T, COS, CHO, and B lymphocytes [46–48].

## 4. Cell Line Development

Once an antibody has been discovered, it is often desirable to move production into a high-producing, well-established cell line. While many cell lines exist that can produce mAbs and biosimilars, none are quite as suited to the task as Chinese Hamster Ovary (CHO) cells. CHO cells are the most prevalent cells used in the production of therapeutic proteins [49,50]. These now invaluable cells were first isolated by Theodore T. Puck and his team in 1958 [51]. CHO cells were originally of interest due to their low chromosome number and applications in genetic studies, but soon proved to be a viable cell line suitable for long-term culture [52].

With an established hybridoma cell line, the next step in transitioning toward CHO cell culture is antibody sequencing. This can be accomplished by using primers targeting highly conserved regions in hybridoma antibody RNA. In combination with a reverse transcriptase, these primers are used to generate complimentary DNA (cDNA) coding for the antibody [53,54]. A variety of methods exist for cDNA generation and purification [55]. The resulting DNA may then be cloned into an appropriate expression vector. This vector may take the form of a transiently expressed plasmid or may be expressed stably [56].

There are many ways to express an antibody inside of CHO cells. Transient transfection offers a temporary platform for antibody expression and can be used as the first step for screening a candidate molecule for further development. An expression plasmid is first transfected into the CHO cell line through techniques such as electroporation, lipid mediation, calcium phosphate, or polymer mediation [57–61]. The

expression plasmid contains multiple components that allow for transient expression in cells often including an SV40 origin of replication, promoter, and a selectable marker [62].

Stable expression of a recombinant protein in CHO cells requires integration into the host genome. The traditional method to achieve integration relies on random integration and selection pressure. Two common methods to achieve integration are the DHFR and glutamine synthetase (GS) systems [63,64]. The DHFR system uses CHO cell lines that have an inactive DHFR gene, thereby forcing the cells to rely on media containing glycine, hypoxanthine, and thymidine (GHT). The plasmid expressing the antibody is transfected with a DHFR gene, either on the plasmid itself or co-transfected as a separate vector [65]. Cells are then cultured in a GHT free medium, forcing reliance on the expression of DHFR. A pool of clones is the result, with varying degrees of integration and expression. To further select for clones with high copy numbers, gradual addition of methotrexate (MTX) may be used. MTX inhibits DHFR function, thereby selecting for clones expressing multiple copies of the DHFR gene [66].

Glutamine synthetase is an enzyme that catalyzes the conversion of glutamate and ammonia to glutamine, which is an essential component in cell metabolism. Similar to the DHFR system, a selection medium lacking glutamine is used as a selection pressure for clones that have successfully integrated the GS gene. Methionine sulfoximine (MSX) is used to inhibit endogenous glutamine production and, much like MTX in the DHFR system, effectively selects for high antibody expression [67,68]. While

both systems have proven historically effective for generating high-producing clones, they are limited in that integration is random, requiring careful selection of clones.

## 4.1 Site-Specific Integration

Integrating a gene of interest (GOI) at a pre-defined site offers many potential advantages over random integration. Loci that are known to have high transcription rates may be effectively targeted to increase productivity. Several tools exist for site-specific integration including recombinase-mediated cassette exchange (RMCE), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS), or clustered regularly interspaced palindromic repeats (CRISPR/Cas9) system [69,70].

### 4.1.1 Recombinase-Mediated Cassette Exchange

Initial attempts to introduce GOIs at high expression loci in CHO cells utilized site-specific recombinases (SSR). This technique takes advantage of SSR's ability to catalyze recombination at a defined site in systems such as the Cre/*loxP* recombination system [71]. RMCE incorporates SSR recognition sites that allow for the addition of a GOI between the sites [72]. This provides a reusable location for site-specific integration. A particular downside to this method is that it requires selecting a high-producing platform cell line through traditional random integration techniques. Once a high producer is identified, it may be used as platform for GOI integration and

expression, however, the integration site is still at its core randomly selected and not a targeted design [73].

### 4.1.2 Zinc-Finger Nucleases

ZFNs offer a method for inducing double-stranded breaks in DNA by combining zinc-finger proteins (ZFPs) with the nuclease domain of the FokI restriction enzyme [74]. ZFPs contain Cys2His2 fingers capable of recognizing a 3 base pair sequence and binding to DNA via the α-helical portion of each finger [75]. For this to be useful in site-specific recognition, several ZFPs must be assembled in a specific order. This necessitates a library of ZFPs that allow for standardized, modular assembly [76–78]. Drawing from these now publicly available protein libraries, a zinc finger array may be assembled that recognizes a specific locus. Recognition and binding are further complicated by context-dependent effects, so-called to illustrate the lack of independence between adjacent ZFPs [79]. While methods exist to design arrays around these effects, these interactions remain problematic for binding efficiency [80]. Additionally, advanced methods for array design require a high level of understanding and expertise.

The activity of FokI is dependent on its dimerization. Therefore, when designing ZFNs, the arrays must flank the cut site with the FokI cleavage domain between them. This results in two monomer subunits that dimerize the FokI cleavage domain across the cut site, initiating a double-stranded break. This break may be resolved through non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is the most

common path and can result in mutations that disrupt the gene. HDR is the less common outcome and requires a supplied repair template to insert a gene at the cut site [74].

### 4.1.3 Transcription Activator-Like Effector Nucleases

TALENs, like ZFNs, use engineered nucleases to perform DNA alterations. Much like ZFNs, TALENs also utilize the FokI nuclease domain and a customizable DNA binding domain [81]. This DNA binding domain consists of transcription activator-like effectors (TALEs), which are found in the pathogenic bacteria Xanthomonas. These bacteria utilize TALEs to alter gene transcription by injecting them into host plant cells. TALEs are then able to bind to host DNA and promote bacterial colonization [82].

TALEs are highly conserved repeats of 33-35 amino acids that each recognize a single base pair, which allows them to be constructed into modular arrays. The customizability of these arrays allows for flexible gene recognition and cutting [83]. TALE-nuclease chimeras can be formed by linking a FokI nuclease domain to the C-terminal of TALE arrays [84]. Like ZFNs, TALENs require recognition sites flanking the cut site. Once recognition occurs at the site, a FokI dimer at the C-terminal of each array forms and initiates a double-stranded break [85]. The DNA may then be repaired through either NHEJ or HDR and a repair template may be provided. Entire libraries of TALENs have been developed, even spanning the human genome [86].

While ZFNs and TALENs dominated genetic engineering methods for several years, their required specialty knowledge and barrier to entry made genetic engineering of eukaryotes a continually difficult endeavor. Mammalian genetic engineering was still in its infancy during this time with the development of CRISPR/Cas systems just on the horizon.

### 4.1.4 CRISPR/Cas9

CRISPR systems are bacterial and archaeal immune defense mechanisms that have been adapted for use as a genetic engineering platform. CRISPRs are characterized by hypervariable spacer sequences that are acquired from foreign invading DNA interspaced between repeat sequences [87]. These sequences are transcribed to produce pre-CRISPR RNA (pre-crRNA), which is then cleaved to isolate individual spacers and a partial repeat. The crRNA is then able to recognize and bind to matching invading DNA (termed the protospacer) to initiate cleavage via recruited Cas proteins.

CRISPR/Cas9 made its debut into mammalian cells in 2013 with the promise of providing a system for mammalian genetic engineering with several advantages over more traditional ZFN or TALENS methods [88,89]. These systems function by guiding an endonuclease to invasive DNA and cleaving the foreign genetic material [90]. Cleavage occurs 3 base pairs upstream of the protospacer adjacent motif (PAM) sequence that must be present for identification of a cleavage site [91]. The Cas9 protein is guided to the target site with the help of two RNAs. The first RNA, called CRISPR RNA (crRNA) is

the genetic material associated with bacterial immunity that is used to identify and cleave previously encountered foreign DNA [87]. The second RNA component necessary for the system to function is known as trans-activating crRNA (tracrRNA). When crRNA and tracrRNA are provided to the Cas9 enzyme the result is targeted DNA cleavage at the site complementary to the crRNA sequence [88]. This targeting process can be simplified by fusing the crRNA with the tracrRNA and incorporating a protospacer adjacent motif (PAM) sequence. The PAM sequence (typically 5'NGG3') is vital to recognition of the cleavage site as its presence initiates the interrogation process [92]. The resulting molecule containing a fusion of the PAM, crRNA, and tracrRNA, referred to as single guide RNA (sgRNA), can be transfected alongside the Cas9 enzyme for a simple, rapid method of genome editing [89]. In practical applications, the sgRNA and Cas9 may be transfected directly into the cell or provided as a plasmid to be produced within the cell. More recently, new CRISPR/Cas systems have been introduced with Cas12 and Cas13 coming under investigation [93]. Like ZFNs and TALENS, following cleavage by Cas9, DNA may be repaired through NHEJ or HDR. When provided with a repair template, HDR allows for gene insertion at the cleavage site [89].

## 4.2 CHO Cell Hot Spots

Site-specific integration allows for the selection of highly transcriptionally active integration sties or "hot spots." Ideally, these sites allow for stable gene expression without inhibiting cellular function. A variety of hot spots have been identified in the

CHO genome that warrant further investigation [94]. In transitioning to a CHO production platform for antibody expression, a valuable consideration is where in the genome allows for the best production. Research on this topic is still very much underway with new sites of interest being discovered regularly [95,96]. Criteria for a useful hot spot include active transcription and a high degree of stability, thereby providing a long-term platform suitable for further development.

Epigenetic events are known to regulate gene expression through chromatin modifications and can be inherited [97]. These modifications include histone methylation, heterochromatic formation, and histone deacetylation [98–100]. Reducing the occurrence of these events or inhibiting the processes that lead to them holds the potential to increase transcription rates. For example, highly acetylated chromatin is associated with higher transcription rates in mammalian cells. Negating the charges in histone tails through acetylation interferes with the winding of the nucleosomal array [101]. This unwound form of DNA is more readily available for transcription than DNA that is tightly wound around the histone proteins. The gene coding for retroelement silencing factor 1 (RESF1) is associated with regulation of epigenetic modifications and may be involved in histone deacetylase regulation [102]. This site represents just one potential target for further investigation.

A variety of stable integration sites have been identified in CHO cells by incorporating recombinase ‘landing-pads’ through random lentiviral integration [103]. These sites were modified to readily allow gene integration using RMCE. The functions of these stable sites seem to vary widely from tumor suppression to cell growth, so

there is further work to be done in determining commonality between stable, highly transcribed loci.

## 5. **Process Considerations**

A well-engineered cell line provides the foundation necessary for antibody production, but a cell line can only flourish as well as its growth conditions allow. Process considerations include basal medium optimization, feed optimization, bioreactor design, and protein quality.

### 5.1 Cell Culture Media Optimization

At the heart of every successful production run lies a reliable, well-characterized cell culture medium. Classical cell culture media are largely based on the early work of Harry Eagle, who developed the combination of amino acids, sugars, vitamins, and salts now known as Eagle's Minimal Essential Medium (EMEM) [104,105]. This led to the development of an optimized version known as Dulbecco's Modified Eagle's medium [106]. While these media supported cell growth and high densities, they required the addition of serum. Serum supports cell growth quite well but requires careful sourcing and carries with it the risk of adventitious agents such as viruses, bovine spongiform encephalopathy, and transmissible spongiform encephalopathy (BSE/TSE) [107,108].

Since serum is a biological product, it is highly subject to lot variability, which can be affected by uncontrolled factors such as seasonal changes and disease.

The desire for a chemically defined medium led to the development of Ham's nutrient mixture F12 (Ham's F12). Ham's F12 still suffered from an inability to promote high cell densities. These challenges promoted further investigation into cell culture media development and the commercial interest in suitable formulations. Currently, several companies offer off-the-shelf proprietary media that are typically tailored to specific types. Companies also offer media development services, which use modern software and Design of Experiments (DOE) to develop chemically defined media custom tailored to specific cell lines [109]. The high variability in nutritional need and response even between clones makes development of platform media a unique challenge.

### 5.1.2 Feed Optimization

Like basal medium optimization, feed design is also an important consideration for fed-batch manufacturing as a pathway to provide high cell densities and productivity. A variety of feed strategies are available, with the needs of individual cell lines varying [50,110]. Pre-made feeds are widely available with many products tailored to suit specific cell lines. DOE may be applied to these products to further optimize parameters at the individual clone or platform level [111,112].

## 5.2 Bioreactor Manufacturing

Industry-scale manufacturing of monoclonal antibodies requires culture in optimized bioreactors. Under these conditions cells must be able to grow under suspension cell-culture. Liberating a cell line from its anchorage-dependence typically involves weaning the cells from serum-dependence, while transitioning from static culture to shaker flasks [113]. This process requires multiple passages and careful monitoring of culture health. Once a cell line is successfully adapted to suspension conditions, bioreactor manufacturing may begin. Bioreactors come in many shapes and sizes from traditional stirred-tank to wave bioreactors [114]. Osmolality, pH, carbon dioxide, and oxygen must all be monitored to ensure maximum performance.

## 5.3 Protein Quality

Antibodies are complex molecules produced in imperfect biological systems. Reducing variability in antibody isoforms helps to ensure that drug products function as intended with minimal off-target binding [115]. Once a cell line has been generated that produces a desired antibody, it follows that the molecule and process must undergo validation to ensure efficacy and reproducibility. Several standards and techniques exist for validation, but the field continues to evolve with debate regarding the best path forward [116]. The introduction of recombinant antibodies manufactured as biosimilars further complicates the standardization of quality control best practices [117].

Biosimilars are molecules with no clinically significant differences from a reference biologic that has already been approved by the FDA [118]. These products must be manufactured under the same conditions as their reference molecule and in some cases may be used as a “generic” in place of the reference biologic. The continued development of biosimilars helps to fill the demand for therapeutics, but this must be tempered with tight standards and quality control to ensure a directly comparable product is made [119]. Comparability attributes as outlined by the FDA include the expression system, manufacturing process, physiochemical properties, and molecule functionality [120].

Validating antibody performance and specificity is crucial to creating a viable product. Non-specific binding has led to the dismissal of potentially promising antibody discoveries [121,122]. This philosophy strongly carries over to biosimilar development, where careful evaluation of physiochemical properties must demonstrate comparability to the reference molecule. The European Medicines Agency (EMA) provides guidelines on demonstrating biosimilar comparability which include physiochemical properties such as amino acid sequence, disulfide bridge modifications, oxidation, glycosylation, and deamidation [123]. Glycosylation is a post-translational modification that involves the addition of carbohydrates to a protein backbone [124]. This process introduces the potential for protein heterogeneity, and can impact the functionality of antibodies [125]. Characterizing the glycan profile of a biosimilar helps to ensure that the product will have comparable characteristics to the reference molecule and can be performed through various methods [126]. Close monitoring of glycan profiles, which can even be

affected by culture conditions, helps to reduce immunogenicity and provide a safer product [127].

## 6. Conclusions

Manufacturing therapeutic antibodies requires significant consideration across a variety of specializations. Care must be taken at the early planning stages with a clear path for development from discovery to manufacturing. The current regulatory environment increasingly favors drug development free from animal-derived components or animal-based drug discovery. Animal-derived material introduces complexity and variability into a process that must heavily rely on strict control.

Evolving technologies have made highly specific gene editing a much easier, accessible process. This opens the door to designing cell lines with productivity and quality in mind during the early stages of development. The days of relying on random gene integration for stable production of mAbs may soon be gone as our understanding of transcription sites develops. Upstream design may allow for quickly choosing a production platform based on specific productivity and quality demands.

## Declaration of competing interest

The authors do not declare any competing interests.

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**Appendix B: Sequences for homology arm design**

Homology arm 1

Nucleotide sequence for homology arm upstream of predicted Cas9 cut site. Primer pairs are highlighted.

CCTTATTTACCACAAAGCTTTGTGCAAGACACTTCTGTTCAGAAACAAAACTTTGTGTCATCTACA
TCATTACAAGTTAAAAATAATCAGCTTCCACCTTCTACACAGACCTTACCATCAAAGCGCCCTGT
ACCTGTGTCGTCATATCAGTATGCTGCAGAAACCAGCAAAAGACTCCCTCCCCCCCCTTACAGCT
GTAGATATGGAAGCCAACATGTGCAAAATTCTCAGTCTGTTTCTAGACACTTGCCTGTGGAAGT
TCCTCAGAGTTCAGAAATGCACTCGTCTGAAAAAAAGAAAGATGCTTACAAAGTCTTTCAACAG
CAGTGGCAGAGCACTAGTAAAAATGTCAGTACAATAGGAAAATTCTGTGAGTTGAAAATTAATA
CAAAACAGTCTTACAATGACTCTGCTGGCTCTTCTGGGGATGGTGTTCATACTCTTGTTCAAAAT
AATCAAGAAGAAAGAAAGTATTCTTATAATCCAAGTACAAATCAAATACTAGACACAAATGTCA
CAAAAGAAAAGCTGGTGAGGGATATTAAATCACTAGTAGAAATTAGCTGGGCCATGGTGGCGC
ATTCTGAATTTTCAGCAAAAGAAATGTCTGCTAAAAGGGACAATCAGTGCTCCATGGAATTGCT
AGCAACATGCCTTTCTCTTTGGAAAAACCAACCTCCAAAAACCACAGAAGAAAATGTTTCAAAA
CCTTTAGAAGAAAAACAATATAATGCATCAAGAACTAGTAC

Homology arm 2

Nucleotide sequence for homology arm downstream of predicted Cas9 cut site. Primer pairs are highlighted with the PAM sequenced indicated in red.

CAGCGGTTGGCCCTTCAAATCCCATGAATGAAGTTCATGTGAAGAATTTTTGTTCAGGTGTTAG

AAATTCTCAGAAAATAACCACCTCGTCACAAACAGTCTTGTCAGTTCTCACACCAGTTTACGATT

CTTCAGATGTAGCTGTTGGAAAAGGAACAGAGCTTCAGATTGCTGTGGTTTCACCTTTAATTCTT

TCAGATGTCAGTACTGTACCTGGGAAAGAGTTAGCTCCTGAAGTCGTATCTGAAACTGTATATC

CAGTTGTGAAGGAAGGCAGTGTTTGTAGCTTACAAAACCAGCAGGCAGAAAATGCAACAGTAA

CTGCTGGTTTGCCCTTTGATGTTATCAGAGCAGTAGCAAGTGCTACTGTATCAGCTGAGCTATCA

CTGCCTGGGCATAAAGAAAAGCAGCACAAACCAACACAGACTGATCTAGATACTGCTGATGGC

AGCCTAGGGAAACACTCTCCCCAGGGTGCTGAAGCTTTGCCTAACCCTAGGGACAGCACCATTG

TGAGTGGGCCTATATTACAGATTGAAAGTATCTGTTCTCTTGCAGAAGGTGATGTATCTTACAAT

TCCCAAATAGCAGAGATATTCAACTCTGTACAAAATGAGCCCCAGAAACCTTCACCTGATCAGC

AAGTAATTAATAGTCAACAAGAAGAACAAGTAGATAAGGTTGCTGAAAATAAAGACTTAAGTTT

TCTGAAAGACAAGTGTATGCAGTGTACAGATGTTCCTCATGAAGTCACTGAACAGCCAGAGCCA

CTGCAGCCTTTAGAGACAACATCTGATGAGTATGTTGAAGC