Overexpression of Transmembrane7 Superfamily member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7) enzymes of Cholesterol Biosynthesis pathway for Enhanced Recombinant Protein Production in Chinese Hamster Ovary (CHO) Cells

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Dedication

This thesis is dedicated to my Son, Daniel Adedolabomi Aderanti. You're my support-system Adedolabomi. I love you my Angel!

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent or any other University or Institute of learning

Folasade Ajayi

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All Glory be to God for making this possible.

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To my family, my adorable Son; Daniel Adedolabomi Aderanti, my darling Parents (Dr and Mrs V.O.O Ajayi), my lovely Siblings (Olufolake, Olatunde, Taiwo, Kehinde, Idowu, Spouses and Children. You all have shown me excessive love and have given me the needed support. I love you all so much. To my friends, thanks for being so supportive.

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Again, to my priceless gift, Daniel Adedolabomi. You have been my greatest source of inspiration. Thank you for giving me your full cooperation always, enduring alot of stressful times, so far.

Folasade Ajayi

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Abbreviations

ATF6 - Activating Transcription Factor 6

BHK - Baby Hamster Kidney

BiP - Binding Immunoglobulin Protein

CAGR - Compound Annual Growth Rate

CD-CHO - Chemically-Defined Chinese Hamster Ovary cell

CHO - Chinese Hamster Ovary cell

CHO-S - Chinese Hamster Ovary cell line

cIAP - Cellular Inhibitor of Apoptosis

CKD - Chronic Kidney Disease

CNX - Calnexin

CO_2 - Carbon dioxide

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CRT - Calreticulin

C14-SR - C14 Sterol reductase

DHCR7 - 7-DehydroCholesterol Reductase

DMSO - Dimethyl sulfoxide

eIF2α - Eukaryotic Initiation Factor

EMA - European Medicines Agency

EPO - Erythropoietin

ER - Endoplasmic Reticulum

ERAD - ER-associated degradation

ESA - Erythropoiesis Stimulating Agents

FA - Fatty Acid

Fc - Fragment Crystallisable

FDA - Food and Drug Administration

GFP - Green Fluorescent Protein

GM-CSF - Granulocytemacrophage Colony Stimulating Factor

GOI - Gene of Interest

HEK293 - Human Embryonic Kidney cells 293

HC3 - Human Complement 3

Hep3B - Hepatoma cell line

HMGCR - HMG-CoA Reductase

HMGCS - HMG-CoA synthase

INM - Inner Nuclear Membrane

IRE1 - Inositol Requiring Enzyme 1

IVCD - Integral of Viable Cell Density

JNK - c-Jun N-terminal kinase

LBR - Lamin B Receptor

LDLR - LDL receptor

mABs - Monoclonal Antibodies

mRNA - Messenger Ribonucleic Acid

Mut-F - Mutant F

MVK - Mevalonate Kinase

NE - Nuclear Envelope

NF-kB - Nuclear Factor kappa-light-chain-enhancer of activated B cells

ONM - Outer Nuclear Membrane

PERK - Protein kinase-like Endoplasmic Reticulum Kinase

PQC - Protein Quality Control

PTM - Post Translational Modification

qP - Specific Protein Productivity

rhEPO - Recombinant Human Erythropoietin

RNA - Ribonucleic Acid

S1P - Site-1 Proteases

S2P - Site-1 Proteases

SCAP - SREBP cleavage activating protein

SLOS - Smith Lemli-Opitz syndrome

SRE - Sterol Response Elements

SREBP - Sterol regulatory element-binding protein

SREBP2 - Sterol regulatory element-binding protein 2

SRP14 - Signal Receptor Protein

TALENs - Transcription Activator-like Effector Nucleases

TG - Triglycerides

TM7SF2 - Transmembrane7 Superfamily member 2

TNF - Tumor Necrosis Factor

TNF-α - Tumor Necrosis Factor Alpha

tPA - Tissue Plasminogen Activator

TPA - 12-o-tetradecanoylphorbol-13-acetate

UPR - Unfolded Protein Response

Vi-Cell - Automated Cell Viability Analyzer

WHO - World Health Organization

XBP1 - X-box Binding Protein 1

YFP - Yellow Fluorescent Protein

ZFNs - Zinc-Finger Nucleases

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Abstract

The biopharmaceutical industry is a multi-billion dollar global market with projections that within the next 5 to 10 years, up to 50% of all drugs in development will be biopharmaceuticals. The majority of these drugs are recombinantly produced protein based therapeutics with the demand for such new therapeutic products continuing to increase, broadening the range of medical conditions that they are being used to treat. Chinese hamster ovary (CHO) cells are the preferred and most commonly used system for large scale production of recombinant glycoproteins for therapeutic use due to their ability to perform complex human-like post-translational modifications required for biological activity. CHO cells also display robust growth, high productivity and stability, attributes that have enabled them to become widely used. Whilst there have been large increases in the yield and quality of recombinant protein that can be obtained from mammalian expression systems over the years, particularly CHO cells, many recombinant proteins remain difficult to express in CHO cells or any other system. Many of these proteins are highly complex, highly glycosylated, large and unstable proteins. As such, there remains a need to develop CHO cell systems that are able to produce such difficult to express proteins rapidly in higher yield and quality. The endoplasmic reticulum (ER) is a key compartment in the secretory pathway of mammalian cells and during the production of recombinant proteins in CHO cells this has reportedly been a potential bottleneck and site of perceived cellular stress as a result of the load imposed on the cell by the recombinant protein.

This study has investigated two sterol reductases; Transmembrane 7 Superfamily member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7). ER resident proteins involved in the biosynthesis of cholesterol that have been reported to influence endoplasmic reticulum (ER) and nuclear envelope (NE) expansion when over-expressed in mammalian cell lines. The work described here therefore set out to determine whether overexpression of these sterol reductases could enhance the secretory capacity of CHO cells by expansion of the ER and hence lead to increased yields of difficult to express recombinant proteins compared to those cells where these were not over-expressed. The TM7SF2 and DHCR7 genes were cloned into a mammalian expression vector with a hygromycin selection marker to allow for the selection of stable cell lines where the genes had been integrated into the genome. Stable CHO cells overexpressing TM7SF2 and DHCR7 were generated to assess the impact on cell growth and stable and transient recombinant protein expression, monitored using western blot techniques and microscopy analysis. Control cell lines over-expressing GFP (but not the sterol reductases) and the pcDNA3.1Hygro (empty) vector were also developed. Stable

expression levels of the sterol reductases were determined in heterogeneous pools and then used for limited dilution cloning to isolate clonal cell lines with differing sterol reductase expression which were further investigated. Growth profile assays were undertaken to monitor any toxicity effect of over-expression of TM7SF2 and DHCR7 on CHO cells. Transient expression studies of the recombinant bio therapeutic protein etanercept, a 150 kDa biomolecule and erythropoietin (EPO), a 36 kDa recombinant protein, were carried out to investigate the production capacity of lipid engineered CHO cells with reference to controls. Immunocytolocalisation studies were also undertaken to investigate pools and clones of lipid targets predicted to be ER-localized or associated. A YFP tag was present on the N-terminal of TM7SF2 and DHCR7 molecules and was used to monitor expression by fluorescence in stable CHO cells.

The data from these experiments show that both TM7SF2 and DHCR7 engineered CHO cells had improved cell growth and culture viability over the course of 10-day experimental study compared to the control cells. Western blot studies showed that both TM7SF2 and DHCR7 could be stably over-expressed in CHO cells and the amount of expression varied among the generated clones. Further, the high expressers of TM7SF2 and DHCR7 were identified and investigated for EPO and etanercept production and expression levels of these recombinant biomolecules were observed to be proportional to the levels of TM7SF2 and DHCR7 expressions in CHO cells. More importantly, TM7SF2 and DHCR7 engineered cells showed increased expression levels of these recombinant products when compared with pcDNA3.1Hygro controls as determined by western blot analysis of the amount of secreted recombinant target protein in the cell culture supernatant. Confocal images also showed clones expressed different levels of YFP signal which related to expression levels observed when western blot techniques were used. In summary, the data presented here shows that the manipulation of cellular circuits such as cholesterol biosynthesis has the potential to enhance cellular growth and recombinant protein yield. By designing new hosts and cellular circuits to reprogramme the CHO cell ER there is the potential of expanding the secretory capacity and/or subsequent secretory vesicle system.

Chapter 1

1.0 Introduction

1.1 Recombinant Bio therapeutic Proteins and the Biopharmaceutical Industry

According to Allied Market Research, 2018, the sales of US $186 billion worth of biopharmaceuticals as at 2017 following an approximate 10% annual growth rate (CAGR) having been valued at US $162 billion in 2014 shows a continuous impressive growth that reflects new opportunities in biopharmaceuticals over the past decade. The global biopharmaceuticals market is projected to reach US $526 billion by 2025, growing at a CAGR of 13.8% from 2018 to 2025 (Owczarek & Gerszberg, 2019). The discovery of recombinant DNA and monoclonal antibody technologies in the 1970s marked the birth of the biopharmaceutical industry by production of recombinant proteins.

The term biopharmaceuticals was coined in the 1980s and refers to pharmaceuticals of a protein based nature produced in living cells utilising biotechnological processes and when recombinantly produced using molecular biology methods. These protein-based drugs are increasingly being used in practically all branches of medicine and have become one of the most effective clinical treatment modalities for a broad range of diseases, including cancers and metabolic disorders (Kesik-brodacka, 2017). Biopharmaceuticals are mostly therapeutic recombinant proteins obtained by biotechnological processes, termed bioprocessing.

According to Faustino et al., (2016) it is predicted that within the next 5 to 10 years up to 50% of all drugs in development will be biopharmaceuticals. So far, recombinant proteins, including monoclonal antibodies (mAbs) have been the top targets and low hanging fruit for biopharmaceutical drug production. The market continues to grow (Figure 1.1). Biopharmaceuticals are derived from biological sources such as organs and tissues, microorganisms, animal fluids, or genetically modified cells and organisms (Faustino et al., 2016) with an ongoing increase in the number of the recombinant proteins produced in mammalian expression platforms (Lalonde & Durocher, 2017). In essence, the biopharmaceutical industry is by far the fastest-growing sector of the pharma industry and growth is expected to continue for the foreseeable future.

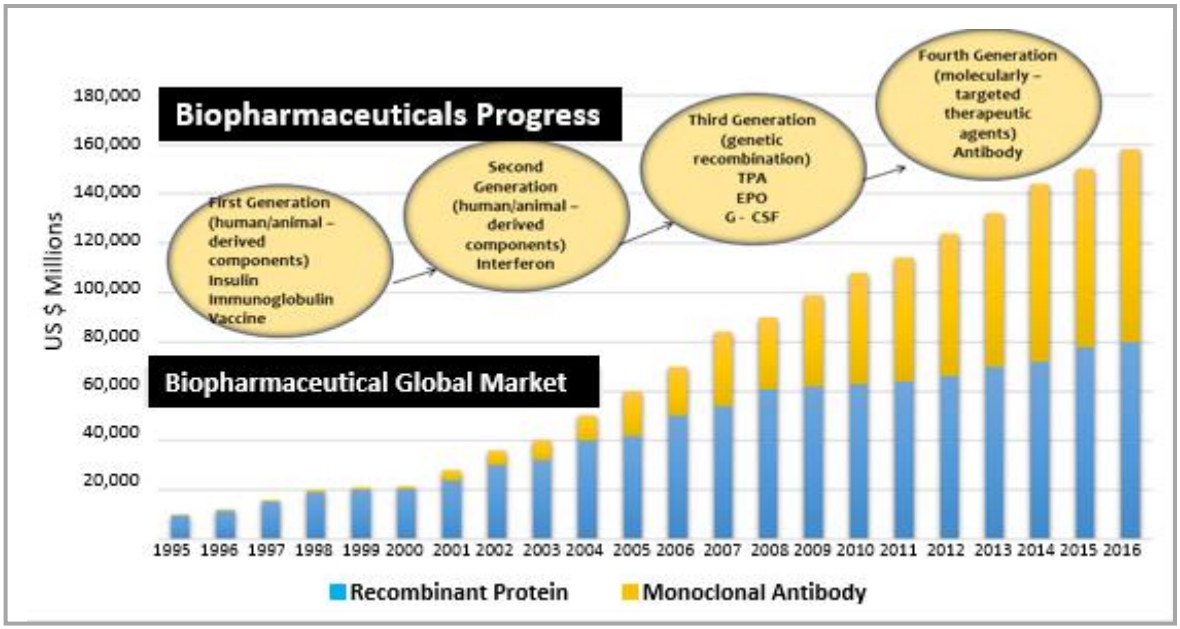

Figure 1.1 The progression rate for recombinant protein and monoclonal antibody production from 1995 till 2016. There continues to be a notable increase in demand and production. Advances in genetic information and associated technologies make it possible for interdisciplinary start-up companies to perform effective bio drug discovery activities. Monoclonal antibodies have constituted an ever increasing proportion of biopharmaceutical drug sales since the year 2000. Source: Evaluate Pharma and Gene Techno Science.

1.1.1 Protein-Based Therapeutics

As described in the section above, the demand for new therapeutic products is continuously increasing in order to broaden the range of medical conditions that are being treated (Marie, 2018). Protein therapeutics (including monoclonal antibodies, peptides and recombinant proteins) represent the largest group of new products in development by the biopharmaceutical industry (Dumont et al., 2016).

Biologics are a rapidly growing segment of the pharmaceutical market with a steady entry of traditional large pharmaceutical companies into this space. Generally, biologics are considered as substances that are derived from living organisms (such as humans, animals). However, they are carefully defined as substances produced in cell culture using recombinant technologies including production in mammalian, plant, yeast and bacterial cells.

Common examples of biologics include proteins; fusion proteins and monoclonal antibodies as well as conjugated proteins. Others include altered proteins while antibody–drug conjugates are recent additions to this group (Kishnani & Squibb 2012). Without doubt, therapeutic proteins are becoming increasingly important as they typically possess low adverse effects and higher specificity compared to the traditional, small molecule drugs. Unfortunately, the high costs of therapeutic protein place a heavy financial burden on the healthcare system and limit the number of patients that are able to be covered (Kaida-Yip et al., 2018). The World Health Organization (WHO), in part of its guidelines, on the evaluation of similar bio therapeutic products, defines a biosimilar as a bio therapeutic product that is similar in terms of quality, safety and efficacy to an already licensed reference product. Such biosimilars include growth hormone, erythropoietin, etanercept and monoclonal antibodies for the treatment of several diseases.

Many of these bio therapeutic products are complex and involve different intricate and expensive development (Lundqvist & Högskolan, 2018). This is so, as most of the established blockbusters face stiff competition from biosimilars, which put more pressure on cost effectiveness in manufacturing processes (Zobel-Roos et al., 2019) as modern industrial production of recombinant proteins in mammalian hosts is challenged by technology, regulatory and market changes and these revolve around factors such as productivity efficiency, quality of bio therapeutics and the resulting cost of production (Figure 1.2) (Bielser et al., 2018).

There is a strong relationship between the manufacturing process and characteristics of the final biosimilar (Mellstedt et al., 2008). According to Amgen Biosimilars, 2017, a small change in manufacturing can result in altered protein stability as well as impact post-translational modifications such as glycosylation hence it is therefore critical that appropriate safeguards be established to protect patients. Process Analytical Technology (PAT) is a key-enabling technology for quality-by-design (QbD) process development approaches such as bioprocess monitoring for product safety and this entails process variables such as (viable) cell, substrate, metabolite, and product concentration, as well as product quality and impurities (Kornecki et al., 2019). However, the main concerns raised regarding biosimilars are immunogenicity, efficacy, adverse effects when switching from a biologic to a biosimilar, and possible long-term effects (Kaida-Yip et al., 2018).

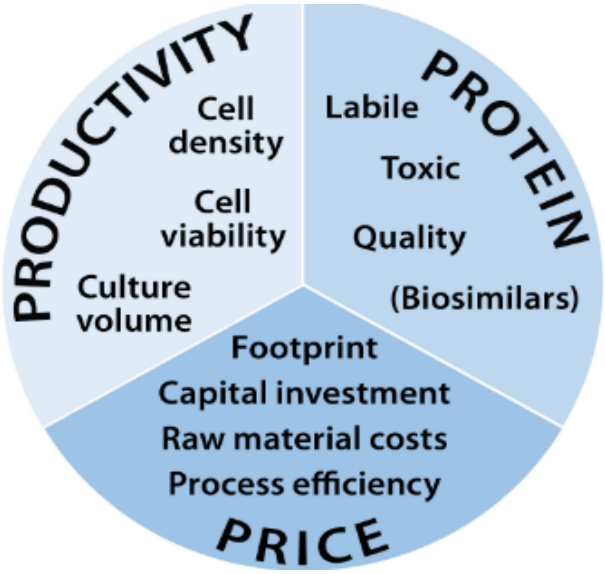

Figure 1.2 Presentation showing successful continuous bio therapeutic protein production revolves around productivity capacity, bio therapeutic protein quality and price of production. Some of the critical parameters to consider for continuous processing as a method of choice for sensitive and complex biologicals. S. R. Schmidt (2017)

As evidenced in the number of successful treatments which have been documented over the years, bio therapeutic proteins have become an important area of pharmaceutical research with several advantages over small biomolecule drugs which include their higher specificity as well as limited adverse effects. The technologies behind their production have changed substantially since bio therapeutic proteins were first approved in the 1980s (Dumont et al., 2016).

The advancement through the establishment of recombinant production for therapeutic proteins was a ground-breaking achievement in the field where living cells are utilised as host systems for protein production. This allows the production of large quantities of human proteins in a controlled environment. This has improved the ability to make such molecules and have helped gain access to these life-changing treatments. However, the development and production of therapeutic proteins can be too expensive in many countries to offer access to such medicines. Perhaps, the heterogeneity of bio therapeutics from groups of proteins allows structures to be sensitive both to the inherent variability of the protein production or expression system, and ultimately to changes in manufacturing processes. Many of these protein products are relatively large, complex entities and therefore are more difficult to characterize than chemical drugs which are simpler (Kang & Knezevic, 2018).

In the production of biopharmaceuticals, a traditional biopharmaceutical manufacturing system consists of a similar sequence of unit operations that are divided into two main parts: upstream and downstream. The upstream process (USP) is the entire process from early cell isolation and cultivation, to cell banking and culture expansion of the cells until final harvest whereas the downstream process (DSP) refers to the part where the cell mass from the upstream are processed to meet purity and quality requirements. The increasing productivity in cell culture of recombinant proteins has led to the investigation of alternate methodologies to boost downstream process productivity as the continuous increase of upstream process productivity continues to place bottlenecks in downstream production processing (Shukla et al., 2017).

Although downstream steps might reach 50 – 80% of the total production costs of proteins, the best step combination can result in significant enhancement in terms of purification and process economy (Lopes et al., 2017) more so, as process development for protein therapeutics is increasingly dependent on high-throughput (HT) technologies to accelerate the screening of many conditions and the optimisation of cell culture process outputs (Rouiller et al., 2013). However, there has been a conscious effort to understand the relationship between the upstream and downstream production processes of recombinant proteins as Upstream processes at low productivity is more costly than downstream processes (Gupta et al., 2017). Stages involved in upstream scale processes include cell culturing, process optimisation, recovery purification, formulation delivery while scale-up, manufacturing/ process monitoring, quality control/ analytical (Figure 1.3).

The inherent complexity of biological molecules and processes has been reported to create challenges for the application of process analytical technology (PAT) to biopharmaceutical manufacturing (Hong et al., 2018) which in turn affects the production processes of bio therapeutics, however, the availability of various expression systems such as Chinese Hamster Ovary cells (CHO) as appropriate host may aid high level of protein expression (Gupta et al., 2017). Process analytical technology is the development of instrumentation for real-time, continuous analysis of manufacturing processes and involves use of mathematical modelling (chemometrics) for monitoring chemical and/or physical critical quality attributes (CQAs) (Pande et al., 2017).

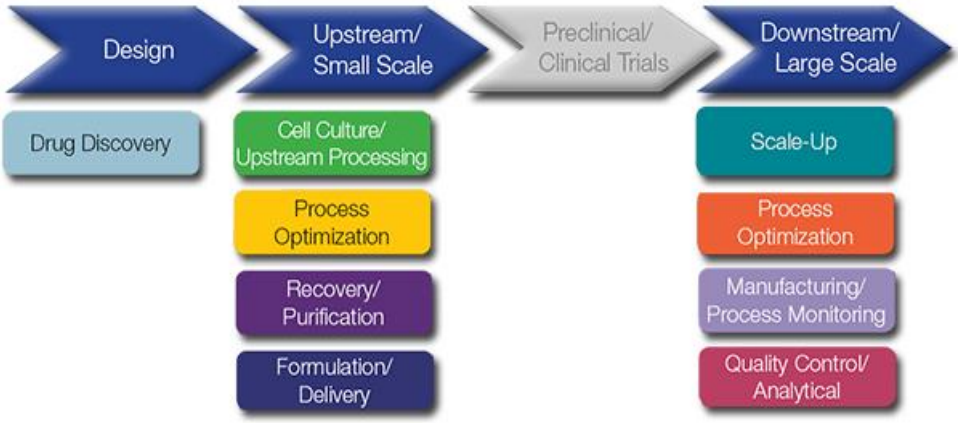

Figure 1.3 Schematics showing processes involved in upstream and downstream - processing. The bottleneck in downstream processing requires new optimisation, technology and development approaches. The optimisation and adaptation vary with every new separation task as development focuses on yield and productivity as well as on purity and process capacities. The various optimisation areas are shown and these are optimised individually with the aim of producing a robust generation of a high product titre, high productivity and defined quality. Source: Emzo Life Sciences.

Demands for biologics will continue to increase in the coming decades and in identifying the challenges of downstream and upstream production processes, bottlenecks involved with innovative technologies must be addressed. According to a review by (Strube et al., 2014), engineering concepts like modularisation and standardisation methods for speeding up process design and filing for regulatory approval must be investigated in an attempt to address the challenges of efficient engineering and production and this is applicable for therapeutic drug production. Some of the strategies proposed for optimisation processes in order to address improved capacity therapeutic drug production include: modelling with a Quality by Design (QbD) approach for a successful and economical process development, advancement of process automation for formulation, continuous production processing with intensification so as to reduce economical production and the use of single-use systems in upstream and downstream processes (Gronemeyer et al., 2014). The QbD approach is recent trend in analytical method development and its helps a lot if properly implemented as the approach suggests looking into the quality of analytical process during the development stage itself rather than testing into final results of analytical process (Pande et al., 2017) and for an efficient QbD, processes such as quality target product information, product and process design, control strategy and continuous improvement (Figure 1.4) have been identified as some key stages for optimising production.

Figure 1.4 Systematics showing the Implementation of Quality by design (QbD) approach for the production of therapeutic drugs. The production of therapeutic protein drugs occurs in a systematic manner which allows both upstream and downstream processes to be optimised for desired production capacity. Such optimisation involves an implementation of the Quality by design (QbD) approach in both the processes for consistency in the continuous process involving high through-put technology for analytical techniques involving production as well

1.2 Host cell physiology and its engineering for therapeutic protein production

The knowledge of the physiology of the host cell is very important and complementary to the genetic engineering for increasing the specific production rates of a desired product. It should also be closely relevant to the bioprocess design and bioprocess engineering which requires a deep understanding of the host cell physiology (Xavier Garcia, 2015). Due to the advances in protein engineering and cell line development, recombinant proteins can be produced in different living standard systems and the type of cell that is best suited as factory depends on the complexity of the protein that will be produced (Lundqvist & Högskolan, 2018). Expression systems such as mammalian cells, *Pichia pastoris*, *Aspergillus niger*, and *Escherichia coli* have been widely used to express recombinant bio therapeutic proteins (Peña et al., 2018), whilst other alternatives such as plant and insect cells have also been investigated or used (Dumont et al., 2016).

1.2.1 *E. coli* cells

E. coli has been a lab-favourite for expression of recombinant proteins for decades. It became popular because it was easy to work with and as a consequence, it is now possibly the most well-understood organism on the planet (Lundqvist & Högskolan, 2018). Without a doubt, *E. coli* is the most widely used host for heterologous gene expression across all scales and uses. It has been used for this purpose for more than 40 years, so there is much accumulated knowledge about its advantages and disadvantages as an expression platform (Rosano & Ceccarelli, 2014). However, there are limitations to the use of *E.coli* for recombinant protein production destined as a biological therapeutic drug in humans. For proteins used in treatment, it is often not the best choice.

Large and complex proteins, such as the commonly used monoclonal antibodies, require the appropriate environment and enzymes to be correctly folded and receive the necessary post-translational modifications (PTMs) (Dalton & Barton, 2014). *E.coli* cells do not have the cellular machinery for undertaking many of the required functional post-translational protein modifications on bio therapeutic protein drugs, such as glycosylation or phosphorylation. Again, the problem of insolubility when using proteins expressed within *E.coli,* because proteins are not folded correctly, remains a challenge. While eukaryotic expression systems favour these needs, *E.coli* remains most suitable for the production of small and less complicated proteins such as insulin (Lundqvist & Högskolan, 2018).

1.2.2 Yeast cells

Yeasts are inexpensive systems to culture with qualities such as easy manipulation and cultivation. They share many biological processes with mammalian cells such as the ability to perform various PTMs, and are therefore more capable when it comes to correctly folding and secreting complex proteins (Wells & Robinson, 2017). However, the glycosylations that occur in yeasts are quite different from those that are carried out in mammalian cells. *Saccharomyces cerevisiae* (baker's yeast) has traditionally been the most commonly used yeast for recombinant protein production, but strains from the *Pichia* genus have recently seen an increase in popularity (Mattanovich et al., 2014). As a host organism, yeast has been used to produce a wide variety of products including sterols, terpenoids, lactic acid, citric acid, alcohols, sugar derivatives, organic and fatty acids, terpenes, aromatics, polyketides, peptides and recombinant therapeutic proteins (Fidan & Zhan, 2015).

However, the major limiting factors of yeasts on protein processing such as folding, assembly, performance of PTMs and secretions are well documented.

1.2.3 Mammalian cells as the preferred expression system

The most preferred platform for expression of the widest range of biopharmaceuticals, including antibodies, has been mammalian cells. This is largely attributed to their ability to produce large volumes of therapeutic antibodies with the required folding, assembly and PTMs and adaptability in large-scale production systems (Kunert & Reinhart, 2016). Mammalian cells from various species such as human, hamster, monkey and mouse have been well studied. Further, advances in cell engineering make it more convenient to engineer the mammalian cells (Dangi et al., 2018). Of these, the most commonly used cell lines for the production of therapeutic proteins are Chinese hamster ovary cells (CHO) and murine myeloma cells, and these rodent cell lines have glycosylation patterns that are similar to humans (Wells & Robinson, 2017).

Close to 70 % of all therapeutic proteins are produced in CHO cells (Kim et al., 2012) and these cells provide high protein yields and are tolerant to changes and fluctuations (e.g. pH, oxygen, pressure, and temperature) during the manufacturing process. Additionally, by becoming a choice of expression system early on, CHO cell lines is now benefitting from a notable knowledge base. One might expect that human cell lines should be the preferred choice of expression system as these would provide true human PTMs (Lundqvist & Högskolan, 2018) and although, HEK cells (human embryonic kidney cells) are the most widely used system, as these cells have shown efficient production in laboratory scale, the application of human cell lines for commercial manufacturing, however, remains limited. This is not without the regulatory hurdles, such as the lack of species barrier which allows easier transfer of adventitious agents making conferring a major limitation on human cell lines. As such, infections with full-blown pathogenic effect in human cells will be easy to detect, whereas such agent may be dormant in rodent cells (Swiech et al., 2012). However, CHO cells have been demonstrated to display reduced susceptibility to human viral infections which represent an additional advantage over other cell lines of human origin (Dumont et al., 2016).

Table 1.1 presents some properties of different mammalian cell lines as reported in literature and these considerable factors may explain why CHO cells remain the preferred choice of host for bio

therapeutic drug production. Human Embryonic Kidney (HEK) cells, originate from neuronal fetal kidney cell as against the initial report that it originated from an endothelial, epithelial or fibroblastic cell of the fetal kidney have proven to be popular and easy to culture and sequencing studies have shown that these cells have a very complicated karyotype with multiple copies of chromosomes as they have four copies of chromosome 17 with no single Y chromosome (Simmons, 2019). With the rising demand for bio therapeutics as many biopharmaceutical drugs are reaching later stage clinical trials, most bio therapeutic drugs which are widely produced in mammalian cell lines for improved post-translational modifications should be at least similar to those obtained in humans.

Table 1.1 General characteristics and genomic information of some mammalian cells as hosts for recombinant protein production.

Mammalian Host	Chinese Hamster Ovary (CHO)	Human Embryonic Kidney (HEK293)	Mouse myeloma cell lines (NSO, SP2/0)	Rat myeloma cell lines (YB2)	African Monkey Kidney (COS, VERO)
Produce large complex proteins with post-translational modifications	✓	✓	✓	✓	✓
Ability to grow in suspension culture	✓	✓	✓	✓	✓
Reduced susceptibility to viral infections	✓	X	X	X	X
Recombinant Protein Yield and specific Productivity	✓	✓	✓	✓	✓
High tolerance to pH, Oxygen level, Pressure or Temperature during manufacturing	✓	✓	✓	✓	✓
Produce N-glycolylneuraminic acid (NGNA)	✓	X	✓	✓	✓
Ability to produce α(2-6) sialyltransferase α(1-3/4) fucosyltransferases glycosylation	X	✓	X	X	X
References	Ghaderi et al., 2012	Goh & Ng (2018)	Dumont et al., 2016, World Health Organisation 2013	Dumont et al., 2016	Dumont et al., 2016

1.3 Chinese Hamster Ovary Cells as an Expression System

Chinese hamsters (scientific name, *Cricetulus griseus*) belong to a family of rodents that are native to the desert of northern China and Mongolia and have been used in life-saving biomedical research ever since they were first introduced in the laboratory in 1919 for typing pneumococci (Jayapal et al*.,* 2007). The original Chinese Hamster Ovary Cell line was derived from a female Chinese hamster by Theodore Puck in 1957 which he obtained from Dr. George Yerganian's laboratory at the Boston Cancer Research Foundation. Theodore Puck first found that a cell line of near diploid karyotype could be generated with relative ease, from a trypsinised sample of ovarian tissue from Chinese hamsters and also that this cell line was able to grow for months without showing signs of senescence, which are normally seen in cell lines derived from humans (Kaas, 2015). With a culture history of more than 60 years, CHO cell populations have been handled by a number of laboratories and have therefore been passed to many researchers in the field with many names generated that are not popular anymore (Wurm & Wurm, 2017). Hence, different lineages of CHO cell line from the original (CHO-ori) have been derived such as the pro3-, K1, DX B11, DG 44, CHO variant (from which the CHO-S is derived) amongst others, always with the prefix CHO.

Literature studies have reported how that Scientists did not give up trying to domesticate and breed Chinese hamsters as they recognized that the hamster was an exceptionally useful animal model for genetic research and that although the CHO-ori cells, described as hardy, very well growing and fast in adherent culture had a high cloning efficiency, even at very low fetal bovine serum concentrations in the culture media there was a need for the addition of proline to the culture media at that time, a nutritional requirement for all CHO cell lines in use today as the loss or inactivation of proline synthesis was an early event in the history of these cells (Wurm, 2013). Figure 1.5 shows the pattern of consistent derivation of variants from the original CHO cell line. Nearly ten years after the CHO-K1 (first derivative) cell line was generated from this ancestral host (CHO-ori), many others have also been derived based on requirement needed in such cell lines.

For instance, to facilitate the creation of stable cell lines producing a gene of interest, a selection system was needed thus resulting in the creation of CHO DXB11 cell line with a DHFR negative phenotype as Dihydrofolate reductase (DHFR) can catalyse the conversion of dihydrofolic acid to tetrahydrofolic acid – an essential cofactor carrier (Kaas et al., 2015), these needs have continued to be addressed and met as required. For CHO-S cells, their derivation dates back to 1973 and as these

cells were passed on from Thompson and/or Gottesman, to Dr R. A. Tobey's laboratory at the Los Alamos National Laboratory, New Mexico and then to a company interested in growing and eventually commercially using such cells, a major advantage in the use of these cells is their capacity to grow in single-cell suspension culture a core requirement for this project (Wurm, 2013).

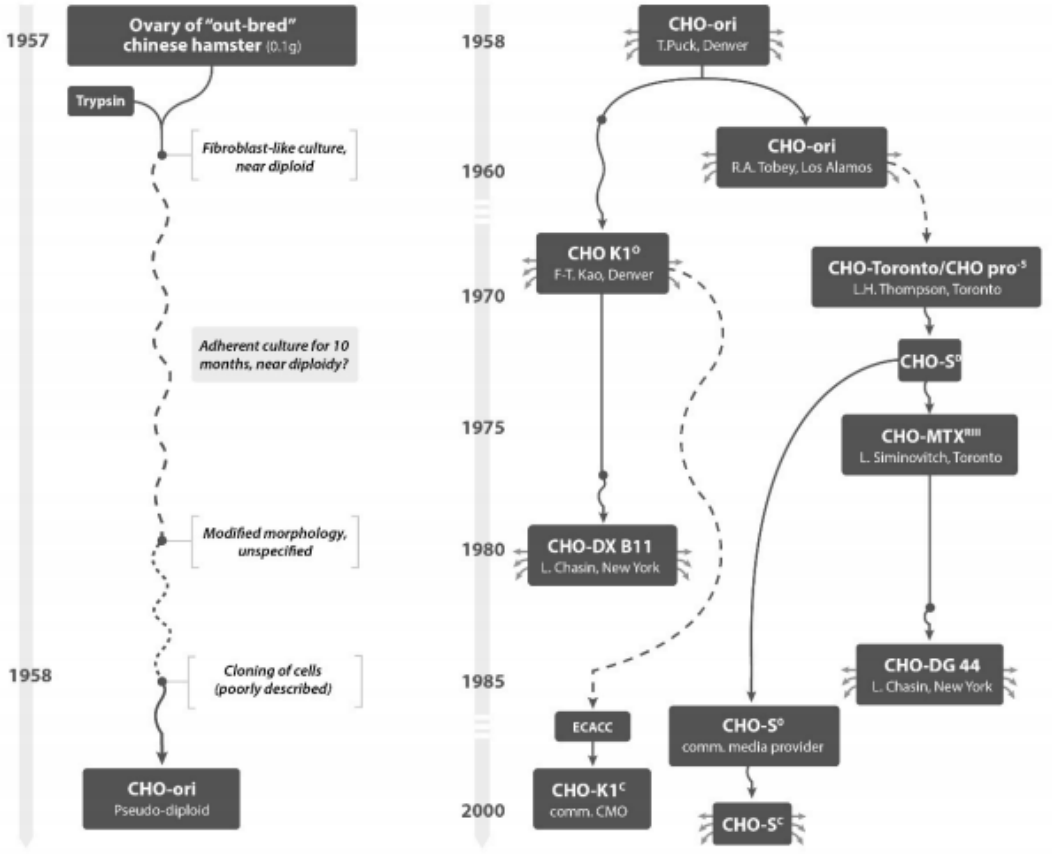

Figure 1.5 Representation showing the lineage of CHO. With a culture history of more than 50 years, CHO cell populations have been handled by a number of laboratories and passed to many researchers in the field. While some names may not be popular anymore, others are still in use today. These include; K1, DXB11, S, DG 44 cells named with prefix CHO. Adapted from Wurm & Wurm (2017).

The approval of CHO-derived tissue plasminogen activator (tPA) in 1986 led to an era of revolutionized medicine which subsequently promoted the possibility of using mammalian cell culture for the manufacturing of protein therapeutic products (Lai et al., 2013). As the preferred system for large scale production of recombinant proteins, CHO cells are fast growing with abilities

to be readily transfected and perform complex human-like post-translational modifications. These cells have several other major advantages over other cell expression systems. For instance, CHO cells are able to grow in suspension culture (which enables large-scale production), they exhibit robust growth, high productivity and stability which allows for consistency in the bioprocesses during the production of biologic drugs (Roy et al., 2018). Also, an optimised CHO host cell could include features such as better growth, no cell aggregation, more efficient metabolism like reduced lactate production, increased tolerance to culture (Gadgil, 2017). Hence, they have become the primary production host for therapeutic proteins, producing seven out of the top ten bestselling therapeutic proteins from 2016 (Kesik-Brodacka, 2017).

Recombinant glycosylated proteins, particularly monoclonal antibodies, hormones, blood-related proteins have been produced in Chinese hamster ovary (CHO) cells. Examples of products expressed in CHO cells include erythropoietin (EPO), indicated for the treatment of severe anaemia, coagulation factors as Factor IX, used as a therapeutic in haemophilia, interferon used for treating multiple sclerosis and monoclonal antibodies (mABs) for treating Crohn's disease, different lymphomas and cancers (Pereira et al., 2018). Generally, there has been tremendous and steady improvement since the first use of CHO cells for recombinant protein expression areas such as production processes, optimisation of basal media, screening and isolation as well as enhanced monitoring and control of processes including feed supplements.

One limitation of the Chinese hamster ovary system until recently was the lack of a Chinese hamster genome. Hammond et al., 2011 had reported that there was little genomic sequence data available for CHO cells and that it limited the application of high throughput molecular tools in gene discovery and cell line engineering. Recently the CHO genome was elucidated, providing high-level information about cell function (Heffner 2017) and this has secured the CHO cell as a tool for industrial applications and manipulations with the potential to develop new CHO cell hosts that could be tailored for the synthesis of specific protein needs, in particular difficult to express proteins. Hence, the publication of the Chinese Hamster (CH) genome (and that of various CHO cell lines), ushered scientists into a new era for the production of biopharmaceuticals (Hefzi et al., 2016). For example, it is now known that the genomes of some cell lines derived from CHO-K1 over the past few decades contain large-scale rearrangements and that even clonal populations are known to diverge into heterogeneous subpopulations.

1.3.1. Recombinant Protein Production in Mammalian Cells

The ability to produce recombinant proteins by utilising different cell factories revolutionised the bio therapeutic and pharmaceutical industry, and consequently influenced health care operations worldwide (Owczarek & Gerszberg, 2019). As there is a continuous increase in the number of biologics entering the development pipelines of pharmaceutical companies, CHO cells have been the preferred production host for therapeutic proteins (Sou et al., 2018). Indeed, recombinant protein therapeutics have become important components of the modern medicine (Bandaranayake & Almo, 2014).

With the multiple expression host system generated from mammalian cells of different species, there is therefore the capacity to function in a reliable and predictable expression system of large numbers of transgenes for a wide range of scientific, industrial and therapeutic applications through improved recombinant protein production for prevailing health issues such as cancer and autoimmune diseases. As hundreds of recombinant protein therapeutics have been approved by the United States Food and Drug Administration (FDA), there is a rapid increase over the last 10 – 15 years in the production of recombinant protein with a total of around 90 now in use for treatment and diagnostic purposes (Kinch, 2015). Production of these proteins have involved several approaches such as improving metabolism, glycosylation, anti-apoptosis and pro-proliferation, molecular chaperones, and protein folding have been successfully implemented (Inwood et al., 2018).

Aside diagnostic and therapeutic applications (Jozala et al., 2016), the use of mammalian cells for smart materials that have also been reported (Hollingshead et al., 2017). Smart materials have properties that react to changes in their environment. This means that one of their properties can be changed in a controlled fashion by external way stimuli or condition such as stress, temperature, moisture, pH, light, pressure or electricity. This change is reversible and therefore can be repeated many times. Improving recombinant protein expression from CHO cells remains a priority in the area and continuing efforts are being devoted to this since the first therapeutic protein, human tissue plasminogen activator, was approved (Inwood et al., 2018).

1.3.2 Difficult-to-Express Recombinant Proteins

Despite the increased yield of recombinant proteins produced from mammalian expression systems, particularly CHO cells, many recombinant proteins remain difficult to express in CHO cells or other systems. The term “Difficult-to-express” is used to refer to recombinant proteins generally considered to be expressed at low titres or quality from mammalian or other expression systems. Many of these difficult to express‘‘ (DTE) engineered proteins, such as fusion proteins and bispecific antibodies, have begun to fill the development pipelines of many biopharmaceutical companies compared to natural protein formats (Johari 2015). As many of these include the highly complex, highly glycosylated, large and unstable proteins, many difficult-to-express proteins require a consistent, homeostatic environment for maximised productivity.

Cell lines producing low protein titres can require long-term production to express enough product to reach required yields. These proteins cover various types of membrane proteins, including ion channels, multi-pass membrane proteins as well as proteins that exhibit cytotoxic effects on the host cell during overexpression (Thoring et al., 2016). They do not emerge in fully soluble, well-folded, and active form in a heterologous expression system and cell culture processes can also affect product quality, especially in the area of glycosylation and post-translational modifications. More relevant for the work here, many of the antibody inspired molecules such as bispecifics and antibody fragments, fusion proteins containing section of antibodies and domain antibodies that are in development as therapeutic molecules are proving difficult to express in CHO expression systems optimised for antibody expression. In order for such molecules to be tested in the clinic and be evaluated it will therefore be necessary to further develop expression systems that can provide the required amounts of these molecules, at a suitable quality.

With regard to bio therapeutic proteins, new formats or novel proteins that could be very beneficial for the treatment of diseases, often diseases with no current treatment options, are also proving difficult to express. These include; TNF Fusion Proteins, Mut-F Recombinant Protein, GM-CSF Fusion Protein, HC3 Recombinant Protein and others. For the scope of this project however, we have used a TNF fusion protein as a model difficult to express molecule and its challenges with secretion.

1.3.2.1 TNF Fusion Proteins

Both the Tumor necrosis factor superfamily (TNFSF) proteins and their receptors (TNFRSF) play critical roles in mammalian biology, including cell growth, survival, and apoptosis, immune responses, and organogenesis of the immune, ectodermal, and nervous systems as the binding of the tumor necrosis factor α (TNFα) to its cognate receptor initiates many immune and inflammatory processes (Lim & Lee, 2018). The focus on developments that will enhance recombinant proteins and monoclonal antibodies (mAbs) in mammalian cells in the recent years, has been on three broad areas: cancer, infectious diseases and immunological disorders. TNF as a signalling molecule controls intercellular communication that mediates inflammation, apoptosis, immunoregulation, and cellular proliferation/differentiation as its main role is in the induction of inflammation and immunity through activating immune cells in reaction to infections and tissue injury (Korani et al., 2019,).

It also functions in many normal physiological functions that cut across antimicrobial immunity as well as in homeostasis and health. Fc-fusion proteins are one of the most intensively investigated, of the proteins that target TNF. The fused partners attached to an Fc-domain have significant therapeutic potential, and the Fc domain contributes additional beneficial pharmacological properties (Chiang et al., 2014). As at 2017, there were five TNF-α antibodies approved by the US Food and Drug Administration (FDA) for clinical applications on the market either chimeric, humanized, or fully human (i.e., etanercept, infliximab, adalimumab, certolizumab and golimumab) (Iz et al., 2018). Many of the commercial TNF antagonists comprise of engineered monoclonal antibodies and fusion proteins reported to have successfully been used for the treatment of a number of chronic inflammatory diseases including psoriasis (Balabashin et al., 2015).

However, TNF is not without its challenges for therapeutic intervention. These include the need to pre-identify anti-TNF treatment responders, the need to specifically target selected TNF producing cells or individual tissue targets, and also the need to discover and produce orally delivered small molecule TNF-inhibitors (Sedger & Mcdermott, 2014). In this project, focus was on etanercept, as model difficult to express molecule whereby difficulty of expression of the Fc-fusion protein impacts on the cost of the molecule.

1.3.3 Etanercept as a model for expression studies

In the early 1990s, Bruce A. Beutler, an academic researcher at the University of Texas South-western Medical Centre, Dallas and his colleagues first reported that the prototypic fusion protein, etanercept, produced by recombinant DNA, when synthesized was highly active and unusually stable as a modality for blockade of TNF in vivo. Etanercept is a soluble protein that binds to and specifically inhibits, tumor necrosis factor (TNF), a pro-inflammatory cytokine (Hassett et al., 2018). As the only TNF blocker commercially available consisting in a fusion protein combining two extracellular ligand-binding portions of the human soluble TNF receptor p75 with the end Fc fragment of human immunoglobulin G1 (Figure 1.6) (Marotte & Cimaz, 2014), it was the first approved biologic disease-modifying anti-rheumatic drug and allowed for a major advance in the treatment of rheumatoid arthritis (Emery et al., 2017) which is used in the treatment of medical conditions such as adult and juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis and spondylitis.

The mechanism of action of etanercept is via binding to TNF, competitively inhibiting TNF from binding to cell receptors and preventing pathway activation. The dimeric structure allows the molecule to bind to two TNF molecules at an affinity more than 50 times that of naturally occurring monomeric forms (Chadwick et al., 2018). This fully human, soluble, dimeric fusion protein has a total of 934 amino acid residues with a molecular weight of 150 kDa (Hassett et al*.,* 2018). As such, this heavily glycosylated molecule with both N- and O-linked oligosaccharides, potentially influences the structure, activity, signalling, clearance and immunogenicity of such glycosylated proteins (Hassett et al., 2018) making the size and complexity of etanercept present an exceptional challenge for companies aiming to produce follow on biosimilar products, which must comprise highly similar structural and functional properties and exhibit no clinically meaningful differences to the original reference product (Lamanna *et al.*, 2017). We explored the secretion levels of etanercept, as a model molecule.

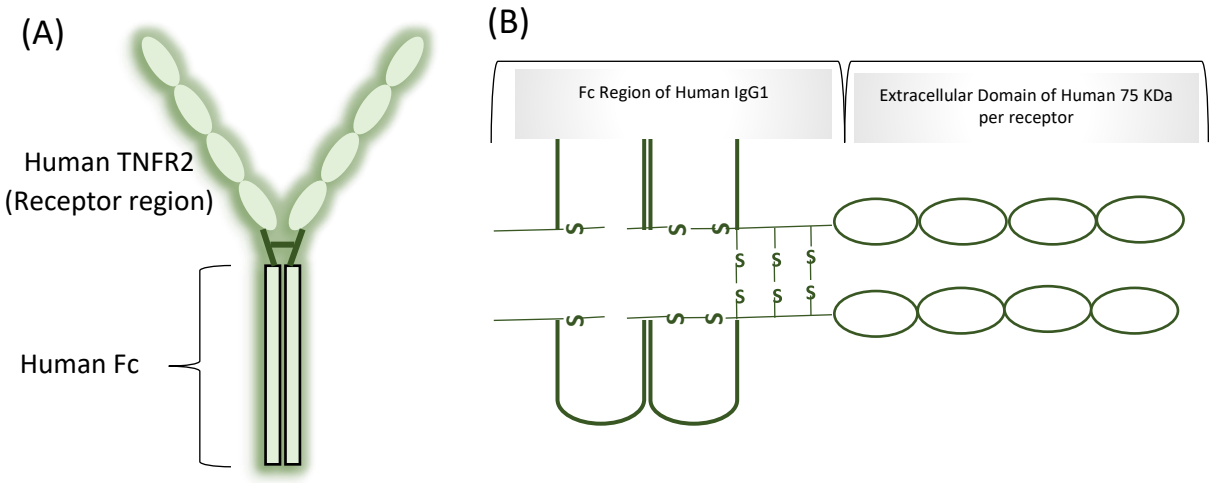

Figure 1.6 Structure of Etanercept**.** Etanercept is a fusion protein with the TNF receptor extracellular domain and the Fc portion of a human IgG1. Its dimeric structure allows it to bind up to two TNF molecules and functions as a TNF inhibitor by competitively binding to TNF and preventing its activation of the inflammatory cascade. (A) Overview of the structure of Etanercept shows two naturally occurring soluble human 75-kilodalton TNF receptors linked to an Fc portion of an IgG1. (B) Chemical structure of Etanercept shows the linkage of the Fc portion of human immunoglobulin G1 (IgG1) by three pairs of disulfide bridges to the extracellular ligand-binding domains of the Receptor region.

1.4 Erythropoietin (EPO)

In 1948, haemopoietin was first discovered in patients with high red blood cell counts living at high altitudes. The name “erythropoietin” (first mentioned by Bonsdorff and Jalavisto) arose from its functional implication in the generation of red blood cells (erythrocytes) for improved tissue oxygen supply (Ostrowski & Heinrich, 2018). For over three decades, recombinant human erythropoietin (rhEPO) has been used in the treatment of anemia. EPO is mainly produced in the kidney that raises blood haemoglobin mass by anti-apoptotic effects on erythroid progenitor cells in the bone marrow (Aachmann-andersen et al., 2018). Therefore, the administration of EPO as a therapeutic is known to improve the quality of life in patients (Shahkillahwati, 2014).

EPO has three N-linked complex oligosaccharides attached to asparagine side chains at positions 24, 38 and 83, and a short O-linked oligosaccharide at serine 126 (Lee et al., 2019). The human erythropoietin which are expressed in mammalian cells, contain substantial amounts of heterogeneous, complex-type oligosaccharide (Čaval et al., 2018), Figure 1.7. The present therapeutic recombinant EPO preparations are manufactured in mammalian host cells as EPO is a complex glycoprotein of 165 amino acids to which four glycans are attached.

Advances in recombinant EPO (rhEPO) production are classified into three types: the development of improved rhEPO molecules by protein engineering; improvement of production host cells by genetic engineering; and culture condition optimisation by fine control of the production mode/system, process parameters and culture media (Lee et al., 2012). A publication comprising a list of over thirty erythropoietin based therapeutics approved in the US and EU for the treatment of anemia (see link; www.fda.com and www.ema.eu). With the approval of erythropoietin based therapeutics, rhEPO became a leading drug for treatment of anemia virtually abolishing the need for RBC transfusion in some types of anemia (Debeljak et al., 2014) and as EPO increases the number of peripheral red blood cells, there has been considerable interest in the therapeutic use of EPO for the treatment of severe anemia (Garcel et al., 2015).

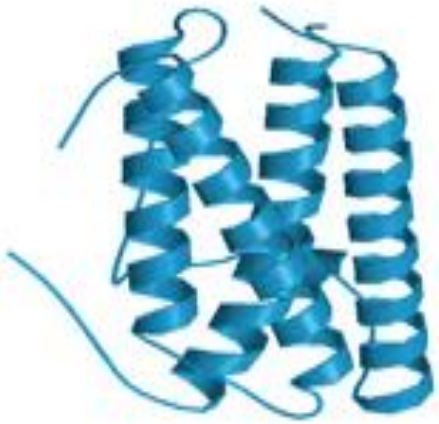

Figure 1.7 Protein structure of Erythropoietin. The three-dimensional structure comprises two immunoglobulin-like domain. Also, erythropoietin has one binding interface of the ligand which allows for high affinity interaction with the receptor. Image source: Proteinkinase.de

1.5 Strategies for Mammalian Cell Engineering

Recent advances in mammalian cell engineering have witnessed significant improvement in production of recombinant protein production and numerous strategies have been used and targeted to enhance cell engineering with growth-promoting and productivity-enhancing factors. These past few years particularly have witnessed reports of many strategies to improve the productivity of mammalian cell factories (Zucchelli et al., 2016) and these mammalian cell lines that support reliable and predictable expression of large numbers of transgenes are an enabling technology for a wide range of scientific, industrial and therapeutic applications such cell lines could be used to improve production of recombinant proteins that can treat autoimmune disorders, cancer and other diseases (Gaidukov et al., 2018).

There are several methods used to select host cells that have attributes which further improve the efficiency or robustness of protein production and the economic footprint of the cell factories ranges in the hundreds of billions of USD/year on the global markets (Davy et al*.,* 2017). For over three decades, vast optimisations of cell productivity for recombinant protein production during bioprocess has yielded powerful clones for bio therapeutic production. While many of the processes used in these advancements are kept as commercial knowhow, there are reviews which detail cell line development technologies. With the fast rising advancement in technology, the next generation may, as well, come from genetic engineering approaches where approaches such as vector engineering and cell regulation through the use of endogenous promoters to overcome bottlenecks that limit increased protein production, a major focus in this work.

1.5.1 Host cell line selection

Every bioprocess design begins with the important step of choice of a suitable host as an expression system for the production of recombinant protein, decision of which may be challenging (Overton 2014). Quite a number of mammalian systems are capable of yielding high-level expression of recombinant proteins that are suitable for use in the manufacture of protein pharmaceuticals. Some of the most commonly used cell lines used are rodent-derived such as Chinese hamster ovary (CHO) cells and baby hamster kidney (BHK) cells. Other examples of mammalian cells used for the production of approved bio therapeutics include human embryonic kidney cells 293 (HEK293) and HT-1080 human cells (Goh et al., 2018) while CHO cells remain the predominant host cell platform of the several non-

human mammalian expression systems, accounting for over 60% of currently approved bio-therapeutics (Butler & Spearman 2014).

1.5.2 Improved screening and selection methods

Methods of screening for the selection of cell lines is critical due to the desire for high specific productivity of stable clone for recombinant protein production. As it has become a valid requirement for commercial production of therapeutic proteins the selection procedures used to identify the desired cell line are often tedious time-consuming but remains an important requisite for the regulated expression or production. A popular method of screening and selection is the limiting dilution of cells which is used to identify monoclonal cell lines, followed by other analysis that favour high production processes. While many screening methods are being developed in order to reduce the time in the generation and identification of high producer cell lines, there remains the challenge of identifying desirable high producers in a transfected pool as low producers are usually the majority of the cell pool whereas high producers often remain scarce. Antibiotics commonly used for selection include hygromycin, geneticin (also known as G418) and puromycin. Table 1.2 shows some examples of the selection methods popularly used to identify clonal cells and perhaps the pros and cons with their choice of use of cell samples which cut across peptides, DNA, membranes as well as intracellular proteins.

Table 1.2 Examples of some clonal selection methods and their advantages/disadvantages.

Method of Selection	Advantages	Disadvantages
Limited Dilution Single Cell Cloning	Simple Inexpensive	Time-consuming Isolation not guaranteed Low level of successful outcome
Fluorescent activated Cell sorting (FACS)	High throughput	Toxicity problems from fluorescent proteins Limited to cells with certain characteristics Expensive instruments
Gel micro-drop technology	High saturation levels Limited Production Diffusion	Needs trained person
Laser enabled analysis and processing (LEAP)	Automated	Expensive Potential Damage to Cells (irradiation)

1.5.3 Cell line engineering

Cell engineering is by far, one of the most effective and powerful ways to improve the production of therapeutic proteins. For two decades, efforts have been devoted to genetically engineer host cells or produce cells with enhanced growth characteristics or to modulate the glycosylation pattern of the product. There have also been efforts to genetically engineer host cells so that the same desirable traits would be carried to new cell lines for different products (Le et al., 2015). Further, advances in cell engineering make it more convenient to engineer mammalian cells (Dangi et al., 2018) as these have continued to enable techniques that cut across various fields of synthetic biology, molecular biology, genetic engineering and many other areas. Mammalian cell lines can be improved by genetic engineering of either enzymatic or regulatory activities for example. The benefits of cell line

engineering are however not limited to antibody production only, but have also been explored in production of other biopharmaceutical products as are fast selling as well as highly effective products

as other recombinant proteins, vaccines, fusion proteins and growth factors (Sandig et al., 2017). Applications of cell engineering have enabled manipulations which favour cell protein production. Various types of cellular and genetic engineering techniques are therefore directed at modifying features specific to the host cells (Dangi et al., 2018). There are approaches used to manipulate the delay of apoptosis (Pistritto et al., 2016), over-express anti-apoptotic genes (Zustiak et al., 2014), down-regulate pro-apoptosis genes (Zhang et al., 2018) in engineered cell lines, manipulate growth of the cell (Vergara et al., 2018), promote post-translational modifications (Alexandris et al., 2017) as well as other cell engineering processes.

For instance, overexpression of the anti-apoptotic Bcl-X(L) gene was reported to improve cell productivity against breast cancer cells (Mimura et al., 2018). Along with these strategies, researchers have engineered cells in order to increase transcription rate, modulate specific posttranslational modifications and decrease protein misfolding, aggregation, and degradation such as the engineering of chaperone network of CHO cells which was reported to improve recombinant protein production (Jossé et al., 2012). The use of micro-RNAs in host-cell engineering is another important strategy used to improve recombinant therapeutic protein production as well as the regulation of apoptosis so as to promote cell-cycle progression for improved cell viability and overall improved cellular productivity (Fischer et al., 2017).

1.5.3.1 Anti- Apoptosis engineering in CHO cells

Anti-apoptosis engineering is an established technique to prolong the viability of mammalian cell cultures used for industrial production of recombinant proteins (Templeton et al., 2014). Cell death is a known critical issue as it affects the viable cell concentration thereby impacting on volumetric titre and ultimately on product quality hence many of the methods used to delay cell apoptosis have been largely considered as a highly promising and advantageous to increase product quantity and quality and also to reduce production costs in medical biotechnology applications (Iz et al., 2017). Due to the morphologically homogeneous processes by which apoptotic cell death are generally characterized, (Inoue & Tani 2014), anti-apoptotic cell engineering therefore remains a potential strategy to improve life cycle of cells for a longer production period. It is believed that nature gives prolonged life span to

some cells like stem cells, tumor cells and memory B and T cells and this has inspired researchers as a Bio Design approach from nature, with this strategy for different purposes (Iz et al., 2017). However, the overexpression of anti-apoptotic genes, such as those in the Bcl-2 family, have been shown to improve cell viability and increase mAb production (Basu & Sridharan, 2017). Another promising strategy towards achieving improved recombinant protein production in CHO cells is the inhibition of the expression of caspases, which functions in apoptosis regulation (Zamaraev et al., 2017).

1.5.3.2 Secretory pathway engineering in CHO cells

The elucidation of the cell biology of the secretory machinery is of great importance, as it drives protein expression for biopharmaceutical industry (Lund et al., 2017). Mammalian secreted proteins and membrane bound receptors often contain obligate post-translational modifications including disulfide bonds and unique glycosylation patterns necessary for proper folding and/or biological activity, preventing their expression in prokaryotes (Dalton & Barton, 2014). It is therefore imperative that the secretory pathway is correctly regulated for the life and health of the organism (Viotti 2016).

The secretory machinery includes various functional modules along the endoplasmic reticulum (ER) and Golgi apparatus. However, dysfunction of the secretory pathway may result in a variety of systemic or developmental diseases, like cancer, diabetes, Parkinson's disease, and congenital neurodegenerative disorders (Wang & Kaufman 2016). Basically, there are two main functions of the secretory pathway. To perform proper folding and post-translational modifications (PTMs) of proteins such as, glycosylation and sulfation, and to sort proteins to their final cellular or extracellular destination (Lund et al., 2017). Protein post-translational modifications (PTMs), such as phosphorylation, acetylation, methylation, and ubiquitination, have been shown to control protein activity, regulate protein stability, promote protein interactions and alter protein subcellular localization, leading to the modulation of crucial signalling pathways and affected cellular processes (Yang, 2018).

The protein secretion pathway is controlled by some regulatory proteins responds to environmental changes, nutrient production as well as stress conditions. Secretory vesicles and secretory granules are distinct vesicular carriers employed in constitutive and regulated secretion, respectively (Cotzomi-

ortega et al., 2018). Recent studies reported that the bioengineering of host cell lines may improve the modification or the secretion of heterologous proteins and of other therapeutics and also of functional proteins involved in the various post-translational steps of the secretory pathway and of exocytosis. Some of these pathways could be successfully engineered to solve bottlenecks and cellular limitations caused by recombinant protein overflow (Fourn et al., 2014) . Although efforts within bioprocess optimisation have led to increased product titres of recombinant proteins expressed in CHO cells, post-transcriptional bottlenecks in the biosynthetic pathway of r-proteins remain to be solved (Hansen et al., 2017a). For instance, Sommeregger et al., (2016) had reported that cell lines expressing seemingly difficult-to-express recombinant proteins had increased levels of ER stress compared to cell lines expressing easy-to-express recombinant proteins.

A non-linear relationship between the transcript level and cell specific productivity (qP or amount of protein made per cell per unit time) in CHO cells shows that there is a post-transcriptional bottleneck in the biosynthetic pathway of recombinant proteins (Hansen et al., 2017). Meanwhile, previous studies had identified several post-translational bottlenecks in the generation of certain recombinant proteins such as the bottleneck in CHO cells upon the transient expression of an Fc-fusion protein (Johari 2015), the post-translational bottleneck of EPO transient expression at high gene doses (Ku et al., 2010).

The overexpression of human signal receptor protein SRP14 and other components of the secretion pathway via proper processing and secretion resulted in the expression of the difficult-to-express protein to high yields (Fourn et al., 2014). This again suggests that the engineering of the secretory pathway may be used to improve the secretion efficiency of therapeutic proteins from CHO cells Indeed, overexpression of transcription factors can boost productivity and the secretory capacity of the cells can be improved (Pybus et al., 2014).

1.5.3.3 Glycosylation engineering

Glycosylation is the most abundant post-translational modification (Burchell et al., 2018) and the most complex post-translational process (Shen et al., 2018) In eukaryotes, the majority of proteins expressed on the cell membrane or that are secreted carry glycans (Burchell et al., 2018). Glycosylation plays an important role in the pharmacokinetics and immunogenicity of recombinant bio therapeutics

(Goh & Ng, 2018). It is a critical and variable factor in the quality of several therapeutic proteins, particularly those with immune-system interactions such as monoclonal antibodies (mAbs) (Blondeel 2018) and it occurs within the secretory pathways of cells, precisely in the endoplasmic reticulum (ER) and Golgi apparatus, where monosaccharide units such as galactose (Gal), mannose (Man), fucose (Fuc), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and sialic acids are covalently attached to specific amino acids of newly synthesized proteins and lipid structures (Goh & Ng, 2018). Glycosylation occurs in two main forms, designated by the functional group of the amino-acids where the glycans are bound; the N-linked and the O-linked (Ghaderi et al., 2012). During the production process various parameters impact glycan structures, including cell culture conditions, protein structure and protein folding (Shen et al., 2018).

In order to improve the pharmaceutical properties of therapeutics, the glycosylation of bio therapeutics is now critically defining glycosylation for each drug to maintain consistent characteristics which affects many general functional factors of a glycoprotein, such as stability, inhibition of proteolysis, solubility, aggregation, as well as other attributes (Butler & Spearman, 2014). Meanwhile, there are many approaches for glyco-engineering, including the introduction of new glycosylation sites to increase carbohydrate content or to block specific binding (Mimura et al., 2018). According to Shen et al., (2018), there was a reduction in glycosylation heterogeneity following changes in culture environment with consistent expression and culture conditions of variants.

These findings agreed with reports of Hossler (2012) on the methods to modulate glycosylation heterogeneity for glycoproteins in mammalian cell culture during the manufacturing process such as optimising pH, temperature, oxygen concentration, the media formulation and feed rate, expression level, and production method (batch, fed-batch, perfusion). Meanwhile glycosylation of recombinant proteins in non-mammalian systems are different from humans and thus have limited applications in the production of therapeutic glycoproteins (Loos et al., 2011).

Further, renewed and increasing attention is being paid to the effect of N-glycosylation on product quality; such efforts are underway to develop analytical workflows to monitor glycosylation profiles throughout the bioprocessing production cycle (Sha et al., 2016). While strategies for generating novel molecules with desirable therapeutic properties are being identified, glycosylation of recombinant protein remains to be fully exploited.

1.5.3.4 Cell Media Formulation and Feed development

Media development is aimed at supporting cell growth, having a robust process with high viable cell density, high product titre and high product quality (Klein et al., 2015). Mammalian cells require a combination of both nutritive (for example, sugars and amino acids) and non-nutritive (for example, trace metals, vitamins and co-factors) components to support cell growth and protein production. One of the most challenging aspects of culturing recombinant CHO cells is, in fact, the optimisation of the diverse nutritional requirements unique to each clone. Cell culture optimisation often involves the development of a customized, chemically defined media along with process-specific feed supplements, all combined with process parameters individualized for each CHO cell clone (Baktur et al., 2016). Additionally, increasing demands on product quality result in higher complexity of processes and analytics, thereby increasing the costs for product work (Gronemeyer et al., 2014).

Producing more proteins with fewer resources and in less time remains a significant challenge when working with mammalian cell expression systems (Lalonde & Durocher, 2017) as cells require a base media to support the early stages of culture followed by nutrient feeds to replace limiting nutrients and to extend the period of protein production, which helps to achieve high titre (Sellick et al., 2011). Therefore, many researchers have tried to optimise the composition of culture media to gain the most suitable conditions for recombinant protein production yield in CHO cells and some of the approaches used include the substitution and supplementation of glucose and/or glutamine, hormone, growth factors, lipids manipulation and nutrient engineering, amongst others (Porncharoennop, 2016).

1.5.3.5 Site-specific targeting techniques

Several site specific recombinases have been described and used for engineering of mammalian genomes (Marie, 2018). While site-specificity may not be a prerequisite for introduction of transgenes into a host for overexpression, there are factors that may improve integration success by using more specific insertion methods. The large-scale engineering of a cell's genome requires the ability to precisely and efficiently integrate large amounts of heterologous DNA into genomic loci that support robust expression of transgenes unlike current genome-engineering (Gaidukov *et al.*, 2018). Targeted integration methods are frequently applied (as reported) for the reduction of the long timelines associated with stable cell line generation. Such approaches use site-specific recombinases,

integrases or transposes for the integration of the expression cassettes (Büssow, 2015). To date, three main types of engineered nucleases have been developed for genome editing: zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (Nishitani et al., 2016) but unlike ZFNs or TALENs, the CRISPR/Cas9 system does not require any protein engineering steps, making it much more straightforward to test multiple single guide RNAs for each target gene (Nakajima et al., 2017). In recent times, site specific gene knock-ins in cell line development have been successfully performed using transcription activator-like effector nucleases (TALENs) (Aida et al., 2015) or CRISPR-Cas9, RNA-guided nucleases. However, these methods are not without setbacks as they often use the error-prone non-homologues end joining (NHEJ) mechanism for double strand break repair, as genome editing still suffered from two major drawbacks as reported by Komor et al., (2017). First, that non-homologous end joining (NHEJ) which also occurs at the site of DSBs, typically more efficiently than HDR, results in stochastic insertions and deletions (indels) of nucleotides at the target locus and a second setback which occurs due to the low probability that a known meganuclease would cleave a particular target locus of interest without having to incorporate either a meganuclease recognition site into the genomic locus of interest or perhaps engineer a meganuclease to cleave the target locus.

1.5.3.6 Cell Cycle engineering

Cell growth, a process that occurs during cell doubling, is defined as an increase in cell mass and size and this process is tightly organised involving cell division with distinct cell cycle phases that transition through phases regulated by several layers of checkpoint control mechanisms (Dolatabadi et al., 2017). A recent review by Duggal et al., (2018) reported on the highly coordinated and regulated process of an ordered series of events of the mammalian cell cycle. It was reported that, though diverse processes, cell growth and proliferation are always linked. Although many studies have compared the metabolic and physiological states of different CHO cell culture phases (Pan et al., 2017), it is however less clear whether intrinsic cell size control plays a large role in regulating cell size in multicellular organisms where cell size may be constrained by tissue structure and changes in cell size are associated with development and morphogenesis (Jones et al., 2017).

In understanding the mechanisms by which molecular determinants of cell cycle progression occurs, analyses of cell populations have revealed well-documented substantial changes in gene expression,

protein abundance, protein post-translational modifications and DNA synthesis as a population of cells moves from one cell cycle phase to the next, or when a population becomes quiescent (Carolina & Carolina, 2018). According to Griffith (2015), the four phases of growth for any cell in culture [lag, log, stationary, decline] can be targeted to increase the longevity of batch cultivation with a short lag phase where cells acclimatise to the environment, followed by a rapid log/exponential growth phase, where the cells grow to maximum density as fast as possible so that you get a longer stationary phase. Unlike cell growth which is defined as an increase in cell mass and size, cell cycle requires the activation of many stage specific signalling molecules as well as that of regulatory cell cycle proteins (Duggal et al., 2018).

For the cell to move from a phase of cellular growth to cellular energy phase where energy is being utilised for protein synthesis, there is the need to extend the stationary phase which is the main production phase, a term called Biphasic (Figure 1.8). During this extended stationary phase cells are able to concentrate their metabolic energy to increasing specific protein productivity and not on other cellular functions Griffith (2015). Some of the strategies required for the growth arrest of cells include chemical agents, decreasing cultivation temperature and changing media PH. These strategies have focused on enhancing time integral of viable cell density (IVCD) as well as specific protein productivity (qP).

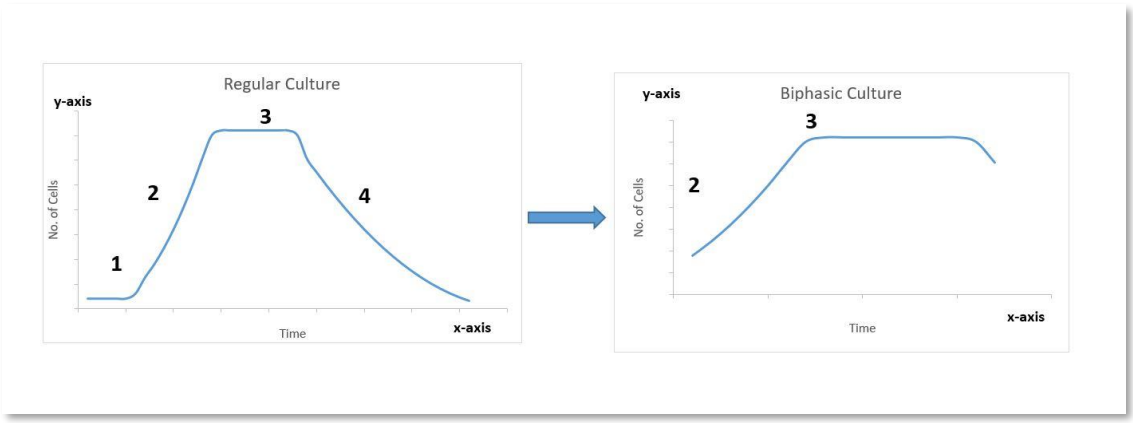

Figure 1.8 Regular and Biphasic growth curve of cells in culture. **1**= Lag phase, **2**=Exponential phase, **3**=Stationary phase and **4**=Decline/death phase. In regular cells, stages 1 to 4 make a complete cycle whereas in a biphasic culture, cells show an extended exponential phase leading to an extended stationary phase where maximal use of energy is observed. Source: (Griffith, 2015).

1.5.3.7 Overexpression engineering

The approach used in this work encompasses techniques of systems biology for cell engineering. Systems biology refers to an integrative discipline that attempts to describe and understand biology as systems of interconnected components and this approach is made possible by the explosion of data from genomic, transcriptomic, proteomic and metabolomic techniques developed within the last decade (Materi & Wishart, 2007). The emergence of Systems biology has span over the past two decades with the aim of examining organisms from a systematic and holistic perspective, characterized by large datasets and modelling (Zou & Laubichler, 2018).

Hence, the data systems biology presents scientific possibilities to understand processes as a whole and to integrate new data and new analytical tools that are intended to solve biological problems by integrating parts and modules (Martins-Santana et al., 2018) and mammalian synthetic biology continues to developing research tools and programmable therapeutics that are based on sophisticated genetic circuit design (Cella et al., 2018). With these developments, many sub-disciplines of systems biology have been established, such as systems pharmacology, which explores the mechanisms behind drugs using systems biology methods, and cancer systems biology, which applies computational modelling to reveal the carcinogenic pathways (Zou & Laubichler, 2018).

Because the secretory pathway primarily relies on local interactions in order to make decisions on the fate of the secretory protein, rather than transcriptional responses (Hou et al., 2012), the need to link genotypes to phenotypes and other associating genes to biological path-ways as gene overexpression is necessary to particularly stimulate a specific activity and mimic gain-of-function mutations via systematic gene overexpression (Znaidi et al., 2018). Also, various metabolic conditions require an enlarged capacity in the ER-localised pathways of intermediary metabolism, and this frequently results in proliferation of the organelle.

Kuo et al., (2018) had reported on the recent advances in systems biology and data-driven approaches aiming to unravel how molecular pathways, cellular processes, and extrinsic factors (e.g. media supplementation) influence recombinant protein production and had summarized these approaches into three namely; Bioprocess and transgene expression optimisation, targeted engineering of CHO cells and the characterisation and Engineering of the CHO Protein Secretion System. While the ER is a

major site of protein synthesis, it is also a site of bulk membrane lipid biogenesis. In this study, we have focused on reprogramming cholesterol biosynthesis which occurs in the endoplasmic reticulum (ER) to target ER expansion.

More studies have continued to overexpress certain genes for induced ER expansion in mammalian cells. For example, some studies demonstrated that overexpression of spliced XBP1 induced ER expansion by increasing the amount of Protein C per milligram of protein and the total amount of phospholipids (Sriburi et al., 2004). Chaperones have been examined in a number of disease models as potential tools for lowering ER stress and preventing the activation of UPR pathways. Literature studies from (Zwerger et al., 2010) reported on the over-expression of three of the investigated LBR mutants as well as TM7SF2 and DHCR7 induced the large-scale separation of the INM from the ONM as well as of ER membranes, implicating an additional function of these proteins in maintaining the integrity of these compartments. Though they stated that assumptions are still highly speculative but over-expression of various LBR constructs which findings have proven to be a valuable tool to elucidate protein function.

1.6 The Secretory Pathway

The secretory pathway of eukaryotic cells is composed primarily of two organelles, the ER and Golgi, responsible for maintaining the fidelity of protein synthesis and maturation (Young et al., 2011) as well as provide both a specific folding environment and appropriate chaperones in the ER for the folding of secretory proteins and organelles for further modification of folded and assembled proteins. This highly complex pathway which directs the nascent polypeptides to the extracellular space, cell wall, plasma membrane, or organelles (Celińska & Nicaud, 2019) where the proteins ferried between compartments via transport vesicles bud off from one donor compartment to fuse with a downstream acceptor compartment, thereby mediating directional traffic of both lipid and protein (Barlowe & Miller, 2013) usually by an N-terminal signal sequence which directs them to the ER. This N-terminal signal sequence is hydrophobic in nature and binds to the signal recognition particle (SRP) (Mercier et al., 2017). In order to maintain homeostasis of individual organelles in the secretory pathway, the forward-moving or anterograde pathway is balanced by a reverse, or retrograde pathway that returns escaped resident proteins and maintains the homeostasis (Barlowe & Miller, 2013).

1.6.1 The Endoplasmic Reticulum

The endoplasmic reticulum (ER) is the entry portal of the conventional secretory pathway where the newly synthesised polypeptides fold, modify, and assemble (Jiang et al., 2018). As the site of synthesis for nearly one-third of the mammalian genome encoded proteins (Pobre et al., 2019) the ER is accordingly endowed with specialized machinery to ensure that proteins deployed to the distal secretory pathway are correctly folded and assembled into native oligomeric complexes (Olzmann et al., 2013) where they further undergo processes such as post-translational modifications, oxidative folding, and maturation to their functional tertiary or quaternary state (Pobre et al*.,* 2019). As the protein folding factory of the secretory pathway, the ER hosts a specialized PQC (protein quality control) system of molecular chaperones and folding enzymes (McCaffrey & Braakman, 2016).

In maintaining its role as a central coordinator for maintenance of cellular homeostasis (Rutkowski & Hegde, 2010), the ER engages in various cellular functions involving the biosynthesis of lipid species such as cholesterol, triacylglycerol, and phospholipids, the degradation of glycogen, detoxification, and the maintenance of Ca^{2+} homeostasis (Reverendo et al., 2019). Upon entry into the ER lumen, newly synthesized polypeptides are folded and modified to ensure precise conformations and function. Impairments of this process lead to ER stress due to the accumulation of misfolded or unfolded proteins in the ER (Byrd & Brewer, 2012). However, abnormal accumulation of proteins in the ER can be a sign of cellular malfunction and stress that triggers a collection of conserved emergency rescue programs (Reverendo et al*.,* 2019).

Lipids are mostly synthesised in the endoplasmic reticulum (ER) and distributed to other membranes by non-vesicular mechanisms through mechanisms which act in conjunction with lipid metabolic networks for the maintenance of the unique lipid profile of the plasma membrane and subcellular organelles, thus enabling rapid membrane lipid remodelling in response to signals and stresses (Quon et al., 2018). Therefore the ER dynamically changes its membrane structure to adapt to the changing cellular lipid concentrations (Almanza et al., 2019). Several studies have investigated the impact of ER expansion on lipid biosynthesis and cholesterol amounts as the ER contains the sterol regulatory element-binding protein family of cholesterol sensors ensuring cholesterol homeostasis (Almanza et al., 2019).

1.6.2 Protein translocation across the Endoplasmic Reticulum

Protein targeting to the ER has been the subject of intensive research for decades (Aviram & Schuldiner, 2017). Translocation of proteins into the endoplasmic reticulum (ER) is the first step in the biogenesis of most extracellular and many soluble organelle proteins of eukaryotic cells (Zimmermann et al., 2011) as proteins destined to enter the secretory pathway typically possess N-terminal hydrophobic signal sequences, which direct them to the endoplasmic reticulum (ER) membrane, where they are translocated into the lumen of ER (Nyathi et al., 2013). Protein transport into the ER can occur co- or post translationally (Zimmermann et al., 2011) where processes such as post-translational modifications (PTMs) occur on a large number of proteins de facto increasing the actual complexity of the proteome (Audagnotto & Dal Peraro, 2017).

PTMs consist in a covalent modification of amino acids of the primary protein sequence whereby the function of proteins is frequently modulated by chemical modifications introduced after translation from RNA and post-translational modifications (PTMs) have been shown to also influence the interaction between proteins carrying them (Duan & Walther, 2015). Therefore, protein folding is the initial step for polypeptides entering the secretory pathway. Protein folding can initiate inside the ribosomal exit tunnel, e.g. through helix formation in the lower tunnel (Ellgaard et al., 2016).

Chaperones are bio molecular complexes that help other proteins (their clients) to fold (Agozzino & Dill, 2018) into their native conformation, thereby preventing their aggregation. These chaperones as well as some folding enzymes which are expressed in the ER function to provide an environment for nascent polypeptide chains to reach their proper three-dimensional configuration. Many of these factors exist in a preformed multi-chaperone complex that may provide for coordinated catalysis of protein folding. It was long thought that signal recognition particle (SRP) binds the ribosome-nascent chain complex only after the emergence of the nascent hydrophobic motif from the ribosome exit tunnel (Aviram & Schuldiner, 2017) which is thought to allow time for a ribosome-nascent chain complex to diffuse to the ER membrane However, recent studies suggest that, for certain substrates, SRP already binds to the ribosome at earlier stages during translation (Aviram & Schuldiner, 2017) (Figure 1.9).

The co-translational pathway uses the signal recognition particle (SRP) to deliver secretory proteins to the ER membrane while they are still being synthesized by ribosomes and as the SRP delivers the nascent secretory protein together with the associated ribosome to the ER protein translocon (Sec61 complex) through the interaction with its cognate receptor (SR) an ER resident membrane protein, the ribosome nascent chain complex (RNC) engages the Sec61 complex protein synthesis thus enabling the nascent chain to be directly conveyed into the lumen of the ER where it can then fold to its final conformation (Nyathi et al., 2013). However, If translocation is much faster than translation elongation, translocation of all of these proteins could be accomplished in the same amount of time it would take to translocate a single protein co-translationally (Cranford-Smith & Huber, 2018).

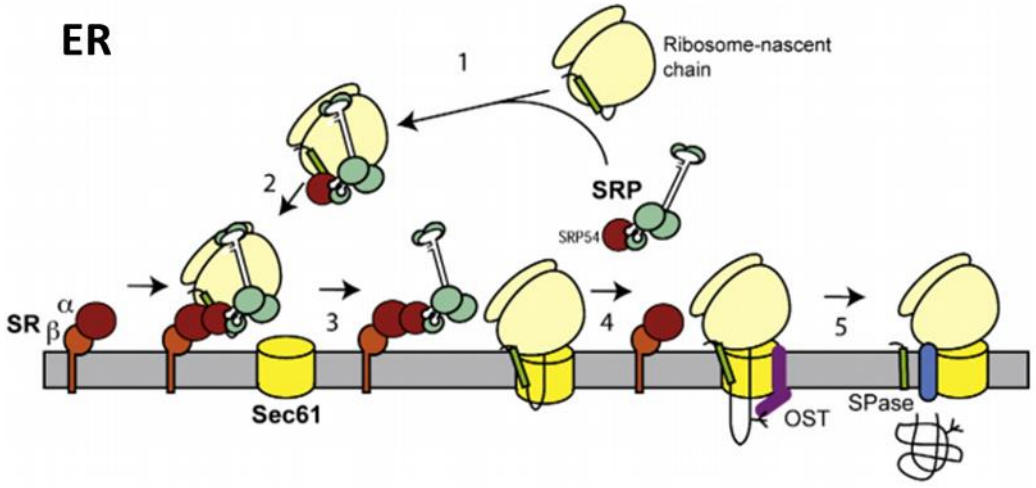

Figure 1.9 Co-translocation into the Endoplasmic Reticulum (ER). Protein targeting pathway involves the binding of the signal recognition particle (SRP) to the signal sequence (green) immediately it emerges from the ribosome nascent chain forming the Ribosome nascent chain – signal recognition particle (RNC–SRP) complex (**1**). Thereafter the newly formed RNC–SRP complex binds to a cognate SRP receptor (SR, consisting of α and β subunits) at the ER membrane (**2**). Next is the transfer of RNC from SRP receptor to the Sec61 translocon resulting in intercalation of the signal sequence in the translocon pore and its opening (**3**) At this point, translocation of the nascent chain proceeds through the pore as SRP now disengages from SR (**4**). Finally, during translocation, the signal peptidase (SPase) and oligosaccharyl transferase (OST) enzyme complexes are recruited to the translocon to cleave the signal peptide and to add N-linked glycans to the nascent chain respectively (**5**). At this point, nascent chain is now released from the ribosome, a process known as termination of protein synthesis. Source: Nyathi et al 2013.

1.6.3 ER Associated Degradation (ERAD) and ER Stress

Under normal physiological conditions, upon entry into the ER lumen, newly synthesised polypeptides are folded and modified to ensure precise conformations and function (So, 2018). Even with the assistance of dedicated protein folding machinery in the ER, a large portion of proteins entering the ER fails to obtain proper conformation due to mutations, unavailability of chaperones, or changes in the amounts of interacting partners and eventually must be eliminated (Yoo et al., 2017). Physiological conditions increase the protein folding demand, which may trigger loss of its regulation (Kadowaki et al, 2018) (Pereira et al., 2016) .

Various stress factors, such as hypoxia, starvation, oxidative insults, changes in pH, Ca^{2+} depletion, hypoglycemia, ATP depletion, and viral infections, can disturb ER homeostasis such that all these aspects can interfere with the correct protein folding, results in the accumulation of misfolded or unfolded proteins leading to ER stress (Limonta et al., 2019). However, what defines ER stress and how ER stress may engender cytotoxicity, are poorly understood issues (Vitale et al., 2019). ER stress is defined as the accumulation of misfolded proteins in the ER although, it is unusual for anyone to attempt measure the load of misfolded proteins in the ER in response to stress (Howell, 2017).

Indeed, the ER-associated degradation (ERAD) is the temporally and spatially coordinated surveillance process charged with clearance of aberrant proteins in the ER (Olzmann et al., 2013) and occurs in three primary steps: (1) recognition and targeting (substrate recognition within the ER and targeting to the retrotranslocon), (2) retrotranslocation (substrate delivery from the ER to the cytosol), and (3) degradation (ubiquitin –proteasome dependent degradation) (Kleizen & Braakman, 2004).

1.6.4 Quality Control in the ER

The best studied quality control checkpoint is the ER system, which identifies non-native features of proteins that have failed to fold correctly and retains them in the ER (Briant et al., 2017). Protein quality control (PQC) is a unique mechanism for adaptation to ER stress. As earlier described, the ER contains an elegant system designed to facilitate the proper folding and trafficking of proteins, particularly those destined for secretory pathways (Kropski et al., 2018), therefore cells rely on a number of protein quality control (PQC) pathways that survey proteins both during and after

synthesis to prevent protein aggregation, promote correct protein folding, and target terminally misfolded proteins to degradation (Peters et al., 2015). To maintain protein homeostasis, cells must ensure the fidelity of protein folding and maturation whereas to maintain ER homeostasis, cells have evolved protein quality-control systems through the action of different pathways, including the UPR, ERAD, and autophagy (Hwang & Qi, 2018).

Basically, there are three axes that define the protein quality control of the ER; acceleration of adequate protein folding, activation of unfolded protein response (UPR), and protein clearance via ER-associated degradation (ERAD) (Yoo et al., 2017). Accumulation of unfolded or misfolded proteins is the trigger of ER stress, which activates the unfolded protein response (UPR) to restore the lost balance (Leitman et al., 2012) which in turn inhibits the proteasomal degradation of misfolded proteins with a coordinated action of chaperone calnexin (CNX) along with calreticulin (CRT), a soluble homolog of calnexin. The ER quality control system therefore senses and disposes of terminally misfolded proteins by ERAD, a process that is conserved in eukaryote (Byun et al., 2014) and the fundamental ERQC factors are the lectin chaperones calnexin (CNX) and calreticulin (CRT) (Volpi et al., 2017), which assist the folding of ER-synthesized glycoproteins (Lamriben et al., 2016).

In the CNX/CRT cycle, the glycan portion attached to the unfolded glycoproteins are modified so as to drive the alternate dissociation and re-binding of these proteins to the lectins, until their proper structure is reached (Figure 1.10). Another major chaperone system in the CNX/CRT complex is the BiP/GRP78 or Hsp70 system. After leaving the CNX/CRT binding cycle, correctly folded proteins will hence be targeted for trafficking to the Golgi via the ER/Golgi intermediate compartment. This implies that through the CNX/CRT cycle, slow- folding or misfolded glycoproteins would be segregated from the peripheral ER to the ERQC and then cycle back for deglucosylation, reglucosylation, and a new folding attempt (Leitman et al., 2012).

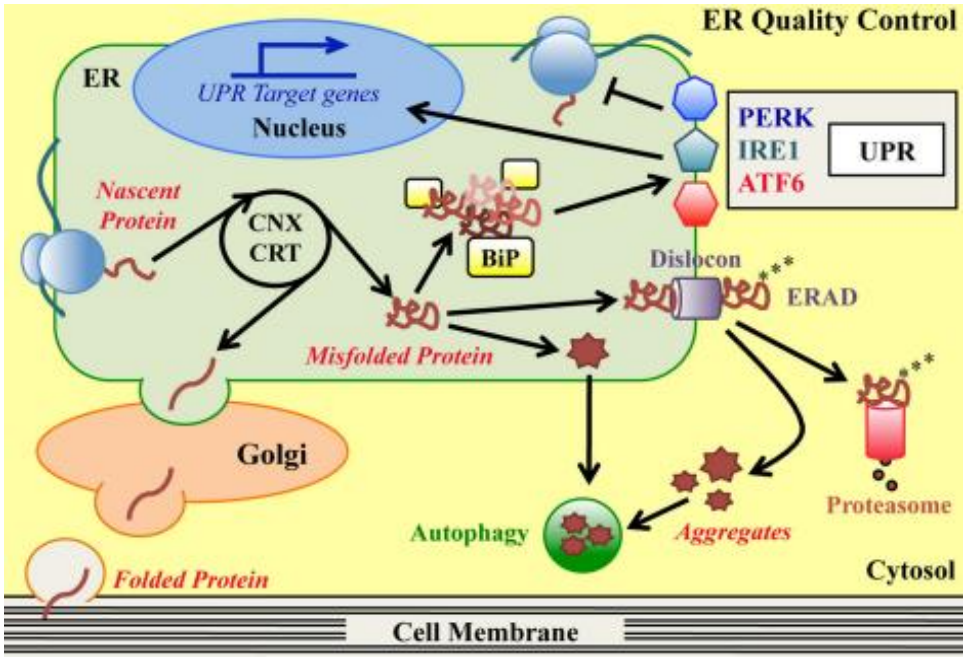

Figure 1.10 ER quality control (EROC) pathways**.** In the ER specialised protein quality control (ERQC) pathways involve the synthesis, folding and maturation of proteins destined for secretion or insertion into the cell membrane. ER-synthesised proteins emerge from ER-bound ribosomes and reach their native structure, guided by ER-resident chaperones, such as calnexin (CNX), CRT (calreticulin) and BiP (Binding Immunoglobulin Protein). Proteins unable to reach their native conformation, designated as terminally misfolded are eliminated to counter potential toxicity. (Volpi et al., 2017).

1.7 The Unfolded Protein Response Pathway

In order for the restoration of protein folding capacity with protein synthesis requirements to be activated, a coordinated transcriptional and translational network termed the unfolded protein response (UPR) is initiated (Adams et al., 2019). This response system elicits specific intracellular signalling pathways to protect against ER stress (So, 2018) with large-scale transcriptional alterations for approximately 400 genes in order to recover the secretory pathway to homeostasis (Bao, 2017). Other conditions that trigger a major defence system as they help cells survive any stress circumstances include inflammatory responses and oxidative stress caused by either physiological, pathological or biochemical stimuli. UPR also enhances ER-associated degradation (ERAD) processes which involves targeting of unfolded proteins to be degraded by the proteasome.

The canonical core of the UPR is composed of three major transmembrane stress sensors; PERK, IRE-1, and ATF6 (Figure 1.11) (Doultsinos et al., 2017a) and these transmembrane proteins are major transducers of the UPR in mammals and mediate multiple distinct signals from the ER to the cytosol (So, 2018).

IRE1α is a functional kinase, and RNase represents the most conserved branch of the UPR. Upon activation, IRE1α catalyses the unconventional splicing of X-box binding protein 1 (XBP1) removing a 26-nucleotide intron (Hetz, 2017). Another arm of the UPR is the ER membrane protein ATF6 (activating transcription factor 6), a fragment of which moves to the nucleus where it functions as a transcription factor to activate genes that mediate protein folding (Mckimpson & Kitsis, 2017). The activated PERK induces a global translation attenuation by phosphorylation of the α subunit of eIF2α, therefore reducing folding requirements in the ER, which occurs by oligomerization and auto phosphorylation as well as for IRE1, unlike ATF6 which is translocated to the Golgi apparatus and then activated via proteolytic cleavage (Galluzzi et al., 2017). Unfolded Protein Response in the endoplasmic reticulum is influenced by several factors associated with lowering protein synthesis and translocation into the ER.

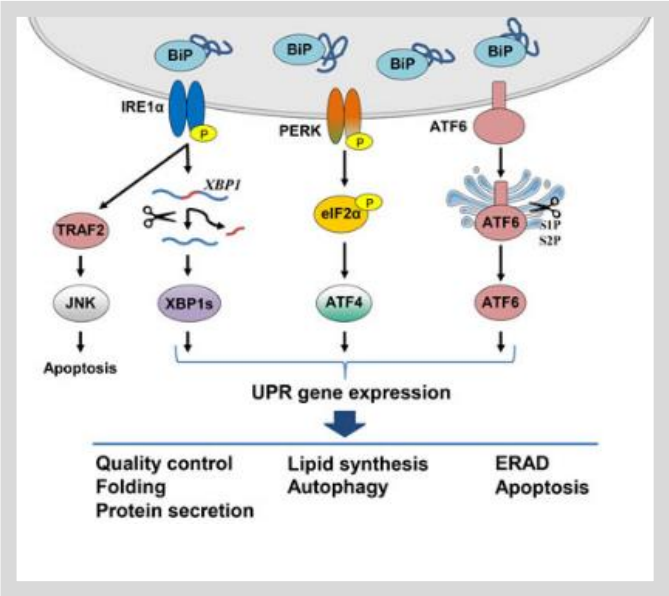

Figure 1.11 Schematic diagram of the UPR pathway. In the event of restoring protein homeostasis (proteostasis), three transmembrane proteins IRE1, PERK and ATF6 are activated by UPR signals. PERK phosphorylates eIF2a, leading to a rapid attenuation of protein synthesis as well as facilitating the non-canonical translation of ATF4 mRNA. ATF4 upregulates proteins involved in protein folding and secretion, ERAD, apoptosis, autophagy and lipid synthesis. The IRE1 and ATF6 branches of the UPR activate the transcription factors XBP1 and ATF6, respectively as target genes encode proteins involved in protein folding, endoplasmic reticulum-associated protein degradation (ERAD) and lipid biosynthesis. (Storm et al., 2016).

1.7.1 Inositol-Requiring Protein 1 (IRE1)

Inositol-requiring enzyme 1 (IRE1) is the most highly conserved signalling node of the unfolded protein response (UPR) and represents a potential therapeutic target for a number of diseases associated with endoplasmic reticulum stress (Volkmann et al., 2011) while the activated IRE1, which has been reported to be associated with tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2), unconventionally splices the mRNA of the transcription factor XBP1/HAC1, activating XBP1, promoting its translation and a conserved XBP1-dependent gene-expression program (Amin-Wetzel et al., 2017).

IRE1 functions as an RNA nuclease whereby it cleaves the primary transcript of Xbox binding protein 1 (XBP1) in order to excise a short inhibitory intron. Thereafter the newly spliced XBP1 mRNA encodes an active transcription factor, XBP1s (s for spliced) (Marciniak, 2019) allowing communication between the ER and the nucleus which results in the increased transcription of binding immunoglobin protein (BiP), during stress. The activated XBP1s then regulates the expression of various chaperones, foldases and other protective molecules (Okazaki et al., 2014). IRE1 in humans exists in the form of two homologues: IRE1α and IRE1β (Doultsinos et al., 2017b). While IRE1α IRE1α is ubiquitously expressed, IRE1β is tissue specific (Ma et al., 2014) (Chen & Brandizzi, 2013).

1.7.2 Activating Transcription Factor 6 (ATF6)

During ER stress conditions, ATF6 is transported on vesicles toward the Golgi apparatus), where it is sequentially cleaved by the Site-1 and then Site-2 Proteases (S1P and S2P, respectively) (Bravo et al., 2013). In the absence of ER stress, Binding immunoglobulin protein BiP/GRP78, a major ER chaperone critical for protein quality control, is associated with all three sensors PERK, IRE1 and ATF6 preventing their activation. ATF6 is synthesised as a type II transmembrane glycoprotein and is embedded in the ER membrane. In response to ER stress, ATF6 is reduced to its monomeric confirmation and translocates from the ER to the Golgi compartment using COP II vesicles resulting in the release of ATF6's cytosolic ATF6 transcriptional activity from its cleaved cytosolic domain, which contains a basic leucine zipper domain that locates to the nucleus where it upregulates.

One of the three canonical arms activated during ER stress sensors, PERK, is an eIF2α kinase whose phosphorylation of eIF2α leading to the preferred translation of selective mRNAs, such as the one encoding transcription factor ATF4. Figure 1.12 shows the functional role played by the stress receptors while ultimately down regulating protein synthesis in order to eliminate misfolded or unfolded proteins during ER stress response.

1.7.3 Protein Kinase-Like Endoplasmic Reticulum Kinase (PERK)

PERK belongs to the eukaryotic initiation factor (eIF2α) kinase family. During ER stress, PERK is activated in a similar manner as IRE1, whereby the activated PERK then phosphorylates Ser51 on the subunit of eukaryotic initiation factor 2 alpha (eIF2α) thus reducing the ER protein folding burden (Cao & Kaufman, 2014). This phosphorylation inhibits the reactivation of eIF2α into its GTP-bound form, resulting in global arrest of protein synthesis that, in turn, prevents further influx of ER client proteins (Ma et al., 2014) thus resulting in a decrease in PERK signalling of the translocation of new proteins into the ER lumen which subsequently prevents protein overload. In addition to reducing translation, PERK-mediated phosphorylation of eIF2α also induces preferential translation of activating transcription factor 4 (ATF4), a transcription activator of the integrated stress response which also induces a subset of ER stress genes involved in inflammation, apoptosis, and oxidative stress by binding to the cAMP-response element (CRE) located in their promoter or enhancer regions (Ma et al., 2014).

While the ATF6 and IRE1 pathways regulate the transcriptional activation of various genes, including those responsible for increasing the translocation, protein-folding, export, degradation, and other functions of the ER (Lin et al., 2019), the ER may also directly activate the apoptotic pathway through ER stress mediated leakage of calcium into the cytoplasm which further activates death effectors, via the ATF4-CHOP-mediated induction of pro-apoptotic genes while supressing the synthesis of anti-apoptotic Bcl-2 proteins (Rozpedek et al., 2016). Due to its role as a type I ER trans membrane protein with serine/threonine kinase activity in its C-terminal cytosolic domain, PERK can recognize the accumulation of misfolded proteins at its N-terminal luminal domain (Kadowaki & Nishitoh, 2013).

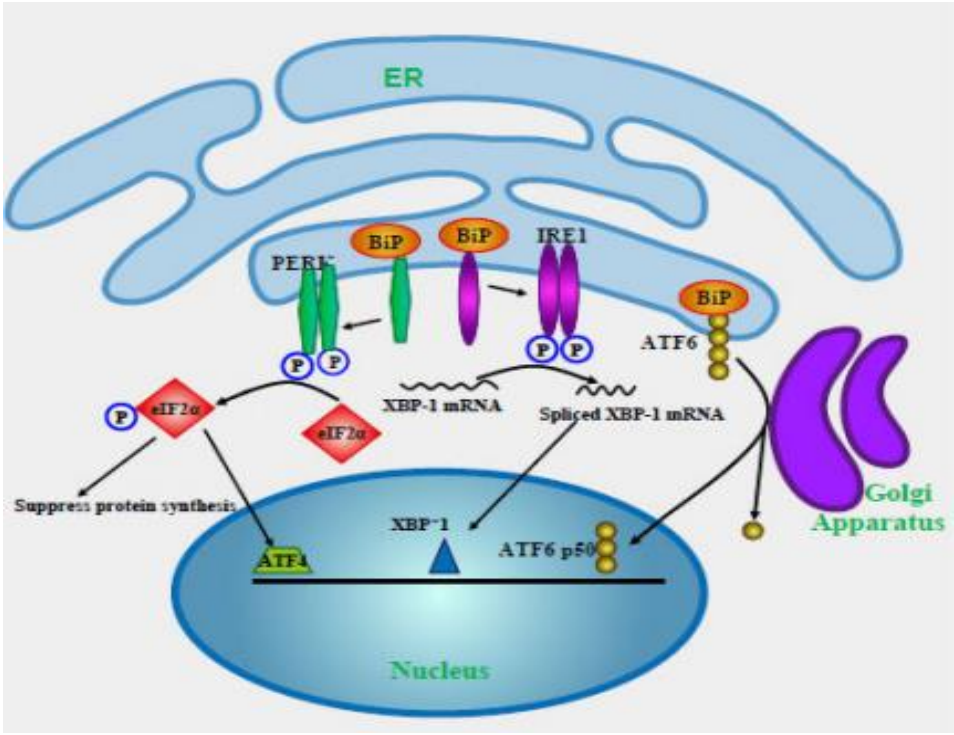

Figure 1.12 The Endoplasmic Reticulum stress receptors. In the absence of stress, the three endoplasmic reticulum (ER) stress receptors, PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) are rendered inactive and remain bound to Binding Immunoglobulin Protein (BiP). However, due to certain cell intrinsic or extrinsic factors leading to ER stress, these three receptors become activated by detaching from BiP/GRP78, as the latter proceeds to bind misfolded proteins, caused by ER stress. To activate the three (released) ER stress receptors, a process of homodimerisation and autophosphorylation take place in PERK, and IRE1 while ATF6 become activated by protease cleavage in the Golgi. PERK phosphorylates eukaryotic initiation factor 2 (eIF2α) to inhibit general protein translation, leading to eIF2α-independent translation of ATF4, reducing protein synthesis in the process. IRE1 catalyses the alternative splicing of XBP1-mRNA leading to expression of the active XBP1 transcription factor via signalling cascades for the translocation of transcription factors such as; ATF4, XBP1s and ATF6f, to the nucleus where they transcriptionally function to restore homeostasis (Source: Jixin Zhong, 2013).

1.8 Biosynthesis of Cholesterol in mammalian cells.

Cholesterol is an important biological molecule because of its dual character: a friend as an essential component of cell membranes and a precursor for the synthesis of steroid hormones, bile acids and vitamin D; a foe as a predisposing factor for various diseases (Bellezza et al., 2013). As an important cellular molecule involved not only in the maintenance of membrane permeability and fluidity, but also in the modulation of transmembrane signalling pathways, cholesterol is a key requirement for the synthesis of steroid hormones, bile acids and vitamin D (Silva Afonso et al., 2018). Cholesterol synthesis occurs in the endoplasmic reticulum (ER) where most of the cholesterol biosynthetic enzymes reside, suggesting the possibility of a specialized metabolon where cholesterol synthesis enzymes exist in an organised system in the ER membrane (Sharpe & Brown, 2013).

From a biochemical perspective, cholesterol is a necessary constituent for eukaryotic cell growth and development and the biosynthesis of cholesterol provides crucial building blocks for cell membranogenesis, membrane fluid regulation, for the synthesis of sterol and non-sterol products. Cholesterol biosynthesis requires more than 30 chemical reactions, with acetate being its precursor (Alphonse & Jones, 2016). The first two reversible reactions, lead to the condensation of two molecules of acetate to form acetoacetyl-CoA which is condensed with a third molecule of acetate forming 3-hydroxy-3methylglutaryl-CoA coenzyme A (HMG-CoA) a reaction catalysed by thiolase and HMG-CoA synthase. The next reaction, catalysed by the enzyme HMG-CoA reductase, a *trans* membrane protein of the ER, reduces HMG-CoA to the key point of regulation in cholesterol synthesis producing mevalonate (Alphonse & Jones, 2016).

Following the production of mevalonate, the next phase promotes the condensation of six molecules of isopentenyl pyrophosphate to form squalene, which is then cyclised and converted to cholesterol through several steps (Berger et al., 2015). Through a series of nineteen additional reactions, lanosterol is converted to cholesterol. These nineteen reaction steps are catalysed by nine different enzymes that are localised either to the ER or to the peroxisomes. One of the nineteen enzymes activated during this stage is Transmembrane 7 Superfamily member 2 **(**TM7SF2), discussed in this work (Figure 1.13). The terminal reaction in cholesterol biosynthesis is catalysed by the enzyme 7-dehydrocholesterol reductase encoded by the DHCR7 gene. Functional DHCR7 protein is a 55.5kDa NADPH-requiring integral membrane protein localised to the microsomal (ER) membrane.

Literature studies have shown, that while cholesterol biosynthesis involves an energetically expensive multi-step process involving over 20 enzymes, the pathway leading to final cholesterol biosynthesis from lanosterol has been directed into two pathways, the Bloch and Kandutsch-Russell pathways. These pathways have common initial steps starting from acetate and then branch out after lanosterol and while these pathways do not have common intermediates after lanosterols. Both routes are not exclusive as same enzymes act in each of the pathways. NADPH-dependent reduction step is catalysed by the sterol reductases; Transmembrane7 Superfamily member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7), among others, which this study focuses on for investigative studies on their role in ER expansion (Figure 1.13). Activities of these terminal enzymes can affect membrane organisation and dynamics, as the addition and position of the double bond in desmosterol and 7-dehydrocholesterol confers very different properties to the cell (Luu et al., 2015).

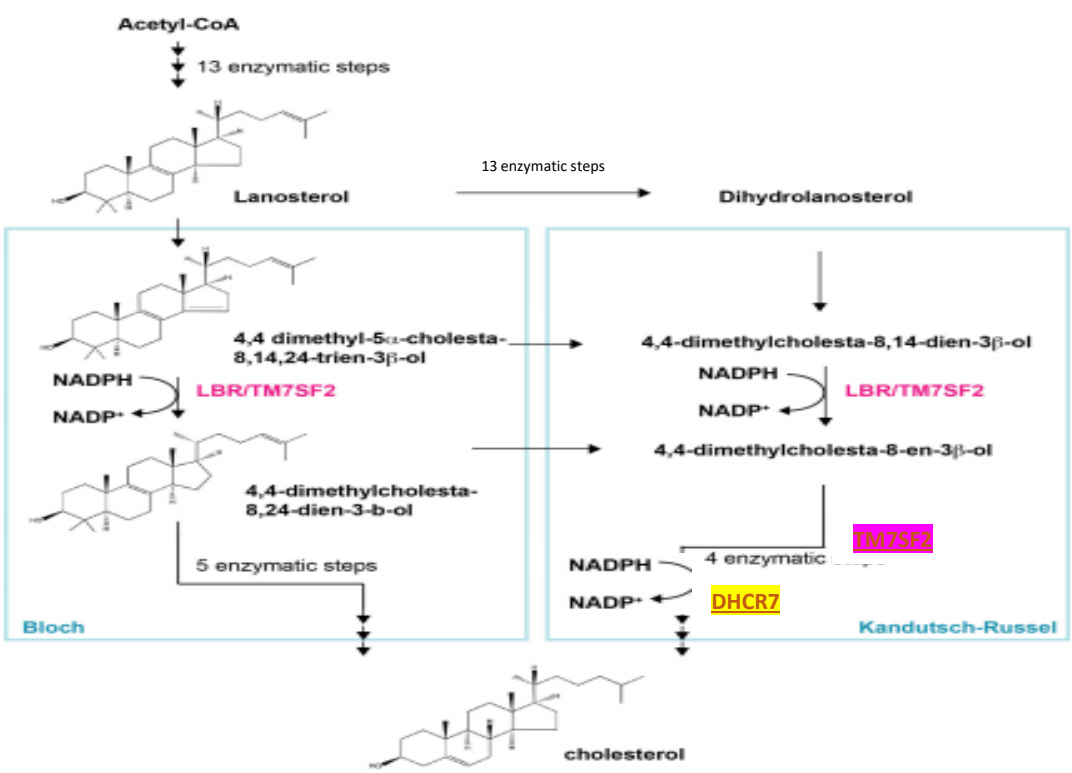

Figure 1.13 A schematic representation showing the link between Bloch and Kandutsch-Russel pathways and Cholesterol biosynthesis Pathway. In a cascade mechanism, cholesterol is ultimately produced following a series of over 30 reaction steps (here briefed). After 13 enzymatic steps, the intermediate Lanosterol can enter one of two parallel pathways designated Bloch and Kandutsch-Russel pathways, respectively, both of which employ an NADPH-dependent reduction step which can be catalysed by the sterol reductases; Transmembrane7 Superfamily member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7), highlighted as shown. Lamin B Receptor is also shown. This study focuses on these two proteins for their investigative role in ER expansion. Adapted, with modifications, from (Tsai et al., 2016).

1.8.1 Transmembrane 7 Superfamily member 2

Transmembrane 7 Superfamily member 2 (TM7SF2), also known as sterol C14-reductase and 3 β-hydroxysterol δ-reductase belongs to the ERG24 family in that, they are C-14 sterol reductases and catalyse the reduction of C-14 unsaturated sterol intermediates in cholesterol and sterol biosynthesis respectively. This 418 amino acid gene encodes 3β-hydroxysterol δ-14-reductase which catalyses the reduction of the Δ14-double bond of sterol intermediates in the post-squalene segment of the cholesterol biosynthesis pathway (Bennati et al., 2006) using NADPH dependant reduction of 4,4-dimethyl-5-α-cholesta8,14,24-trien-3-β-ol to 4,4-dimethyl-5-α-cholesta-8,24-dien-3-β-ol and $NADP^+$.

TM7SF2 exacts its functional activity according to the structural organisation of its nine transmembrane domains (TMD) using TMpred prediction program which was reported by Roberti et al., (2002). As a protein coding gene, TM7SF2 presides over an anti-inflammatory loop and its absence correlates with an inflammatory phenotype, which are NF-kB activation and TNFα up-regulation (Bellezza et al., 2013). Although located in the ER having nine transmembrane segments (TM), its catalytic domain comprises the C-terminal domain which is 58% identical to LBR (Zwerger et al., 2010) hence exposed to the cytoplasmic part of the cell (Figure 1.14). This research was conducted following findings by Zwerger et al., 2010 and some of the samples used by their group had been obtained for this work with the intention that our report will serve as a basis for further discussions on the impact of the genes on cellular modification.

Figure 1.14 shows the site of C-terminal truncations that corresponds to LBR disease mutants as constructed by the Zwerger et al., 2010, some of which were further exploited in this work. As an ER localised protein, its role in lipid metabolism - steroid biosynthesis, as a membrane protein has been widely reported. Several reports suggest that modifications of the cholesterol biosynthetic pathway directly affect cell proliferation (Singh et al, 2013). TM7SF2 has its expression regulated by the sterol regulatory element binding protein (SREBP) in response to cellular cholesterol levels (Tsai et al., 2016).

For instance, Bellezza et al., (2015), have implicated TM7SF2 in the driving of a host of protective responses after topical 12-O-tetradecanoylphorbol-13-acetate (TPA) challenge, centred on increased cholesterol and cholesterol sulphate levels when they applied 12-o-tetradecanoylphorbol-13-acetate (TPA) to the skin of TM7SF2(+/+) and TM7SF2(-/-) mice. TPA increased skin cholesterol levels by inducing *de novo* synthesis and up-take only in TM7SF2(+/+) mouse, confirming that the gene maintains cholesterol homeostasis under stress conditions. Functional redundancy suggests that TM7SF2 is also involved in other physiological functions (Bennati et al., 2008). Reports have also shown that TM7SF2 deficiency during liver regeneration alters lipid metabolism and generates a stress condition, which, in turn, transiently unbalances hepatocytes cell cycle progression (Bartoli et al., 2016). Another study also reported on the role of the TM7SF2 gene as an anti-inflammatory agent aside being involved in cholesterol biosynthesis, thereby confirming the existence of cross talk between metabolic pathways and inflammatory response after a significant increase in renal TNFα

expression following tunicamycin exposure and in the oedematogenic response in TM7SF2(+/+) was observed (Bellezza et al., 2013).

1.8.2 7-dehydrocholesterol reductase

The human 7-dehydrocholesterol reductase (DHCR7) protein is predicted to have a molecular weight of 54,489 kDa with 475 amino acids (Correa-Cerro & Porter, 2005). It encodes δ-7-sterol reductase, the penultimate enzyme of mammalian sterol biosynthesis that converts 7-dehydrocholesterol (7-DHC) to cholesterol, which catalyses the final step in cholesterol synthesis, by a reduction of the sterol C7 - C8 double bond, using NADPH as an electron source (Zou et al., 2013). With a gene that spans 14,100 base pairs (bp) of genomic DNA, consisting of nine exons transmembrane domains (Figure 1.14), DHCR7 is ubiquitously expressed and its transmembrane protein localises to the ER membrane as well as the nuclear outer membrane. Cholesterol, though harmful in high levels, is essential to life since it is involved in membrane structure and permeability, synthesis of steroid hormones and proper fetal development (Peng et al., 2018), hence a lack of functional DHCR7 results in an accumulation of 7DHC and reduced cholesterol synthesis, resulting in the devastating developmental disorder Smith-Lemli-Opitz syndrome (SLOS) (Prabhu et al., 2016).

SLOS is an autosomal-recessive syndrome of multiple malformations and intellectual disability resulting from a genetic error in cholesterol synthesis in all cells and tissues, including brain. The first DHCR7 mutations were identified in 1998 by several groups and the early years of the 21st century resulted in more advanced molecular tests to rapidly identify DHCR7 mutations. Most mutations are identified through sequence analysis of coding exons and flanking intronic sequences (Waterham & Hennekam, 2012). When the activity of 7-dehydrocholesterol reductase is unusually low, there tends to be abnormally reduced levels of plasma and tissue cholesterol which in turn results in the accumulation of the cholesterol precursor, 7-dehydrocholesterol (cholesta5,7-dien-3b-ol), and its isomer, 8-dehydrocholesterol (cholesta-5,8-dien-3b-ol). Because cholesterol is essential for the functioning of every mammalian cell and is required for normal embryonic growth, developmental abnormalities could result markedly via the inhibition of its synthesis or by substituting another sterol. Thus far, over 140 DHCR7 mutations have been reported (Tamura et al., 2017).

1.8.3 Lamin B Receptor (LBR) is a related sterol reductase to Transmenbrane 7 Superfamily member 2 and 7-dehydrocholesterol reductase

The description of Lamin B Receptor (LBR) was first published in 1988 from the Blobel laboratory (Worman et al., 1988). The human lamin B receptor (LBR) is a fascinating multi-span integral membrane protein of 615 amino acids in the inner membrane of the nuclear envelope (NE) (Herrmann & Zwerger, 2010) with an N-terminal lamin B and chromatin-binding domains and a C-terminal sterol δ-14 reductase domain (Tsai et al., 2016). The relationship that exist between LBR and TM7SF2/DHCR7 has been widely reported (Figure 1.14). Prediction sequencing have shown, that LBR shares a unique relationship with Transmembrane 7 Super family member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7), with the identification of two paralogs in the genome for the C-terminus only of human LBR whereby TM7SF2 has 58% identical amino acids residues with LBR while DHCR7 has 37% identical and amino acids with LBR (Olins et al., 2010).

TM7SF2 has its expression regulated by the sterol regulatory element binding protein (SREBP) in response to cellular cholesterol levels; whereas LBR is an inner nuclear membrane protein, and the LBR gene lacks an SRE consensus sequence, thus being constitutively expressed (Tsai et al., 2016). The nuclear envelope (NE) is highly dynamic during the cell cycle whereby it disassembles into membranous vesicles or tubules and disperses into the cytoplasm at the onset of prometaphase and reassembles around the newly replicated chromosomes at the end of mitosis (Lu et al., 2010). The structural organisation of the LBR shows an N-terminal nucleoplasmic region, which acts as the interface for interactions with many binding partners, followed by eight putative transmembrane regions, which have homology with cholesterol reductases such as human TM7SF2/DHCR7 although, bioinformatics searches and TM prediction tools provide both inconsistent predictions on the motifs and domains as the total number of TM segments have been predicted as eight (TM 8) in some cases and nine (TM 9) in others (Figure 1.15) (Olins et al., 2010).

Therefore predictions have shown that 208 amino acid amino-terminal part binds to B-type lamins, chromatin and chromatin-associated proteins, whereas 407 amino acids carboxyl-terminal part of has been predicted to exhibit 8 transmembrane domains that anchor the protein into the membrane (Herrmann & Zwerger, 2010). LBR is composed of double lipid bilayers referred to as the outer nuclear membrane (ONM) and the inner nuclear membrane (INM) (Figure 1.15). While the ONM connects to the endoplasmic reticulum (ER), the INM contains a specific set of

transmembrane proteins termed INM proteins (Iwamoto et al., 2019). Genes (TM7SF2 and DHCR7) used in this work were obtained from Monika Zwerger. Diagram is shown to highlight the amino-acid lengths of these genes. TM7SF2 was truncated at (1- 418) while DHCR7 at (1- 475) amino-acids. Zwerger et al., (2010) publication described extensively, the influence of all the truncated genes shown, particularly on Lamin B receptor (LBR).

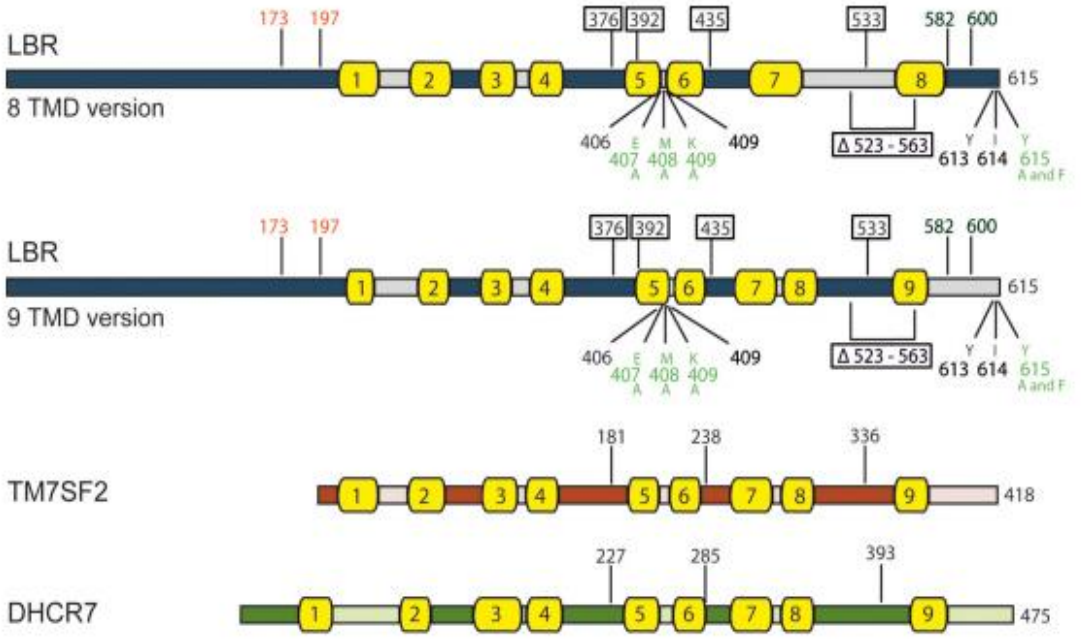

Figure 1.14 Structural Organisation of human Lamin B Receptor (LBR) and human sterol reductases Transmembrane 7 Superfamily member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7). Inconsistent predictions have led to the generation of two transmembrane domains for LBR; TM 8 and TM 9 using TM prediction tools. Effects of LBR disease mutations were investigated by Zwerger et al., 2010 on the organization of the nuclear envelope (NE). Mutations were caused at the C terminal regions of TM Domains of truncated LBR with variants (in black boxes) of known disease-related mutations as described in PHA and Greenberg skeletal dysplasia patients. The Transmembrane Domain are represented with yellow-numbered boxes (1-8) and (1-9) for LBR, and (1-9) for each of TM7SF2 and DHCR7. While the yellow colours indicate lumenal stretches, the dark grey colours indicate the domains exposed to nucleoplasm/cytoplasmic part of the cell. Highlighted numbers with grey background indicate the amino acid length (418 for TM7SF2 and 475 for DHCR7) while 181, 238,336, 227, 285 and 393 are the corresponding sites of C-terminal truncations that relate to LBR disease mutants. red numbers specify the amino acid that N-terminally truncated LBR variants initiate with; and green numbers identify single amino acid changes introduced in the full-length LBR. Zwerger et al., (2010).

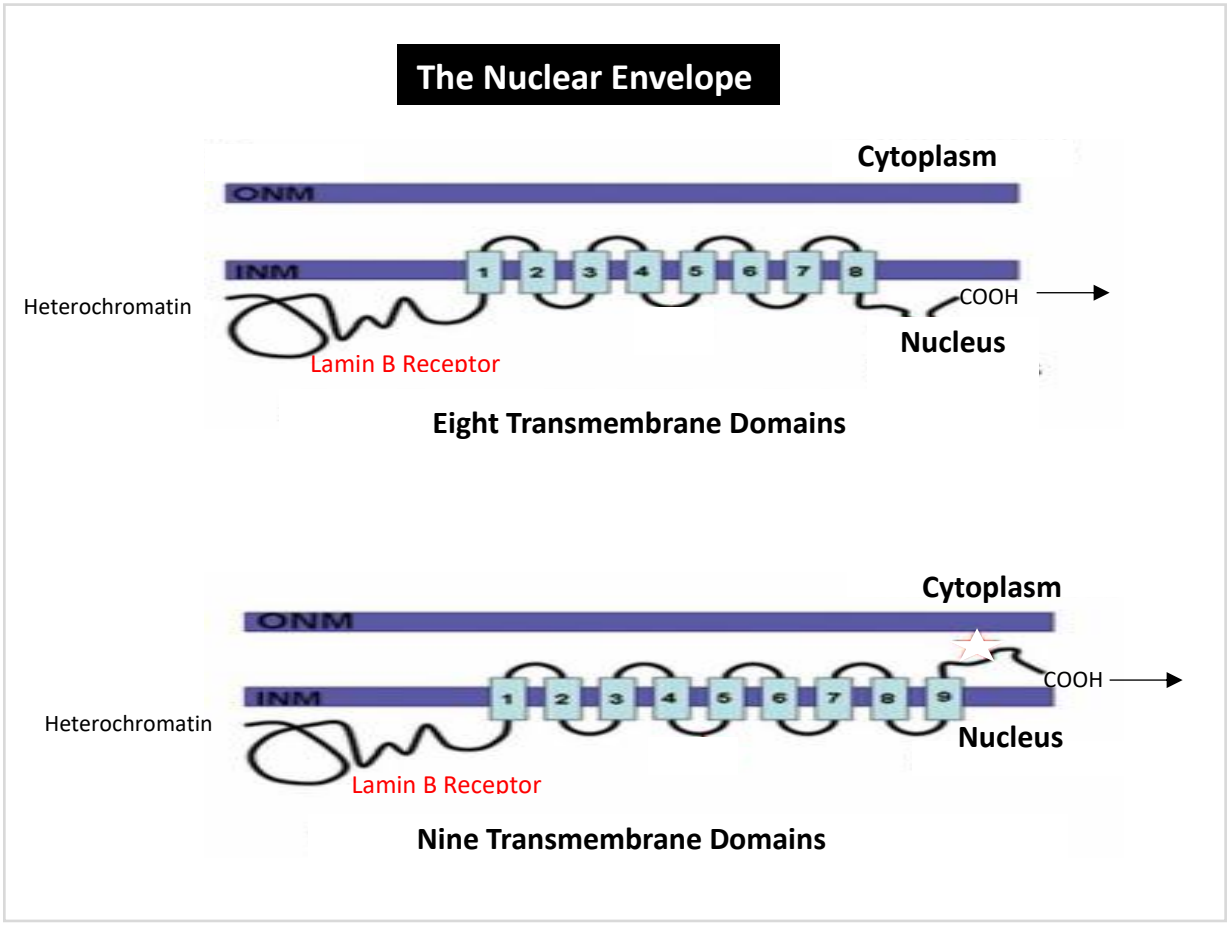

Figure 1.15 Schematic diagram of the Nuclear Envelope (NE) showing link between Transmembrane domains and full-length Lamin B Receptor (LBR) in the inner nuclear membrane (INM). The Nuclear Envelope consists of the inner nuclear membrane (INM) and the outer nuclear membrane (ONM). While the ONM is embedded in the cytoplasm and continuous with the rough endoplasmic reticulum, the INM which consists of over 100 highly organized transmembrane proteins complexes interact with heterchromatin where the LBR tethers the lamina. Lamin B Receptor (LBR) is predicted to have two transmembrane domains (TM 8 and TM9) to the disposition of the C-terminal tail. Adapted with modifications from (Olins et al., 2010).

1.8.4 Transcriptional Regulation by SREBP of Cholesterol Biosynthesis

Cholesterol biosynthesis is tightly regulated by a mechanism of negative feedback that senses cholesterol and oxysterols. Literature studies reveal that the SREBP (Sterol regulatory element binding protein) family of transcription factors are activated in response to low sterol status and help coordinate the cholesterol synthesis pathway (Sharpe & Brown, 2013). Therefore, nearly all the genes encoding cholesterol synthesis enzymes are SREBP targets. The transcription factor sterol regulatory element-binding protein-2 (SREBP-2) is the key regulator of genes involved in cholesterol synthesis such as HMG-CoA reductase (HMGCR), HMG-CoA synthase (HMGCS), and mevalonate kinase (MVK), as well as the LDL receptor (LDLR) which is responsible for cholesterol uptake (Brown et al., 2019).

SREBPs are synthesised as inactive proteins and become active after forming a complex with SREBP cleavage activating protein (SCAP). They bind to sequences upstream of specific genes involved in lipid biosynthesis, called sterol response elements (SRE), and activates transcription. Mammalian cells produce three SREBP isoforms, called SREBP-1a, SREBP-1c, and SREBP-2 (Cheng et al., 2018) (Figure 1.16). As lipid biosynthesis is key to cellular activity, the ER itself is encased by a large membranous structure and lipids are required for formation of other secretory pathway components including vesicles for trafficking, the Golgi and plasma membrane, an alternative approach to increasing the ER capacity of the cell to up-regulating components for the UPR may be to expand the ER and secretory pathway itself by manipulating lipid biosynthesis.

Further, other than the widely studied SREBP, numerous other transcription factors have been implicated in the transcriptional control of the various enzymes in cholesterol synthesis. Liver X Receptor (LXR) has been reported to regulate cholesterol biosynthesis by directly silencing the expression of two cholesterogenic enzymes (FDFT1 and CYP51A1) (Wang et al., 2008). Liver X Receptors (LXRs) are members of the nuclear receptor superfamily comprising several ligand-activated transcription factors and play a crucial role in regulating the expression of genes involved in lipid metabolism (Fiévet & Staels, 2010). Gene transcription is modulated by LXRs, which heterodimerise with the retinoid X receptor and bind to LXR-response elements in the transcriptional regulatory regions of their target genes (Kim et al., 2019).

The work in this thesis is driven by the hypothesis that manipulation of the CHO cell ER and secretory capacity can be achieved by manipulation of proteins known to impact on ER expansion. In particular, there are reports that manipulation of the proteins; transmembrane 7 Super family member 2 protein (TM7SF2) and 7-dehydrocholesterol reductase protein (DHCR7) can impact positively on ER expansion (Zwerger et al., 2010).

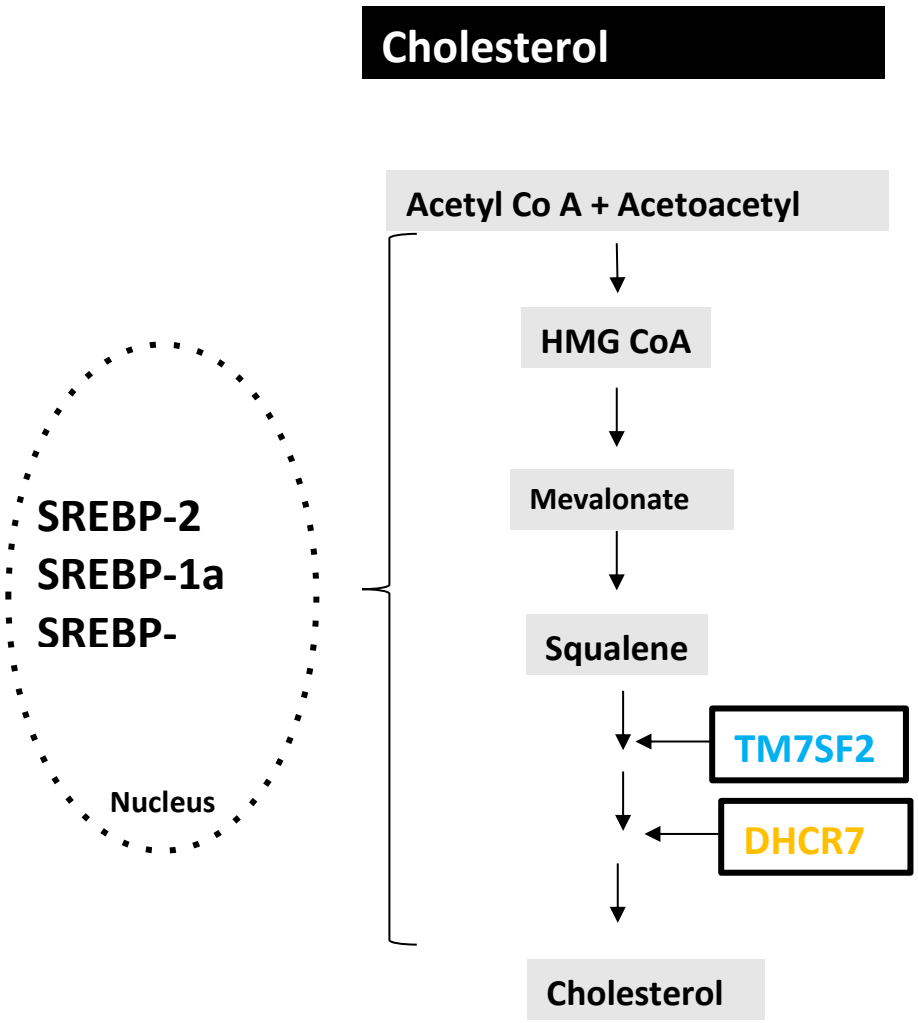

Figure 1.16 SREBP regulation of TM7SF2 and DHCR7. A transcription factor termed sterol regulatory element binding protein (SREBP) which binds to the sterol regulatory elements (SREs) and enhances transcription. However, the SREBP is held captive bound to the endoplasmic reticulum membrane. Activation of SREBP 1a, 1c and 2, results in an activation of TM7SF2 and DHCR7 which favour cholesterol production. In other words, the SREBPs when it is released by proteolytic cleavage travels to the nucleus, where it regulates sterol-responsive genes.

1.9.0 Rationale and Hypotheses of the Work undertaken in this Thesis

In 2010, researchers attempted to cause fundamental influence on the endoplasmic reticulum and nuclear membrane expansion by manipulating Lamin B receptor (LBR), Transmembrane 7 Super family member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7) amounts (Zwerger et al., 2010). Although the primary topic for research and discussion was the N-terminal domain of LBR, it was reported that the overexpression of intact TM7SF2 and DHCR7 as well as mutant LBR C-terminal mutations resulted in peculiar cellular phenotype where by the large dilations were observed as an aftermath throughout the entire cell and especially at the nuclear periphery. When the distance of enlargement measured created was about 50 nm – 150 nm compared to the 30 nm – 50 nm observed in normal cells.

These findings are indeed remarkable knowing that both the inner nuclear membrane and the outer nuclear membrane have a constant thickness and diameter across cells (Shibata et al., 2006). The notable findings also suggest an endoplasmic reticulum expansion was also impacted on some phenotypic characteristics. For instance, Zwerger et al., (2010) had again reported, that the overexpression of the wild-type forms of TM7SF2 and DHCR7 but not wild type LBR mimicked the phenotype observed for mutant LBR, suggesting therefore, that even fully functional proteins can critically affect the endoplasmic reticulum (ER) and nuclear membranes (NE) morphology. These findings were observed in different cell lines with different reactions/responsiveness leading to the dilation of the ER and NE nuclei compacted markedly and “cytoplasmic vacuoles” (CVs) as they developed and increased in size.

Our interest in this research was to investigate further the two sterol reductases as their overexpression have been hypothesised to cause ER expansion. We wanted to corroborate the findings as published by Zwerger et al., (2010) with the overexpression of TMSF2 and DHCR7 as targets in CHO cells and note if the findings are same or similar to those observed when other mammalian cell lines were used. From the literature studies discussed earlier (section **1.3**), CHO cells have been widely reported to show a remarkable post translational modification qualities and if these would in any way influence the impact on endoplasmic reticulum size and nuclear membrane size at large is yet to be discovered.

While the main focus in the report by Zwerger et al., (2010) focused on the LBR, with a report that the two wild-type sterol reductases (Transmembrane 7 Superfamily member 2 and 7-dehydrocholesterol reductase) also mimicked mutant LBR with the phenotype observed, suggesting that even fully functional proteins can critically affect the ER and NE morphology. This is interesting knowing that both Transmembrane 7 Superfamily member 2 and 7-dehydrocholesterol reductase are endoplasmic reticulum localized proteins and key enzymes of the cholesterol biosynthesis and their role in lipid generation has been widely reported. The Lamin B receptor protein, however, is known to have dual function, as it functions both as a sterol reductase and has also been implicated in the structural organisation of heterochromatin.

Our interest therefore was to investigate if these sterol reductases, when overexpressed in stable expression system CHO cell line can show phenotypic characteristics which may result in endoplasmic reticulum expansion. Further, to investigate whether the manipulation of these targets can also impact on recombinant protein production, two difficult-to-express model proteins were also assessed in engineered CHO cell lines over-expressing wild type TM7SF2 and DHCR7 and their expression compared to control cell lines. The hypothesis to be tested therefore, is that controlled manipulation of cholesterol biosynthesis by overexpression of Transmembrane 7 Superfamily member 2 and 7-dehydrocholesterol reductase will result in expansion of the endoplasmic reticulum and also an enhancement of the efficiency of the CHO platform as a recombinant protein expression system by improving cell growth, yield and quality of recombinant protein production.

1.9.1 Project Aims & Objectives

This project set out to address the hypothesis that an alteration of key cellular components by over-expressing target genes that play key functional role in cholesterol biosynthesis at the molecular level would result in a controlled systemic manipulation of cholesterol (lipid) biosynthesis and hence ER expansion for an improved secretion processes of the CHO platform as a recombinant protein expression system. Prior to the commencement of this research, studies had reported on the effect of overexpression of the carboxyl-terminal domain of Trans membrane 7 Super family member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7) as described above in section 1.9, which critically affected the membrane integrity by causing a dilation and nuclear compaction and that when overexpressed in U2OS cells, an identically induced phenotype was observed in both the truncated and the full length variants. At the onset, the aim of this thesis was to use this information to investigate the CHO mammalian cell secretory machinery for ER expansion by manipulating the expression of these cholesterol (lipid) biosynthesis genes thereby investigating the influence on the phenotypic traits as it affects the growth, yield and whether this resulted in an overall improvement of the CHO cells secretory pathway capacity for recombinant protein production. Therefore, the aims of this project was to:

1) To generate a series of CHOS-derived cell pools and clonal cell lines from stable heterogeneous pools over-expressing TM7SF2 and DHCR7.

2) To investigate whether overexpression of these genes individually will influence phenotypic characteristics such as growth and viability that favour CHO-S secretory machinery.

3) To investigate and determine if the manipulation of cholesterol (lipid biosynthesis) by altering cellular pathway processes can be used to enhance secretory productivity from CHO-S cells, using EPO and etanercept as model molecules.

Chapter 2

2.0 Materials and Methods

2.1 Chemical Reagents and Preparation of Solutions

All reagents used for the work as described in this thesis were of the highest grade and obtained from standard sources (such as; Sigma Aldrich, Fisher Scientific). All solutions were prepared using MilliQ water except where otherwise stated. Solutions were sterilised using an autoclave.

2.2 Generating Competent DH5α *E.coli* cells and bacterial transformations

DH5α *E. coli* competent cells were prepared by initially streaking cells onto Luria Bertani (LB) agar plates and placed in an incubator overnight at 37°C. A single colony from the plate was then picked and incubated overnight in 2 ml of liquid LB broth in a 15 ml falcon tube at 37°C with orbital shaking at 200 rpm. 0.5 ml of the starter culture was then used to inoculate 40 ml of liquid LB broth and incubated at 37°C with shaking at 200 rpm, until an OD_{600} nm of between 0.3 - 0.5 absorbance was obtained. The DH5α cells were then pelleted (2500 g for 5 min) and then re-suspended in 20 ml of 50 mM $CaCl_2$ and incubated on ice for 30 min. The cells were then pelleted again (2500 g for 5 min) and resuspended in 4 ml of ice-cold 50 mM $CaCl_2$ and incubated on ice for a further 30 min. 100 µl aliquots of the cell suspension were then placed into sterile 1.8 ml cryovials on dry ice. Aliquots of the competent cells were then stored at -80°C until required.

The introduction of foreign DNA into a bacterial cell was performed to generate copies of the required plasmid DNA. The protocol was as follows;

1. A vial (200 µl) of competent cells (DH5α) was removed from storage at -80°C and thawed on ice. Transformation tubes were also chilled on ice during this time.
2. Once thawed, 50 µl of the bacteria was added to a transformation tube along with 2.5 µl (5% total volume) of plasmid sample or ligation reaction mixture.
3. This mixture was left on ice for 10-15 min, heat shocked in a 42°C water bath for 30- 40 sec then placed back on ice for a further 1-2 min.

4. 500 µl of Super Optimal broth with Catabolite repression (SOC) media (prepared in the laboratory) was immediately added, and this mixture incubated at 37°C for 1 h at 200 rpm agitation speed.
5. The sample was then transferred to a fresh 1.5 ml tube and centrifuged at 3000 rpm in a bench top microfuge for 3 min to pellet the bacterial cells.
6. The majority of the supernatant was removed and the pellet resuspended in 40 µl of SOC.
7. This was then spread on LB agar plates (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl (Sigma, cat # S7653), 20 g agar select, to 1 L with MilliQ water, autoclaved before use) containing ampicillin or kanamycin depending on plasmid used in ligation (100 ng/ml working concentration) and incubated overnight at 37°C.

2.2.1 Plasmid DNA recovery from *E.coli* cells

DH5α *E.coli* cells transformed with DNA constructs were cultured in 6 ml or 250 ml liquid LB broth overnight with 50 µg/ml ampicillin antibiotic selection at 37°C with shaking at 200 rpm. DNA was extracted and recovered from transformed DH5α *E.coli* cells using the commercially available mini prep/high speed maxi prep kits from Qiagen following the protocols provided with each kit.

2.3 DNA Restriction Enzyme digestion

All DNA digestions were undertaken using Promega or Fermentas restriction enzymes and the appropriate buffers as instructed by the manufacturers. Each restriction digest enzyme was used with the appropriate restriction site sequence as specified in the results section (Chapter 3). Digests were undertaken following the protocols provided with the specified enzyme by each manufacturer.

2.4 DNA Ligation reactions

Ligations of DNA inserts amplified by PCR or of DNA fragments released from donor vectors by restriction digestion was generally achieved using a commercially available T4 DNA ligase (Promega) following the protocol provided with enzyme (Table 2.1). The ligation was carried out using amounts of insert to acceptor vector as calculated below.

$$\text{Insert mass (ng)} = \frac{\text{Insert length (bp)} \times \text{Vector Mass (ng)} \times 5 \text{ (ratio)}}{\text{Vector length (bp)}}$$

Table 2.1 Typical example of ligation components and volumes

Components	Control (No ligase) (1:0)	Insert Ratio (1:3)	Control (No Insert) (1:0)	Insert Ratio (1:3)
Plasmid Backbone (Vector)	2.0	2.0	2.0	2.0
Insert DNA	0	Variable*	0	0
Ligase Enzyme	0	0.5	0.5	0.5
Buffer (10x)	1.0	1.0	1.0	1.0
H_2O	to 10	to 10	to 10	to 10
Total Volume	10	10	10	10

2.4.1 Alkaline Phosphatase (AP) Treatment

This procedure precedes ligation as an intermediary step. After digestion and purification of the plasmid backbone and the PCR product (eluted with nuclease-free water into ~45 µl), it was important to ensure that there was no re-circularisation of digested plasmid backbone samples (Table 2.2). The purified plasmid samples were treated with alkaline phosphatase (AP) which dephosphorylates the phosphodiester bonds in DNA decreasing the probability of sticky ends (generated from restriction digests) from re-joining undesirably. This was performed to improve the efficiency of future ligation reactions.

Table 2.2 Typical alkaline phosphatase (AP) treatment of ligation components and volumes

Component	Volume
Purified Plasmid	44.6 µl
AP Buffer (10x)	5 µl
AP	0.4 µl
Total volume	50 µl

2.5 Quantification of DNA and RNA Samples

Measurements of isolated DNA and RNA sample concentrations were performed using 1 µl of the sample on an ND-1000 spectrophotometer (nanodrop). After initialisation with water appropriate solvents (as described in the following sentences) were used to zero the device; for plasmid samples isolated using the Qiagen miniprep kit, 1 µl of the kit's elution buffer was used. For plasmid or DNA samples extracted from an electrophoresis gel using the QIAQuick gel extraction kit or otherwise, 1 µl of the kit's elution buffer was used. DNA concentrations were recorded as the ratio of absorbance at 260 nm and 280 nm. DNA/RNA was then quantified in ng/ µl.

2.5.1 Large Scale Preparation of Plasmid DNA (Maxiprep/Midiprep)

For generating larger quantities of plasmid DNA, a Maxiprep/Midiprep kit was used (Qiagen). A larger volume of Luria Broth (LB) media was used to facilitate larger scale proliferation of the cells. The following protocol was performed as outlined in the operating manual;

1. After successful generation of transformed colonies, a starter culture of LB and single colony, equivalent to a miniprep (5-10 ml universal) was made.
2. This starter culture was then added to a further 200 ml of LB broth containing ampicillin and incubated overnight at 37°C, 170 rpm.

3. After incubation this culture was then centrifuged at 6000 g for 15 min, 4°C, and the supernatant discarded.

4. The resulting cell pellet was then processed using a Qiagen Plasmid Maxi kit (see manufacturers protocol for details), with a final DNA resuspension step carried out using 250-500 µl of TE buffer.

5. The concentration of this DNA prep was determined using a Nano Drop 1000 spectrophotometer. This was then used in subsequent DNA manipulation reactions or stored at -20°C.

2.5.2 Small Scale Preparation of Plasmid DNA (Miniprep)

Commercial kits were again used for minipreps of plasmid DNA from bacterial colonies (Qiagen Mini kits) following protocol as outlined in the operating manual;

1. After successful generation of transformed bacterial colonies, a single colony was picked and grown overnight at 37°C, 170 rpm in a 5-10 ml aliquot of LB broth (autoclaved before use) containing appropriate antibiotics.
2. This culture was then processed using a Qiagen QIAprep Spin Miniprep kit (see manufacturer's protocol for details), with a final DNA elution step carried out using 30-50 µl of TE buffer.
3. The concentration (ng/ µl) of this DNA prep was determined using a Nano Drop 1000® spectrophotometer. This could then be used in subsequent DNA manipulation reactions or stored at -20°C. Typical yields were ~200-450 ng/ µl.

This miniprep sample was then used in downstream processes; typically small scale minipreps were suitable and used in initial restriction digest tests to ensure cloning was successful and for sending a sample away for sequencing to Beckman Coulter, thereby verifying that the correct fragment went in to the respective plasmid.

2.6 DNA Sequencing

Aliquots of prepared plasmid DNA constructs with genes of interest (TM7SF2 and DHCR7 in pcDNA3.1 Hygro vector) were sequenced using a Sanger sequencing service provided by Beckman Coulter Genomics in Danvers, Massachusetts. Samples (templates) were prepared at concentration of 100ng/10 µl in PCR tubes. Therefore samples were prepared and made up to 10 µl volume using nuclease free water in order to prevent EDTA in the plasmid DNA samples. Primer T7 and BGH were used as forward and reverse primers respectively. The technique is used to determine the precise order of the four nucleotide bases – adenine, guanine, cytosine and thymine that make up a strand of DNA. Therefore plasmid DNA to be sequenced served as a template for DNA synthesis where T7 forward primer starts the process of synthesis on the strand of DNA constructs to be sequenced. Basically, the process involved four reactions of Adenine, Guanine, Cytosine and Thymine deoxynucleotide triphosphates (dNTPs) and each would have a low level of one of four dideoxynucleotide triphosphates (ddNTPs): ddATP, ddGTP, ddCTP, or ddTTP which included four ddNTPs. Since, the ddNTP molecule lacks a 3' hydroxyl group, which is required to form a link with the next nucleotide in the chain, synthesis terminates at the incorporation of ddNTP. The cycle continues till cycle for nucleotides have been done. Then, an autoradiogram of the four reactions can be read to yield the linear DNA sequence of all four bases and this would provide information on the presence or absence of genes of interest, TM7SF2 and DHCR7 needed in pcDNA3.1Hygro for this project.

2.7 Mammalian Cell Culture

2.7.1 Cell culture maintenance

All cell culture work was carried out in a laminar flow hood and cell culture sterile disposable plastic materials, manufactured by Sarstedt Incorporated, Fisher scientific, UK used unless otherwise indicated. When sterile glass or other materials were used, these were sterilised in an autoclave for 45 min at 120°C. The host Chinese hamster ovary (CHO) cell line used throughout this study was the industrially relevant CHO-S cell line (ThermoFisher Scientific/Life Technologies). CHO cell lines were regularly maintained in suspension culture in flasks in chemically defined, protein free CD-CHO media (ThermoFisher) at 37°C in a 5% CO_2 atmospheric condition with shaking at 125 rpm. This process of rigorous agitation allows for proper mixing and shearing of cells such that air is uniformly

circulated within the cell cultures thereby allowing mass transfer due to adequate mixture of oxygen, nutrients and heat as this discourages cells clustering which occurs due to inadequate circular air. By shaking at 125 rpm, following protocol, it was observed that cells were not too agitated which may result in collision within cells, collision with vessel walls or with impeller from such turbulence shear forces. Again, inadequate agitation increases viscosity within cells which impacts on cell efficiency. Cells were regularly passaged where spent media was aspirated off and cells passaged at minimal 0.2 x 10^6 viable cells/ml in fresh growth media. CHO-S engineered or variant cell lines, were routinely cultured while ensuring cells were allowed to grow and after 5-6 passages, were suitable for experimental use. Control mechanisms used during cell culture involved spacing flasks in infors to prevent collisions. Also cells continue to shake in flasks during culturing as infors remained on and only cells being cultured under same experimental conditions were used for research. CHO-S cells were grown in serum-free chemically defined CD-media. All engineered CHO-S cells were cultured with antibiotic selection prior to experimentation following the generation of stable cell lines. These stable cell lines generated in CHO-S cells were cultured with 50 µg/ml hygromycin B (Invitrogen) in order to maintain the selection for the gene of interest. Cells were harvested by centrifugation at 1000 rpm in a MSE bench top centrifuge. For cryopreservation cells were prepared in 10% DMSO (Sigma), 90% CD-CHO at a concentration of 1 x 10^7 viable cells/ml. This freezing mixture containing the cells, which had been taken from mid-exponential phase of growth, following the assessment for cell numbers and viability state (from a commercially available ViCell machine by Beckman Coulter), was then aliquoted into 1.8 ml cryopreservation tubes (Nunc, Roskilde, Denmark) in 1 ml aliquots, placed in a polystyrene box and stored at -20°C for 3-5 h then -80°C overnight or stored in a cryo 10°C freezing container (NALGENE, 5100-0001), after which they were transferred to liquid nitrogen storage until required.

2.7.2 Recovery of cells from cryopreservation

Cell revival was achieved by rapidly thawing one vial of frozen cells by placing the tube in a 37°C water bath until completely thawed. The vial contents were then added to 10 ml of appropriate media to wash cells by removing traces of DMSO. The solution was then centrifuged at 1000 rpm for 5 min and the supernatant discarded. The cell pellet was then re-suspended in 10 ml of fresh growth media, put in a culture flask and gassed with 5% CO_2 in air before incubation for 1-2 days at 37°C in an orbital shaking incubator model Infors FIT (Rittergasse, Bottminger) at 100 rpm.

2.7.3 Monitoring of CHO-S cell growth

Prior to experimentation, exact cell numbers to work with, was a necessary requirement for research work. Cells were counted for cell concentration determination and culture viability using an automated ViCell instrument (Beckman Coulter). When using a haemocytometer, the percentage culture viability was calculated by dividing viable cells by total cells of a sample population. Counts were made by analysing the number of cells in four large squares followed by application of the equation:

$$\text{Culture viability (\%)} = \frac{\text{Total no. of living cells} \times 100}{\text{Total no. of cells}}$$

2.8 Generation of Stable CHO-S Cell Pools and lines expressing a Target Gene of Interest

2.8.1 Linearisation of YFP-YM7SF2, YFP-DHCR7, GFP, pcDNA3.1HYGRO plasmids

Prior to the generation of stable cell lines, plasmid constructs were linerarised in order to enhance productive integration. Fsp1 restriction digest enzyme (From New England Biolabs) was used to linearise the plasmids. 40 µg DNA plasmid was used in a 1: 1 ratio of DNA: Restriction Enzyme. Following preparation of DNA with Milli-Q water and appropriate buffer as directed by manufacturer, mixture was placed at 37°C over night as Fsp1 is a known slow reactive enzyme. At 12 h post reaction, sodium acetate washes were done with 100% cold ethanol of appropriate volumes. This follows a series of centrifugation stages at 4°C with the careful removal of supernatant. With a repeat of ethanol wash, the residual fluid is collected using nuclease-free water of 50 µl and allowed to settle on ice for about 1 h before the concentration is noted using Nanodrop analyser.

2.8.2 Determination of optimal selection Antibiotic concentration: THE KILL CURVE

To generate stable cell lines, a first critical step is determining the optimal antibiotic concentration for selecting stable cell colonies. A "Kill curve" was therefore undertaken, which is a dose-response experiment where the cells are subjected to increasing amounts of antibiotic in order to determine the minimum concentration of antibiotic needed to kill all the cells over the course of one week. Cells were plated in 24-well tissue culture plate of 0.5ml growth media/well one day prior to introducing hygromycin B antibiotic selection from Thermofisher Scientific. Cells of confluents between 70-80% were then seeded at $0.2x10^6$ viable cells/ml. Therefore, for 0.5 ml media, cells were seeded at $0.1 x10^6$. Next, 50 mg/ml hygromycin B was added in an increasing amount/volume, into duplicate wells of cells plated in complete media. There was a plate with no hygromycin B added to serve as control. Thereafter, hygromycin B was added into duplicate wells as 0, 250, 500, 750, 1000 ng/ µl. Media containing selection was replaced every 2-3days for up to 14 days. A decision on the optimal dose was made by considering the lowest hygromycin B antibiotic concentration at which all cells were dead after one week of antibiotic selection. The kill curve was also plotted (Figure 2.1). For the purpose of this study, 750 ng/ µl was considered the optimal hygromycin B concentration and was used for this study. It was observed that cells responded to drug at 750 ng/µl concentration with some resistance, tolerance and persistence over a 10 day period and this antibiotic sensitivity of cells appeared to have been retained across culture media with 3 independent experiments unlike the response to 500 ng/µl where cells were observed to be more susceptible to the effect of hygromycin B antibiotic as same pattern of decline was observed from day 2 till day 10.

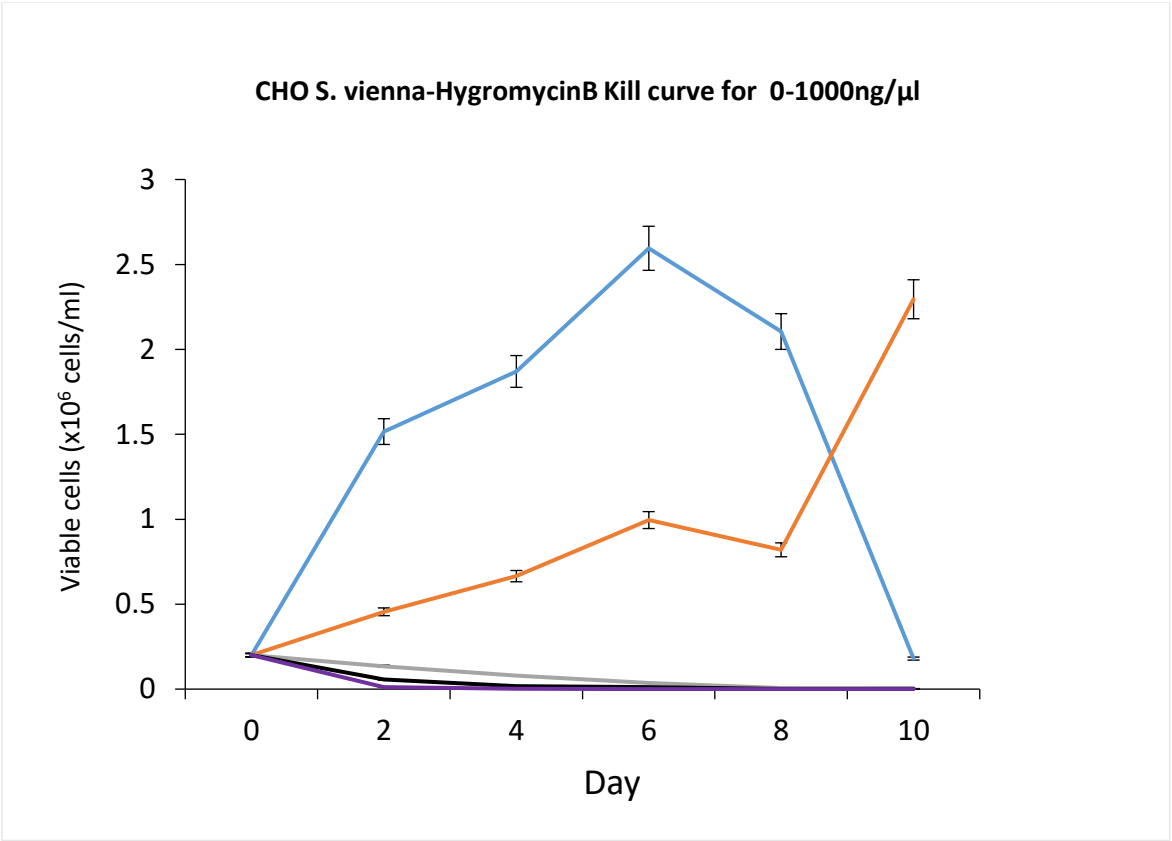

Figure 2.1 Hygromycin B Kill Curve for stable cell line generation. Selected transfected cells were selected using optimal antibiotic concentration, which is the lowest concentration that kills cells with 14 days. Here, 750 ng/ µl was the optimal concentration used for this study. Error bars represent percentage among biological triplicates (where n = 3).

2.8.3 Transfection protocol for stable cell line generation

Stable CHO-S cell pools and lines overexpressing exogenous target genes of interest (YFP-TM7SF2 and YFP-DHCR7) were generated and cultured in the laboratory for research purposes. The target genes were obtained from Monika Zwerger, School of Biological and Biomedical Sciences, University of Durham, South Road, Durham (Zwerger et al., 2010) which had been cloned with YFP at the N-terminal into a Clontech plasmid. The following sub-cloning strategies were carried out which involved releasing the target genes (YFP-TM7SF2 and YFP-DHCR7) from the donor pEYFP-C1 vector into pcDNA3.1Hygro and pcDNA3.1CAT vectors.

For transfection protocol by Thermofisher, cells were initially seeded to be 70-90% confluent. Then a dilution into four, the amount of Lipofectamine® Reagent in Opti-MEM® media was done. 4 µg/DNA plasmid of YFP-TM7SF2 and YFP-DHCR7 was each diluted in Opti-MEM® Media and allowed to mix for 5min. After the incubation of DNA with Opti-MEM® Media, the diluted DNA was subsequently added to the diluted Lipofectamine® 2000 Reagent in a 1:1 ratio. Again, this was allowed to incubate for 5-10 min before the DNA-lipid complex was introduced to cells and allowed to settle at 37°C for 4-6 h before, removing the transfection media and now adding more of cell media and allowed to incubate for 24 h.

Tube1: 300 µl optimum + 14.4 µl lipofectamine 2000 (obtained from Thermofisher Scientific)

Tube2: 300 µl optimum + 4.8 µg plasmid (YFP-TM7SF2 or YFP-DHCR7 respectively)

The tubes were then kept in an upright position for 5 min before mixing tubes 1 and 2 together by gentle pipetting. The mixture was then allowed to remain at room temperature for 20 min before adding 100 µl of final solution/mixture to each well while gently rock to mix. This was followed by incubation at 37°C for 4-6 h. Transfection media was then removed and replaced with fresh pre-warmed (37°C) media (600 µl/well). Cells were usually allowed to grow for a week before monitoring and counting. Usually, cells were moved to T25 flasks with 10 ml fresh media and selection antibiotics (Hygromycin stock 50 mg/ml, Invitrogen). By regular passages, successful stably engineered cells were subsequently cultured in 20 ml cell media at 0.2 x 10^6 viable cells/ml with regular selection antibiotics. For control cell pools, stable CHO-S expressing GFP and pcDNA3.1Hygro were also generated using the same procedure.

2.8.4 Flow Cytometry

Flow cytometer assay was conducted on stable cells using Becton Dickinson FACS Calibur Cell sorter, a multi-laser, bench top flow cytometer which has the ability to acquire parameters for a large number of colours. The technique utilises laser-based technology to count, sort and profile cells in a heterogeneous fluid mixture through the optimisation of the optical and fluidics system so as to maximise the detection of fluorescence and ensure optimal sensitivity and resolution for forward

scatter channel (FSC) and side scatter channel (SSC) signals. Hence, it interrogates cells with fluorescent markers, classifying them into groups. When stable engineered CHO-S cells expressing GFP were analysed along with CHO-S the aim was to investigate the expression of eGFP/YFP stable cells so that clonal cells can be generated from the heterogeneous population. 10 000 cells/1000 µl sample were gated for each sample as stable engineered cells were analysed and compared with GFP-expressing control at 532 nm wavelength. While 10000 cells were acquired, the gating strategy to identify the cells of interest leads to only 1000 cells in the final gate, hence a stringent fluorescent gating cut-off was applied using CHO-S/GFP negative cells as autofluorescence/control with threshold number of events was set at 10^1 mark, below which a frequency is deemed negative. This would allow the Becton Dickinson software discriminate between positive and negative GFP expressing cells in the populations to be sorted.

2.8.5 Limited Diluting Cloning to obtain Clonal Cell Lines

In 96-well plates, cells from a culture of a viability >90% were then placed in well A1, following the protocol from Corning Inc™ as outlined below;

1. Fill the reagent dispensing tray with 12 ml of the appropriate culture media, then using an 8-channel micropipettor add l00 µl median to all the wells in the 96-well plate except well A1 which is left empty.
2. Add 200 µl of the cell suspension to well A1. Then, using a single channel pipettor quickly transfer a 100 µl aliquot from the first well to well B1 and mix by gently pipetting. Using the same tip, these 1:2 dilutions were repeated down the entire column, discarding the original 100 from H1 so that it ends up with the same volume as the wells above it.
3. With the 8-channel micropipettor add an additional l00 µl of media to each well in column 1 (giving a final volume of cells and media of 200 µl /well). Then using the same pipettor quickly transfer l00 µl from the wells in the first column (A1 through H1) to those in the second column (A2 through H2) and mix by gently pipetting.
4. Using the same tips, repeat these 1:2 dilutions across the entire plate, discarding l00 µl from each of the wells in the last column (A12 through H12) so that all the wells end up with 100 µl of cell suspension.

5. Bring the final volume of all the wells to 200 µl by adding 100 µl media to each well.
6. Incubate plate undisturbed at 37°C in a humidified CO_2 incubator.
7. Clones should be detectable by microscopy after 4-5 days and be ready to score after 7 to 10 days, depending on the growth rate of the cells. Check each well and mark all wells that contain just a single colony. Typically the number of single clonal events was around 10 clones per plate.
8. After 1-2 weeks, these clones should have spread out into larger single colonies visible under the microscope. These colonies can then be sub-cultured from the wells into larger vessels. Usually each clone is transferred into a single well in a 12 well or 24-well plate for further growth.

2.9 Transfection of transient DNA into Stable cell lines

Transfection was carried out in several ways depending on the particular experimental strategy used and the scale of transfection required. However, a general procedure used to transfect cells is described here for transfection carried out in the 6 well experimental plate. One day prior to transfection (Day 1), cultures of high viability generally about 95% were seeded at 0.2 x 10^6 viable cells/ml in the appropriate volume of fresh cell media usually in 6 well experimental plate of 2 ml. On the day of transfection (Day 2), the cells were transfected by mixing 300 µl optimum with 14.4 µl lipofectamine 2000 in one tube and another 300 µl optimum with 4.8 µg plasmid in another tube at a ratio of 1:3 for plasmid/lipofectamine2000. After preparation of solutions, tubes were placed upright at room temperature for 5 min after which solutions in both tubes were mixed together by pipetting. The final mixture was left for 20 min before adding 100 µl plasmid mix to each well and the plate rocked gently to mix. The plate was thereafter placed in an incubator at 37°C for 4-6 h. After incubation, transfection media was replaced with fresh pre-warmed (37°C) media (600 µl /well).

2.10 Agarose DNA Gel Electrophoresis

Preparation of agarose gels was achieved by dissolving agarose powder in 1x TAE buffer to a final concentration of 1% (w/v) gel. This mixture was heated and allowed to cool to approximately 55°C before ethidium bromide or SYBR® Safe (0.1 µg /ml final concentration) was added to the liquid agarose. The mixture was poured into a prepared horizontal gel tank. Electrophoresis was performed with Tris/Borate/EDTA (1x TBE buffer), sufficient to cover the gel. Samples were mixed with 5x sample loading buffer (Promega) and 10 µl of the mixture was loaded into wells on the gel, with a size marker (Hyperladder™ I or V) for comparison. Before being loaded into the gel, 1xloading dye (Promega) was added into each sample and a Quantitative DNA (Promega) or RNA ladder (New England Biolab). An electrical current was applied to the gel tank at 100 V (Bio-Rad) until nucleic acids were appropriately separated. The gels were then visualised under ultraviolet light and recorded the images using a VersaDoc 3000 image system.

2.10.1 Gel extraction and purification

Agarose gels containing PCR products or DNA sequences/inserts release by restriction digest from plasmids were placed under UV light (minimising the time under the UV) to select the bands of interest and isolate them using a sterile disposable scalpel. DNA bands were visualized using a Transiluminator Bio view UV lamp (Biostep) in a Gel logic 100 imaging system (Kodak). Isolated bands from gels were transferred into new tubes and purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. After subjecting the gel to a clean-up using Wizard® PCR Clean-up System kit by Promega, to recover the DNA, the material was used for ligations or it may be kept for further use.

2.11 Protein Extraction and Analysis

2.11.1 Lysis buffer preparation

Mammalian cells were lysed in a buffer consisting of 200 mM HEPES-NaOH pH 7.2, 100 mM NaCl, 1% v/v TX 100 and 10 mM Na-β-glycerophopate. Before use a complete mini, EDTA free protease inhibitor tablet (Roche) was added to 10 ml of the lysis buffer as well as 50 mM NaF and 1 mM NaV (Na-Vanidate) (New England Biolabs).

2.11.2 Protein extraction from mammalian cells

Protein from cells cultured in 6 well plates were extracted by washing the cells in 1 ml of PBS for each well and then the cells were incubated in 200 µl of cell lysis buffer per well on ice for 5 min. The wells were then scraped and the contents put into a sterile 1.5 ml eppendorf tube. An additional 200 µl cell lysis buffer per well was used to wash each well and this was then collected and placed into the corresponding eppendorf tube so that a combined 400 µl of cell lysate was collected per 6-well plate. This was then stored at -20°C until analysed.

2.11.3 Bradford assay quantification of total protein concentration in cell lysates

Protein quantification of cell lysates was determined using a Bradford assay with each lysate being measured in duplicate. Total cell lysates were centrifuged for 1 min at 13000 rpm and then kept on ice. Following the centrifugation step, supernatant was carefully pipetted into a new eppendorf tube as lysate supernatant hence separated from the cell pellet (which was discarded). 5 µl of the lysate supernatant was then diluted in 45 µl with ddH_2O and then 1 ml of Bradford reagent (BioRad) was added to the solution for protein estimation. The Bradford solution was then mixed and incubated for 10 min. The total protein was then quantified by reading the absorption of the solution at a wavelength of 600 nm using an eppendorf biophotometer. The concentration of protein in each lysate was then calculated by comparison to a standard curve generated using known amounts of bovine serum albumin (BSA).

2.11.4 SDS-PAGE Analysis of Protein Extracts

SDS-Polyacrylamide gels were routinely used for protein analysis. SDS page gels were prepared using *www.changebioscience.com/calculator/sdspc.htm* for dilution calculations; 30% 29:1 polyacrylamide (BioRad), ammoniumpersulphate and tetramethlelthylenediaine (TEMED), all sourced from BioRad. SDS gels were prepared in Novex 1 mm gel cassettes (Invitrogen). Protein lysate samples for analysis were diluted in NuPAGE LDS sample buffer x4 (Invitrogen) to the volume appropriate for the well size used or concentration of protein required (typically 5-20 µg/well) and number of runs per sample (well volume (µl) x number of runs = total volume of sample). Prior to loading, samples were boiled for 5 min at 85-95°C, then loaded into the SDS gel for analysis. Gels

were assembled in a Novex mini cell tank (Invitrogen) and loaded with the prepared samples and electrophoresis undertaken at 125 V for 1 h and 30 min or until the dye front of the samples had reached the bottom of the gel. Gels were removed from the gel cassettes and both stained with Coomassie blue R-250 for 20-60 min and then destained until protein bands were visualised or prepared for transfer onto nitrocellulose for western blot analysis.

2.11.5 Western Blot Analysis of Proteins

Proteins separated by SDS-PAGE were transferred to 0.45 µm nitrocellulose blotting membrane (Amersham Hybond ECL) via electrophoresis using a Tris EDTA (T.E) series transfer electrophoresis unit (Hoefer) for 1 h at 4°C. After transfer, the blots were incubated in 20-30 ml of blocking buffer (5% dry milk (Marvel) in tris-buffered saline with 0.1% tween 20 (0.1% TBST) for 30 min-1h. Blots were then washed in 0.1% TBST three times for 5 min per wash, then incubated overnight in a 10 ml dilution of the appropriate primary antibodies (section 2.14.1) prepared in 3% BSA-TBS. After overnight incubation, the blots were washed 3 times with 0.1% TBST for 10 min and then incubated in the eGFP primary antibody with Anti mouse 1:5000 (from sigma) for YFP detection dilution in blocking buffer for 1 h. Blots were then washed again 3 times with 0.1% TBST for 10 min and incubated for 5 min in ECL western blotting detection reagents (Amersham), following the instructions provided with the reagents by the manufacturer. The blots were then exposed to high performance chemiluminescence film (Amersham Hyperfilm ECL), which was subsequently processed using a Compact X4 processor (Xograph imaging systems).

2.11.5.1 Coomassie Brilliant blue staining/destaining of SDS-PAGE gels

For staining with coomassie, the gel was carefully transferred to a staining box and rinsed with deionised water. Enough staining solution was poured in to the staining box to cover the gel and then kept rocking position for 1-2 h or overnight to stain the gel. For destaining, the gel was rinsed with de-ionized water after discarding the used staining solution. The gel was destained in destaining solution (I) for 1-2 h and then destaining solution (II) until the gel bands became clear. Table 2.3 shows the composition of some reagents used to prepare the coomassie brilliant blue stain.

Table 2.3 Table showing composition of reagents for preparation of gel, washing, staining and destaining gel.

Reagent	Composition
Coomassie Blue Stain	0.25% (w/v) Coomassie Blue R-250 20% (v/v) isopropanol
Coomassie Blue Destain	7% (v/v) acetic acid (4x)
Separating Buffer for SDS-PAGE:	1 M Tris-HCl pH 8.8 (4x)
Stacking buffer for SDS-PAGE	1.5 M Tris- HCl pH 6.8
(10x) running buffer for SDS-PAGE:	0.25 M Tris, 1.9 M glycine 1% (w/v) SDS
(2x) Loading buffer:	25% (V/V) 4x stacking buffer 20% (v/v) glycerol 0.5% (w/v) Bromophenol Blue 4% (w/v) SDS 10% (v/v) 2-mercaptoethanol
Blocking solution for western blot (1X)	0.1% (v/v) TBST 5% (w/v) dry milk (Marvel)
Washing solution for western blot:	0.1% (v/v) TBST

2.12 PCR Amplification of Target Genes

2.12.1 RNA Extraction from Mammalian cells

Engineered CHO-S cell total RNA lysates were analysed from $2x10^6$cells. Each sample was firstly homogenized in a Lysing Matrix D tube (FastPrep® Kits) with 1 ml of Tri-regent (Sigma) using FastPrep® instrument for 20 sec at speed setting of 4.0. The homogenised sample was then centrifuged at x 12,000 g for 5 min at 4°C and the aqueous upper phase transferred into a new tube. An extra 5 min incubation at room temperature for 5 min was then undertaken to allow complete dissociation of nucleoprotein complexes. RNA was further separated from DNA by adding 300 µl of chloroform, vortexing for 10 sec, and incubating for 5 min at room temperature before centrifuging the mixture at 12,000 g for 15 min at 4°C. Following the centrifugation, the mixture was separated into an upper aqueous phase (colourless), an interface phase (white), and a lower phenol-chloroform phase (red). The aqueous phase of mixture was transferred into a new 2 ml tube for further RNA precipitation. 500 µl of pre-cooled 100 % (v/v) ethanol (Sigma) was then added and incubated for at least 1 h at -20°C before centrifuging at 12000 g for 5 min at 4°C. Following the centrifugation, a white RNA pellet was formed in the bottom of the tube. The supernatant was discarded and 750 µl of pre-cooled 75 % (v/v) ethanol (Sigma) added to wash and resuspend the pellet. To suspend the RNA centrifuged at 12000 g for 5 min at 4°C, the supernatant was discarded and the pellet air-dried at room temperature for 5-10 min. The pellet was finally dissolved with 30-50 µl of RNase free water and incubated at 50°C for 10 min or until the pellet resuspended completely. To remove potential genomic DNA all RNA samples were further treated with DNase using TURBO DNA-free™ Kit (Ambion) according to manufacturer's instruction. All RNA samples were stored at -80 °C until use. The total RNA extracted from the lysate using a QIAGEN Shredder kit and the amount of RNA extracted determined by A260 nm measurement. Total RNA was stored at -80°C.

2.12.2 DNase I Treatment of RNA Isolates

The amount of RNA in each sample was quantified using a Nano Drop® ND-1000 UV/Vis spectrophotometry. Using the DNase I Kit (Sigma-Aldrich). RNA was treated with DNase I to remove any genomic DNA contamination. All the reagents were supplied with the kit unless otherwise stated. DNase treatment was carried out by adding 1 µl of RNA (1 ug/µl), 7 µl of 0.05% (v/v) DEPC-

treated ddH_2O, 1 µl DNase (10x) reaction buffer and 1 µl of DNase enzyme. The reaction was incubated at room temperature for 15 min prior to addition of 1 µl of 0.2 mM EDTA stop solution. The mixture was then heated at 70°C for 10 min and chilled on ice for 2 min prior to use (with storage at -80°C).

2.12.3 Assessment of total RNA quality

A NanoDropTM 1000 spectrophotometer (Thermo Scientific) was used to directly quantify 1 µl of RNA sample. The purity of all RNA samples were confirmed by having the optimal ratio of absorbance at 260 nm/280 nm (1.9 - 2.3) and 260 nm: 230 nm (>2.2).

2.12.4 Quantitative PCR Analysis (qRT-PCR) of Target mRNA Amounts

qRT-PCR analysis was carried out to determine the relative amount of target mRNAs (relative to a house keeping mRNA) between samples was undertaken using the commercially available iScript TM One-Step RT-PCR kit with SYBR Green kit (BioRad) and an Eppendorf Realplex Master Cycler instrument. GFP primers were used for mRNA assay of samples and were designed using Primer Blast (available from: www.ncbi.nlm.nih.gov/tools/primer-blast). RNA samples were diluted to a concentration of 25 ng/µl in sterile nuclease free water (sNF). Separate master mixes (25 µl per a reaction) for each experimental and housekeeping gene primer pairs were prepared on ice under sterile conditions. Table 2.4 shows the reaction mixture volumes used for the qRT-PCR analysis.

Table 2.4 Reaction mixture volumes for qRT-PCR analysis

Reagent	Volume (µl) x1 RT reaction
Sodium Nitrate flotation (sNF)	8.5
2x SYBR Green	12.8
Forward Primers (10 µM)	0.75
Reverse Primers (10 µM)	0.75
Reverse transcriptase	0.5
Template RNA	2

25 µl per reaction of the master mixes were then pipetted into a Bio Rad 96-well optical plate on ice. The plate was then sealed and placed in the Eppendorf Realplex Master Cycler (Thermocycler) and the following program used:

- 50°C for 10 min
- 95°C for 5 min
- 40 cycles of
- 95°C for 10 min
- 55°C for 30 sec, SYBR emission reading
- 95°C for 15 sec
- 55°C for 15 sec
- Melting curve: 55°C to 95°C with 0.5°C
- 95°C for 15 sec

The Eppendorf instrument excites the fluorescent dye used and the software calculates when the fluorescence reaches a critical threshold (C_T). The C_T value is that when the fluorescence becomes detectable above the background, the C_T value is inversely proportional to the logarithm of the

initial copies of template DNA (Livak and Schmittgen 2001). Calculations using the C_T values of the sample were normalised to that of the housekeeping gene (the endogenous control GFP). This comparative method follows the comparative C_T method equation shown below:

$$\text{Relative Expression} = 2^{-\Delta\Delta C_T}$$

2.12.5 Analysis of qRT-PCR Results

The qRT-PCR data was analysed using Opticon Monitor v3.0 software. Blanks and baseline fluorescence (as calculated as an average over cycle range 1 – 10) were subtracted from fluorescent plots. The threshold setting was raised to 0.05 and the cycle C(t) value at which samples reached this fluorescence value was measured. A standard curve graph of log (cDNA) value versus C(t) value was plotted. The relative concentration of cDNA was achieved from the standard curve. The total amount of mRNA was normalised against GFP qPCR-value. The melting curve was used to assess the quality of the amplified product, where single peak at 80°C to 90°C reflected a pure product, whereas double peaks would reflect primers dimers and impurities.

2.13 Fluorescence imaging of live cells expressing YFP-TM7SF2 or YFP-DHCR7

For live cell imaging, mammalian cells were seeded into glass bottom petri dishes, with coverslips that have a collagen coating at 2.5 x 10^5 viable cells in 2.5 ml of media per dish. Cells were left overnight to adhere, then transfected with the appropriate gene of interest using the same transfection protocol as described for 6 well plate transfections. The plates were then incubated for 24 h then imaged using a temperature controlled LEICA DMIRE2 confocal microscope (TCSSP2 AOBS).

2.14 Immunofluorescence imaging of fixed CHO cells

For immunofluorescence imaging of cells, prior to seeding cells before transfection, coverslips were sterilised and placed 1 per well in 24 well plates. Cells were then seeded at $6x10^4$ viable cells per well and incubated for 24 h. 0.8 µg of the selected DNA plasmid was then added per well in triplicate wells using the 24 well plate transfection protocol (section 2.11) and incubated for 24 h. Cells were then prepared according to the following protocol:

Paraformaldehyde (PFM) fixing of cells

Cells were required to be fixed to the cover slips within each well:

1. Siphon media from wells
2. Wash the cells with 1 ml warm PBS (37°C)
3. Siphon off PBS
4. Add 1 ml 4% PFM (-20°C)
5. Incubate the plate for 15 min on ice
6. Siphon off PFM and air dry (about 5 min)
7. Wash cover slips with PBS
8. Keep plates at 4°C (for up to 1 week)

2.14.1 Antibody probing of the coverslips

Once the cells were fixed to the coverslips, the slips were probed using antibodies to visualise specific targets. This was achieved using the following protocol:

1. PFM fixed cells were rehydrated with 1 ml of PBS (room temperature, RT) for 5 min
2. PBS was then siphoned off.
3. The coverslips were then incubated with 250 µl blocking buffer w/v sterile-filtered 3% BSA in PBS per well for 15 min at room temperature
4. 25 µl droplets of 1° antibody (diluted 1:500), anti-Rabbit (sigma) in w/v sterile-filtered 3% BSA were placed on a sheet of parafilm in a damp box and the slips applied 'cell side' down. The slides were incubated overnight at 4°C in the damp box.
5. 4 sets of 100 µl PBS with Tween wash droplets were prepared on sheets of parafilm.
6. Coverslips were then transferred from the damp box to the 1st wash droplet (being dipped in PBS and 0.1% Tween between 1st antibody and 1st wash droplet). The

coverslips were then transferred 'cell side' down through the wash droplets, allowing 5 min per wash.

7. Whilst the coverslips were on the 1st droplet the 2° antibody was prepared: TRITC antibody (sigma) was diluted 1:100 in BSA dilutions prepared in light aggregates; the 'supernatant' was then transferred to a fresh light protected tube.
8. 25 µl droplets of 2° antibody were then aliquoted on to parafilm in a light protected damp box. Cover slips were transferred cells side down onto the 20 antibody droplets; blotting off any excess liquid from previous washes.
9. The coverslips were then incubated in the dark for 2 h at RT.
10. The coverslips were then washed again in 100 µl PBS with 0.1% Tween as they were after incubation with the 1° antibody.

2.14.2 Nuclear staining of DNA

1. 25 µl droplets of DAPI (10 mg/ml) were prepared on parafilm in a light protected box.
2. Coverslips were then placed cell side down on the DAPI droplets and incubated for 1 min.
3. Coverslips were then washed twice for 10 min in 100 µl PBS droplets, prepared on parafilm.

2.14.3 Anti-fading and preparation of coverslips for imaging

1. During the washing process, either after incubation of 2° antibody or post staining with DAPI, 900 µl of Mowiol was mixed with 100 µl of phendinediamine.
2. 5 µl of the Mowiol/Phendinediamine mixture was then dripped onto a microscope slide and the cover slip applied on top of the 5 µl cell side down.
3. Cover slips were then left at 4°C overnight to set in a light protected box.

2.14.4 Coverslip preparation for imaging

1. The edges around coverslips were sealed with clear nail varnish and the top of the cover slip washed with ddH_2O and a cotton bud.
2. Cells were then imaged on a LEICA LEITZ DMRD or a LEICA DMIREZ confocal microscope (TCSSP2 AOBS).
3. Wavelengths of excitation and emission of each colour visualised are shown in table 2.5.

Table 2.5 Wavelengths used for excitation and emission of colours visualised during immunofluorescence studies

EXCITATION WAVELENGTH (nm)	EMISSION WAVELENGTH (nm)
340-380 (Blue)	LP 425 (Blue)
450-490 (Blue)	LP 515 (Green)
515-560 (Green)	LP 590 (Red)

2.15 Statistical Analysis

All assays were repeated at least three times to ensure reproducibility and three replicates of each sample were performed in each test. Error bars represent standard of the mean (SEM) (which relates the closeness of the sample mean to the population mean) for triplicate biological samples. Statistical significance of differences for triplicate samples/groups was evaluated by students T-Test with Probability value significant at P value < 0.05.

Chapter 3

Isolation, Cloning and Characterisation of Transmembrane 7 Superfamily member 2 (*TM7SF2*) and 7-dehydrocholesterol reductase (DHCR7) Genes and Expression

3.1 Introduction

Research on the role of Transmembrane 7 Superfamily member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7) in the ER and peri nuclear space expansion has gained momentum in recent years. We have explored the possibility of expanding the ER membrane and reprogramming of lipid biosynthesis by over-expressing TM7SF2 and DHCR7. The different strategies used to isolate, clone, and characterise YFP tagged *TM7SF2* and *DHCR7* from plasmid vectors in which they were provided (a eYFP-C1 plasmid vector backbone) into the commercial mammalian expression vectors pcDNA3.1CAT and pcDNA3.1Hygro for transient and stable expressions in Chinese hamster ovary (CHO) cells have been detailed in this study.

This was undertaken in order to attempt to manipulate lipid biosynthesis and expansion of the endoplasmic reticulum for increased secretory pathway capacity and enhanced culture life (growth, viability and phenotypic characteristics) as described in the introduction chapter of this thesis. As the biosynthesis of cholesterol represents one of the fundamental cellular metabolic processes (Bietz et al, 2017), we have investigated the manipulation of this pathway by overexpression of TM7SF2 in CHO-S and the feedback effects on secretory pathway capacity and ER.

The cloning and characterisation of the TM7SF2 and DHCR7 genes in CHO cells provides yet another example of how basic research in mammalian cells can dramatically advance the understanding of over-expression engineering in the secretory systems. Zwerger et al., (2010) had reported on a peculiar comparable phenotype induced in different mammalian cell lines observed to impact upon the regular organisation of the nuclear envelope and the endoplasmic reticulum (ER).

Indeed, the exogenous expression of these appears to result in an expansion of the ER and thus potentially the ER capacity. An increased ER capacity could be useful for the enhanced expression

and quality of bio therapeutic proteins from mammalian cells, however whether the ER can be expanded in such cells using this approach, and whether this results in enhanced secretory recombinant protein expression has not been elucidated.

Because abnormal protein accumulation can be toxic to cells, it should be of no surprise that mammalian cells have evolved an elaborate system to monitor the status of protein folding in the ER and eliminate misfolded proteins when they accumulate (Romero & Summer, 2017). Proper regulation of protein homeostasis in a cell is critical for the health of the organism. Proteins, intracellular or secreted, need to be produced and maintained at the right quantity, folded into their three dimensional conformation with necessary post-translational modifications, targeted to their correct destinations to insure their optimal function, and degraded efficiently and disposed when needed (Yalcin & Hotamisligil, 2013).

Upon detection of an unfolded protein load in the ER, an ER-nuclear signalling pathway is activated to protect the cell from the cytotoxic effects resulting from the accumulation of misfolded proteins subsequently leading to a disruption of homeostasis which in turn triggers stress responses, or unfolded protein responses (UPR), in these organelles (Bernales et al., 2012). The folding process is not completely accurate. In mammals, 30% of all newly synthesised proteins are estimated to be incorrectly folded. In order to improve recombinant protein production, it has been suggested that an optimum expression level exists for any foreign protein, which may be due to saturation of protein folding capacity of the ER as the maximum productivity is determined by the capacity of the ER to express the protein when this is saturated.

However, even though in a specialised secretory cell unfolded proteins do trigger the UPR to increase folding capacity, this signal is not necessary for every step of ER expansion (Christis et al., 2009). Recombinant protein production can also be improved through genetic manipulation. The overexpression of an individual gene in a host cell line can often achieve the effect of amplifying its activity, although it should be noted that the effect of gene overexpression is not always predictable or beneficial. A number of gene manipulation strategies have been published with the aim of increasing the productivity of an exogenous protein from a host cell line e.g. see (Stoyle et al., 2017). Many of these genes are involved in certain functions associated with the endoplasmic reticulum (ER) such as, phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (PERK) Cnop et

al., 2017), protein translocation and folding in the ER (ATF6) **(**Gardner et al., 2013), ER stress sensor and cell fate executor (IRE1) (Chen & Brandizzi, 2013), critical effector of the mammalian UPR whose activity is regulated by IRE1 (XBP1) (Acosta-alvear et al., 2007), among others. Different situations that induce ER stress also lead to autophagy induction.

As discussed above, the ER stress response is activated to protect cells from different alterations affecting this organelle. However, when the intensity or duration of ER damage cannot be restored by this response, ER stress can also lead to cell death (Verfaillie et al., 2010). It is possible to adapt the target protein and host organism to prevent ER stress; a protein that correctly folds more readily is less likely to cause stress to the host and the benefits of producing a host more able to withstand stress is obvious (Wyre, 2014). N-terminal fusions have been shown to be more effective for *in vivo* folding, as a correct initial folding of the fusion partner can impact positively on the folding of the recombinant protein products.

This chapter describes an alternative approach to enhancing mammalian cell expression systems for the expression of recombinant proteins, specifically the expansion of the ER by manipulation of two targets of lipid biosynthesis; TM7SF2 and DHCR7 (which are both predicted to reside in the cytoplasm and are not believed to be in prolonged direct physical contact with chromatin (Holmer et al., 1998). These were to be exogenously overexpressed in order to manipulate the secretory pathway capacity by causing an expansion of the ER which may cause an improved quantity and quality in the output of recombinant materials of the cell and also may impact on growth. Such an approach of over-expression of target proteins can lead to cellular expansion as shown when high abundance of transmembrane proteins in the ER were shown to induce extensive membrane growth, whereas low expression did not alter ER morphology (Borgese et al., 2006).

Our hypothesis therefore was that controlled manipulation of cholesterol biosynthesis by over-expression of TM7SF2 and DHCR7 will result in an enhancement of the efficiency of the CHO platform as a recombinant protein expression system via expansion of the endoplasmic reticulum (ER) capacity of the cell. This chapter reports on the isolation, cloning, characterisation and engineering of the CHO cell using these lipid biosynthesis targets where overexpression of these proteins are used to drive increased secretion of difficult-to-express recombinant proteins

(described in chapters 4 and 5). Preliminary studies on LBR mutants of this were also undertaken with an mCherry tag at the N-terminal, along with the YFP-TM7SF2 and YFP-DHCR7.

3.2 Results

3.2 Vector Engineering

3.2.1 Cloning strategies for the insertion of mCherry-LBR, YFP-DHCR7 and YFP-TM7SF2 genes into pcDNA3.1CAT and pcDNA3.1Hygro Mammalian Expression Vectors

Samples of mCherry-LBR, YFP-DHCR7 and YFP-TM7SF2 constructs were provided by Monika Zwerger (University of Durham, South Road, Durham). Information on clonings confirmed Lamin B Receptor plasmid was amplified and cloned into pEYFP-C1 using BspEI/EcoRI double restriction sites. The human DHCR7 was amplified and cloned into plasmid pEYFP-C1 using restriction digestion sites; BspEI/SalI while YFP-TM7SF2 plasmid construct, containing amplified coding sequence of human TM7SF2 had been cloned into pEYFP-C1 using BspEI/SalI or with a stop codon in pEGFP-N1 via BglII/SalI restriction sites. Initial cloning strategies involved removing the genes of interest (mCherry-LBR, YFP-DHCR7 and YFP-TM7SF2) from plasmid EYFP-C1, using restriction digestion followed by the introduction of these inserts into pcDNA3.1CAT and pcDNA3.1Hygro vector plasmids.

The ultimate aim of this work was to generate constructs for the expression of the target proteins into plasmid vectors; 3.1CAT and 3.1Hygro for transient gene expression studies and stable cell line generation of these targets in CHO mammalian cells, for studies on the impact of manipulating these lipid engineered cells on the CHO ER membrane and also on bio therapeutic recombinant protein expression. pcDNA3.1CAT plasmid with Chloramphenicol Acetyltransferase (CAT) at the multiple cloning site with a size of 660bp was to be released following the double restriction digestion of Nhe1/Xba1 and Nhe1/Apa1 for pcDNA3.1CAT-mcherry-LBR and pcDNA3.1CAT-YFP-DHCR7 respectively although that wasn't the case for pcDNA3.1CAT-YFP-TM7SF2 (Figure 3.1). All transient expression studies for construct plasmids of YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR were carried out using pcDNA3.1CAT while pcDNA3.1Hygro vector was used for the stable cell line expression of these lipid target genes.

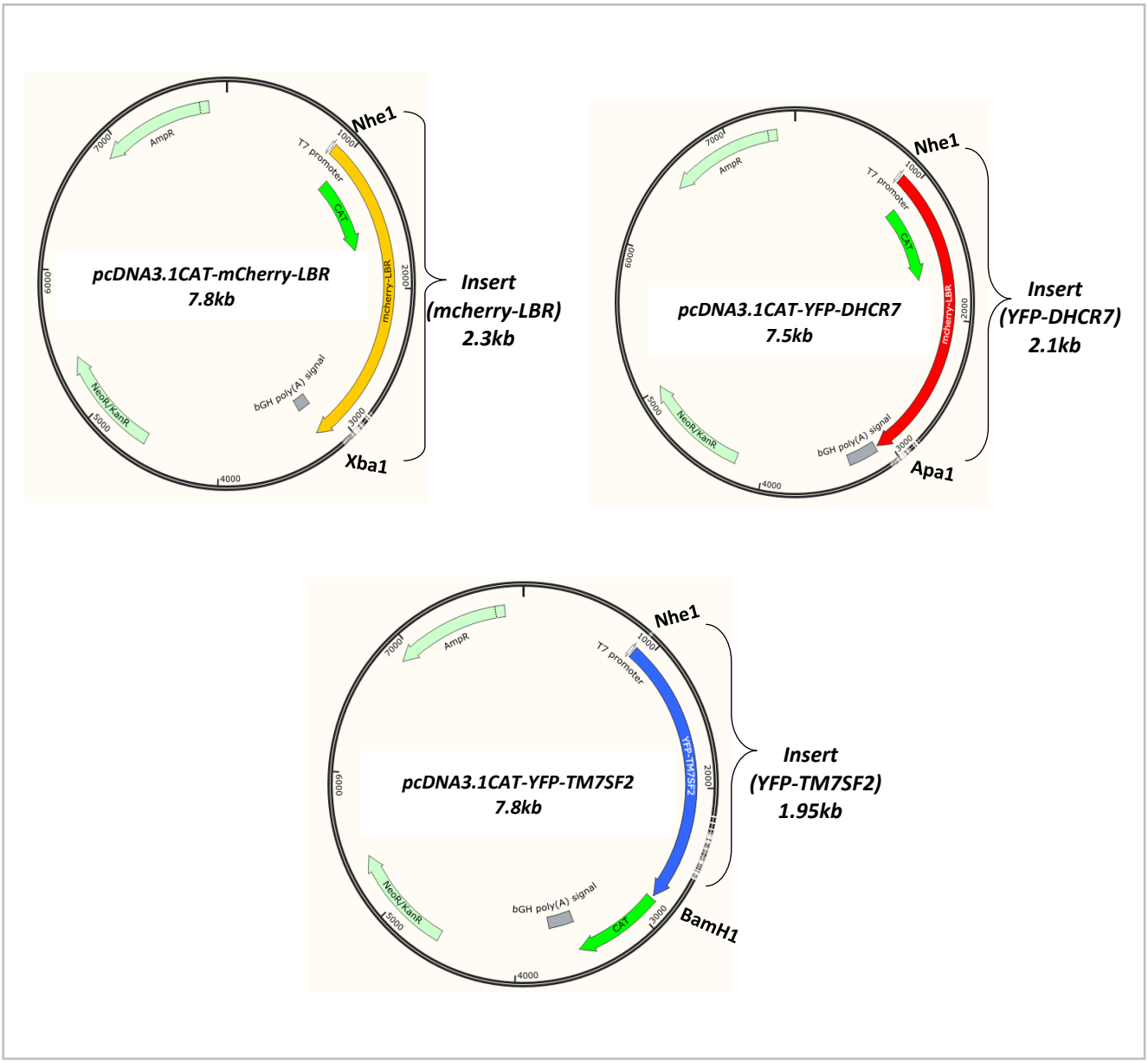

Figure 3.1 pcDNA3.1CAT plasmid vector containing the mCherry-LBR, YFP-DHCR7 and YFP-TM7SF2 genes. The respective genes were cloned from pEYFP-C1 plasmid vector using restriction digest into plasmid vector pcDNA3.1CAT. The restriction sites used are indicated in the vector maps.

When pcDNA3.1CAT constructs were generated, the expectation was to undertake transient studies such that, the effect of over-expression of the lipid engineered genes on recombinant protein

expression can be monitored over a course of time. In order to investigate the impact of overexpression, under stable conditions, the generation of stable cell lines was imminent. Figure 3.2, shows the constructed plasmid map of the lipid engineered genes in pcDNA3.1Hygro, which serve as a 'vehicle' for protein expression in CHO cells.

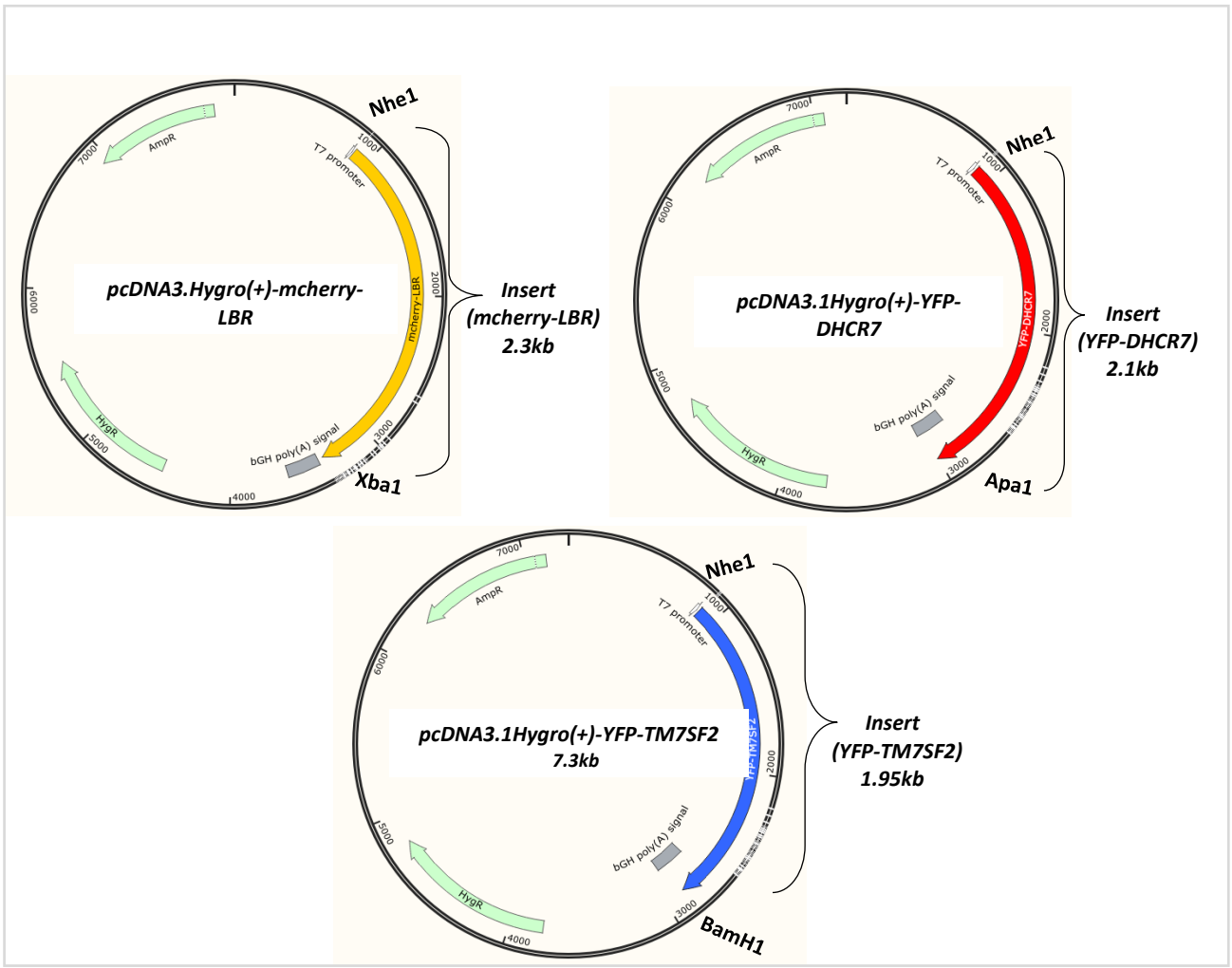

Figure 3.2 Schematic displaying the principle of the sub-cloning strategy. mCherry-LBR (yellow), YFP-DHCR7 (red) and YFP-TM7SF2 (blue) genes were cloned from pEYFP-C1 plasmid vector using restriction digest into plasmid vector pcDNA3.1Hygro. The restriction sites used are indicated in the vector maps. Constructs were generated for stable cell generation.

The human YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR were cloned out of pEYFP-C1 using restriction digest of enzymes on the restriction site to release the genes of interest. The predicted TM7SF2 protein has 418 amino acid residues in length and a molecular mass of about 46.4 kDa. The DHCR7 and LBR

proteins consist of 475 and 533 amino acids with the molecular mass of both proteins, 54 kDa and 70 kDa respectively (Table 3.1). The expected size of YFP-TM7SF2 was cloned out using Nhe1/BamH1 restriction sites which releases a 1.95kb fragment containing the YFP reporter gene tag. YFP-DHCR7 was cloned out using Nhe1/Apa1 with a released fragment of 2.1kb, having the YFP reporter gene tag. Also, mCherry-LBR was cloned out of pEYFP-C1 plasmid using Nhe1/Xba1 (Figure 3.3).

Confirmation of the YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR inserts being released was done using gel electrophoresis. Plasmid vectors pcDNA3.1CAT and pcDNA3.1Hygro were also digested each with restriction digest enzymes Nhe1/Xba1, Nhe1/Apa1 and Nhe1/BamH1. A single band size of 6.2 kb was seen on gel electrophoresis image (Figure 3.4) for plasmid vector pcDNA3.1CAT for each of double digest cut while pcDNA3.1Hygro showed a single band size of about 5.6 kb for each of the three double restriction digest. These fragments of interest were excised both for insert and vectors.

Table 3.1: Predicted Sizes for YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR constructs and genes.

Gene	Amino Acids	Total Nucleotides	Reporter (bp)	Total Insert (bp)	Total Plasmid (bp)
TM7SF2	418	1254	YFP (720)	1974	5954
DHCR7	475	1425	YFP (720)	2145	6125
LBR	533	1599	Mcherry (711)	2310	6299

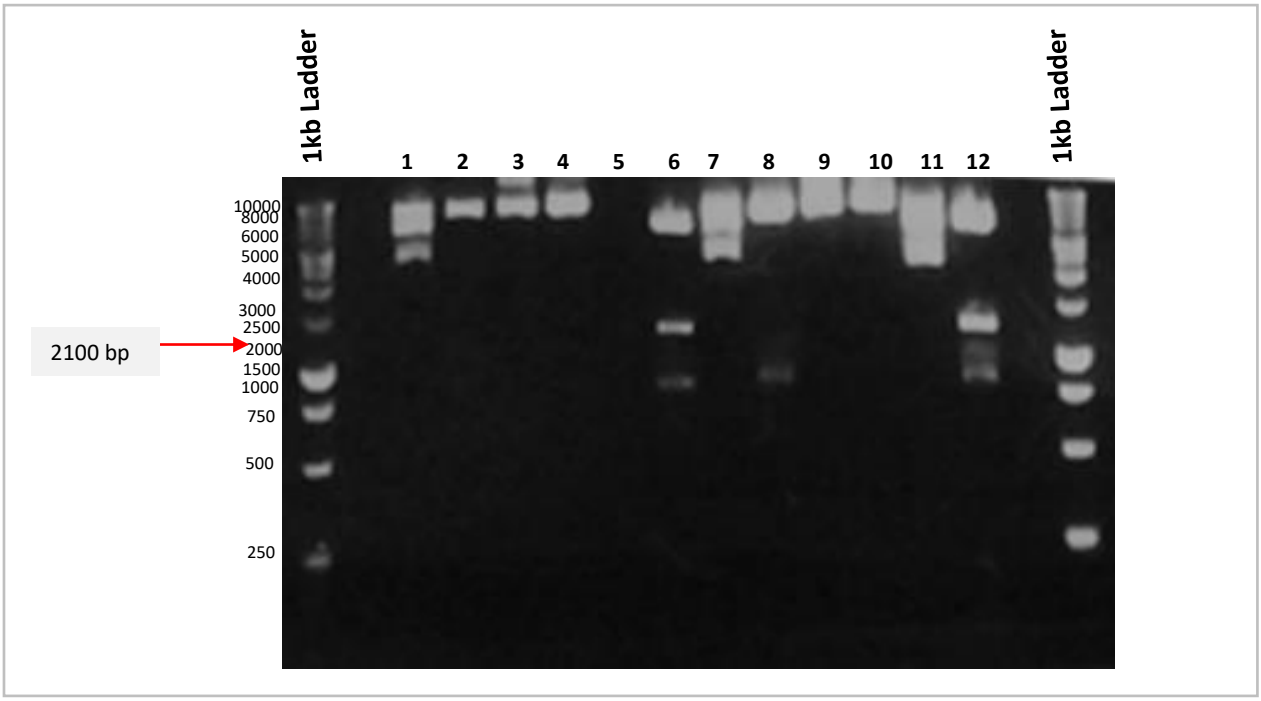

Figure 3.3. 1% Agarose Gel Electrophoresis showing restriction double digest check for two different minipreps (A and B) of mCherry-LBR, YFP-DHCR7 and YFP-TM7SF2 loaded on lanes 1-6 and 7-12 for the enzymes that will release the genes of interest. Nhe1/Xba1 for mCherry-LBR (A) (Lane 1), Nhe1/Xba1 for YFP-DHCR7(A) (Lane 2), Nhe1/Xba1 for YFP-TM7SF2 (A)(Lane 3), Nhe1/Apa1 for mCherry-LBR(A) (Lane 4) , Nhe1/Apa1 for YFP-TM7SF2 (A) (Lane 5), Nhe1/Apa1 for YFP-DHCR7 (A)(Lane 6), Nhe1/Xba1 for mCherry-LBR (B) (Lane 7), Nhe1/Xba1 for YFP-DHCR7(B) (Lane 8), Nhe1/Xba1 for YFP-TM7SF2 (B)(Lane 9), Nhe1/Apa1 for mCherry-LBR (B) (Lane 10) , Nhe1/Apa1 for YFP-TM7SF2 (B) (Lane 11), Nhe1/Apa1 for YFP-DHCR7 (B) (Lane 12) to release the gene of interest. Here, lanes 6 and 12 show YFP-DHCR7 released at 2100 bp with Nhe1/Apa1.

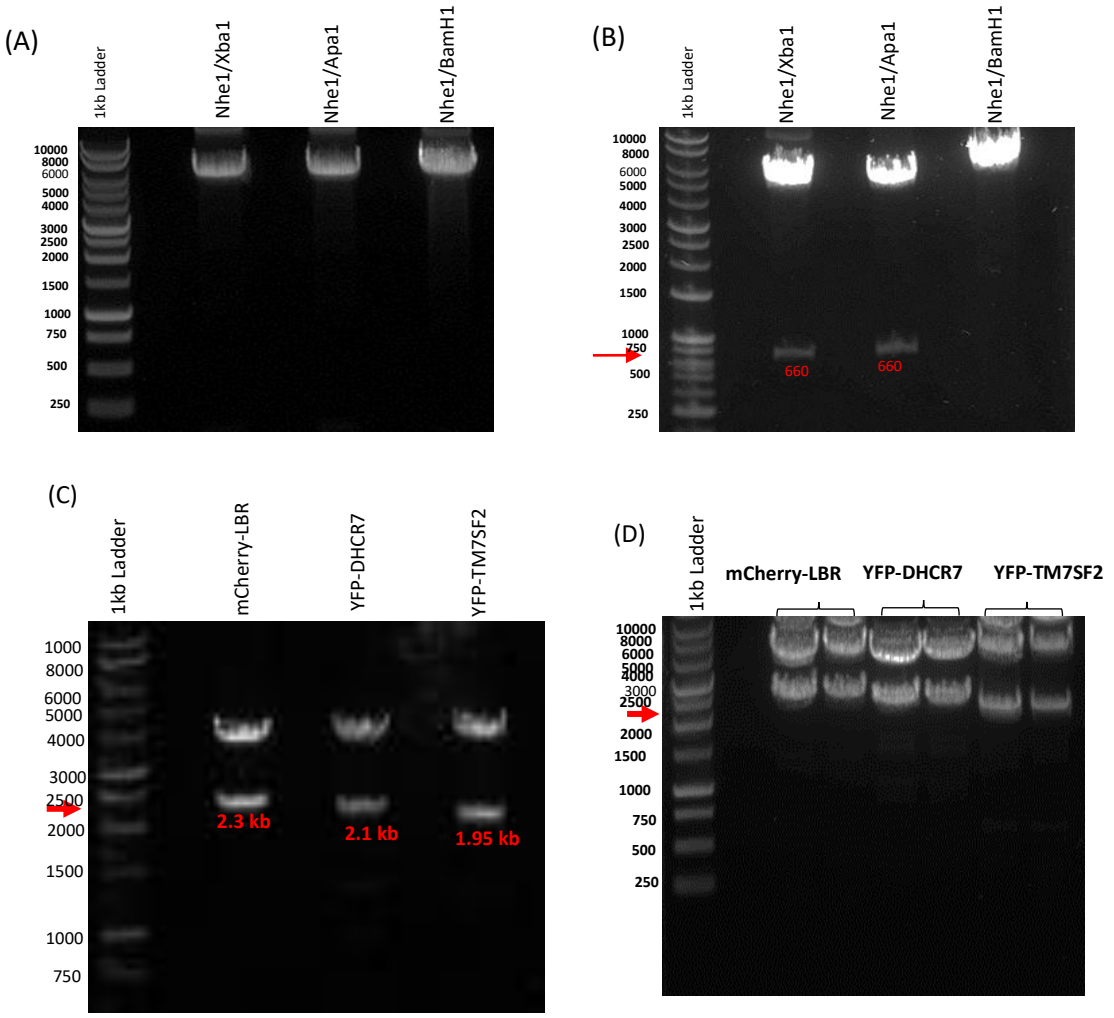

Figure 3.4. Agarose gel electrophoresis analysis of restriction double digest of plasmid vector pEYFP-C1 to release the genes of interest. mcherry-LBR is released at the expected size of **2.3 kb** using Nhe1/Xba1, YFP-DHCR7 was released as a **2.1 kb** fragment using Nhe1/Apa1 while YFP-TM7SF2 was released as a **1.95 kb** fragment using Nhe1/BamH1 (**A**) Gel image shows restriction double digest of plasmid vector pcDNA3.1Hygro cut with Nhe1/Xba1, Nhe1/Apa1 and Nhe1/BiamH1 for subsequent ligation procedure with inserts cut with corresponding restriction enzymes. (**B**) Gel electrophoresis image shows single band expressed at expected size of **6.2 kb** following double digest procedure using Nhe1/Xba1, Nhe1/Apa1 and Nhe1/BamH1 for pcDNA3.1CAT plasmid vector. Chloramphenicol Acetyltransferase reporter gene (CAT) was released at 660 bp when cut with Nhe1/Xba1 and Nhe1/Apa1 only. (**C**) Gel image shows the predicted/calculated sizes for the insert fragments cut with Nhe1/Xba1, Nhe1/Apa1 and Nhe1/BamH1 from pEYFP-C1 plasmid for ligation with vector of corresponding restriction digest enzymes as pcDNA3.1Hygro. (**D**) Gel electrophoresis showing double loading of insert fragments of predicted sizes cut with Nhe1/Xba1, Nhe1/Apa1 and Nhe1/BamH1 from pEYFP-C1 for ligation with pcDNA3.1CAT vector with corresponding restriction digest enzymes.

3.2.2 Transformation and selection

A ligation mixture consisting of the prepared plasmid vectors pcDNA3.1CAT and pcDNA3.1Hygro each with the gene fragments for YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR was carried out following DNA purification procedure of the excised fragments from p EYFP-C1 plasmid. The purified fragments of YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR were thereafter cloned into the corresponding pcDNA3.1CAT and pcDNA3.1Hygro vectors using T4 DNA ligase. Ligation was performed at room temperature overnight resulting in the recombinant plasmid constructs pcDNA3.1CAT-YFP-TM7SF2, pcDNA3.1Hygro-YFP-TM7SF2, pcDNA3.1CAT-YFP-DHCR7, pcDNA3.1Hygro3.1-YFP-DHCR7, pcDNA3.1CAT-mCherry-LBR and pcDNA3.1Hygro-mCherry-LBR. Transformation was undertaken using chemically competent *E.coli* host cell strain. Putative transformants were selected using antibiotic selection (ampicillin). Screening of positive clones was based on an analytical restriction digest analysis. The plasmids with expected inserts released were considered as positive clones and further sequenced to confirm their identity with the experimental sequences compared with the human sequences available at NCBI GenBank database and that for YFP or mCherry. The positive plasmids were grown up and isolated using both plasmid Miniprep and Maxi prep kits following the manufacturer's instructions (Figure 3.5).

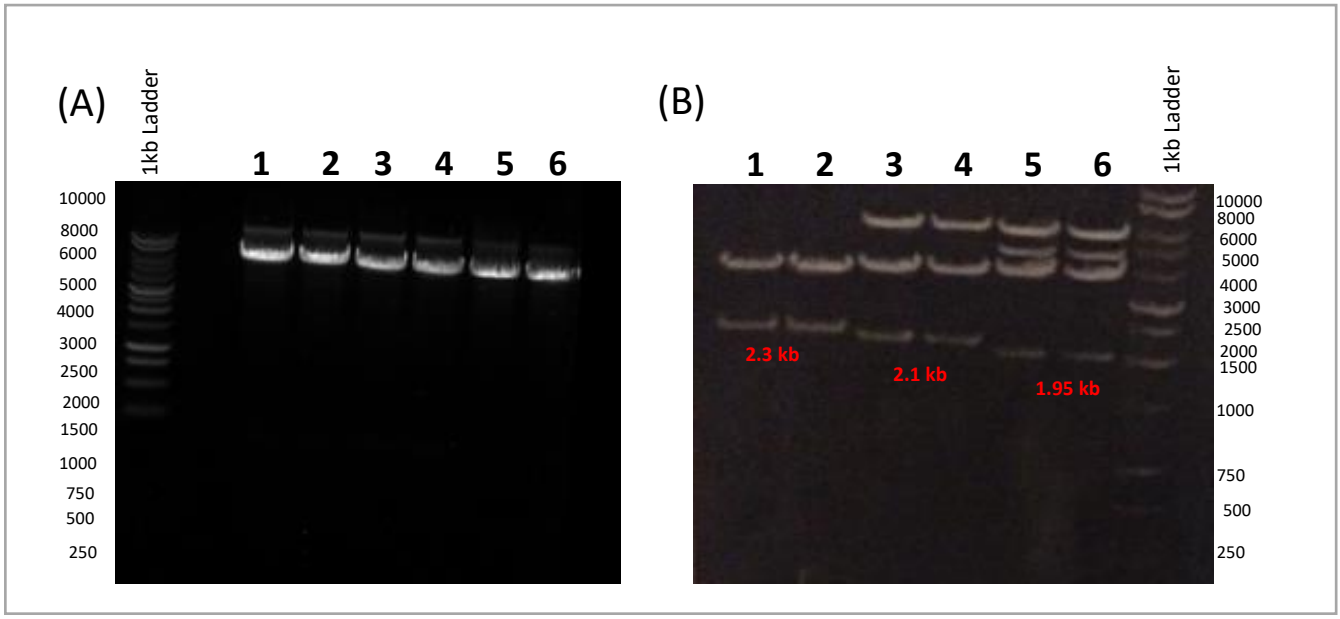

Figure 3.5 (**A**) Agarose gel electrophoresis analysis showing a representation of single bands of 2 µg concentration of plasmid vector pcDNA3.1Hygro cut with Nhe1/Xba1 (lanes 1 and 2), Nhe1/Apa1 (Lanes 3 and 4) and Nhe1/Xba1 (Lanes 5 and 6) prior to bands being excised and subsequent DNA purification procedure using a commercially available Promega clean up kit for ligation with corresponding inserts with same restriction digest. (**B**) Gel image shows the release of inserts YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR cut with double restriction digest enzymes of Nhe1/BamH1, Nhe1/Apa1 and Nhe1/Xba1 respectively. Fragments of inserts were then excised and cleaned-up using the commercially available Promega DNA purification kit for ligation with appropriate vector of same double restriction digest. For example, lane 1A ligates with lane 1B, 2A ligates with 2B and so on.

3.3 DNA Sequence Analysis of YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR Constructs

Prior to identifying positive constructs with the required inserts, a random screening of colonies which had grown on Luria Bertani (LB) Agar plates with ampicillin antibiotic selection was undertaken. Several screenings revealed some colonies that appeared to have been cloned successfully and containing the genes of interest by gel electrophoresis assay (Figure 3.6). Colonies that appeared to have the inserts were isolated, prepped and thereafter samples were sent off to be sequenced by Beckman Coulter Genomics. The sequencing was undertaken for plasmid constructs with the inserts using T7 forward and BGH reverse primers which flank the multiple cloning site of pcDNA3.1Hygro and pcDNA3.1CAT plasmid vectors. The incorporation of the sequence was also verified by gel electrophoresis with mCherry-LBR released at 2.3 kb, YFP-DHCR7 at 2.1 kb and YFP-TM7SF2 at 1.95 kb (Figure 3.6).

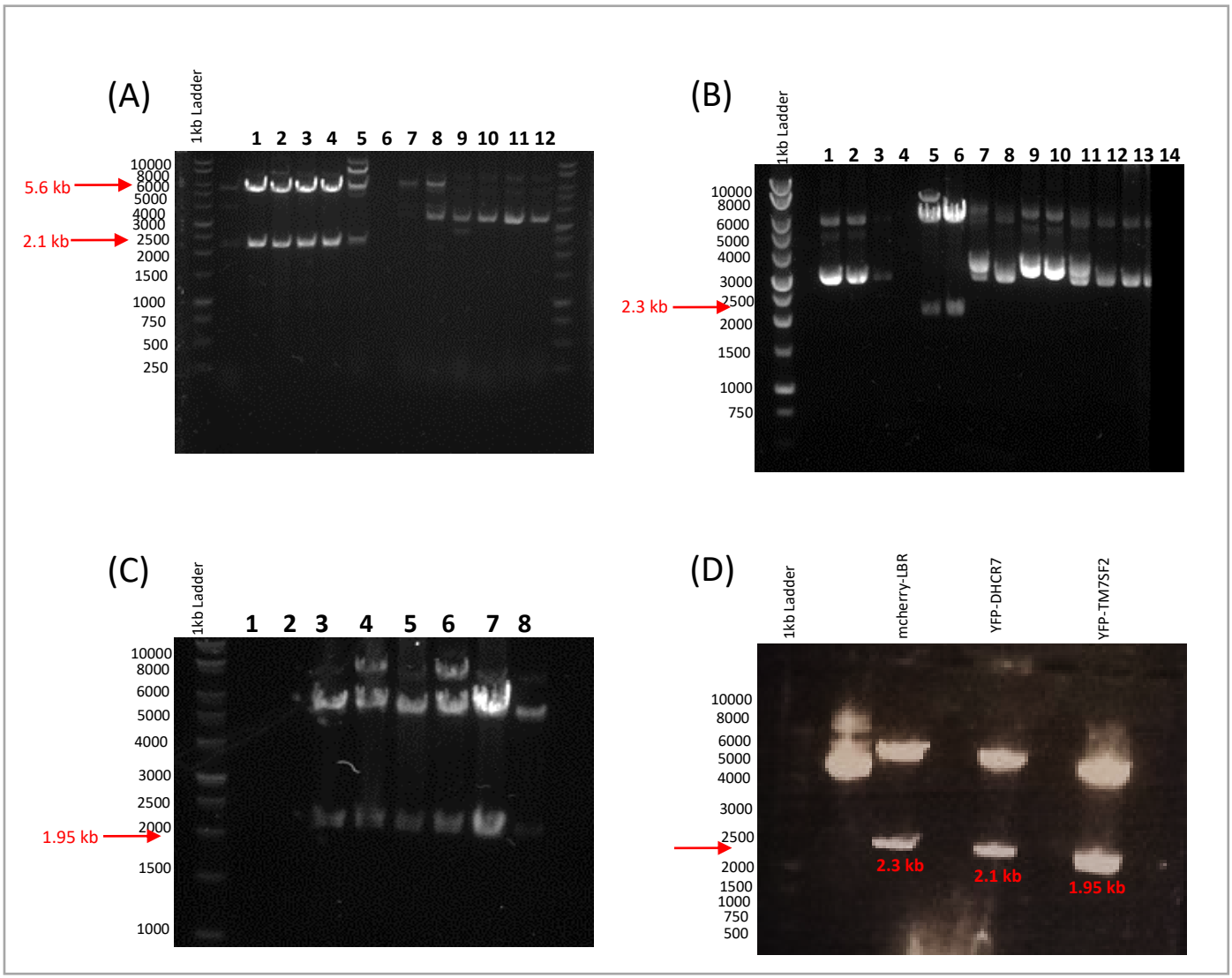

Figure 3.6 (**A**) Analytical agarose gel electrophoresis showing **the** screening of constructs of pcDNA3.1Hygro-DHCR7. Lanes 1-4 show YFP-DHCR7 at **2.1 kb** with pcDNA3.1Hygro vector at **5.6 kb** as expected. (**B**) Analytical test digest showing screening of pcDNA3.1Hygro-LBR. Lanes 5 and 6 show mCherry-LBR insert at **2.3 kb** while vector pcDNA3.1Hygro is at **5.6 kb**. (**C**) Gel electrophoresis showing analytical test digest for pcDNA3.1Hygro constructs of YFP-TM7SF2. Fragment released at **1.95 kb** indicate constructs are positive. All positive constructs were further analysed by DNA sequencing. (**D**) **1%** Agarose gel electrophoresis showing constructs of pcDNA3.1Hygro with mCherry-LBR **(2.3 kb),** YFP-DHCR7 **(2.1 kb)** and YFP-TM7SF2 **(1.95 kb).**

3.4 Transient and Stable Expression of YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR Constructs in CHO Mammalian Cells

3.4.1 Generation of cell lines over-expressing target genes

For biochemical and microscopy experiments aimed at investigating the subcellular localisation of YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR fusion proteins and whether their overexpression improved the production capacity of the secretory pathway for recombinant proteins, generation of cell lines expressing these proteins was a necessary process. The approach was to generate cells stably expressing these targets in CHO.S cells where manipulative investigations could be undertaken whereby all cells would be expressing the target fusion protein. This process takes months and hence, these proteins were also transiently expressed in CHO-S cells over different periods of time and analysis of the impact of transiently over-expressing these engineered cells undertaken. Confirmation of expression was achieved using western blot and comparing the observed protein band(s) against those of the calculated molecular weight of these proteins including the reporter genes YFP and mCherry (Table 3.2). Note that all transient transfection studies of YFP-TM7SF2 and YFP-DHCR7 were carried out with pcDNA3.1CAT vector while stable expressions of these sterol reductases occurred in pcDNA3.1Hygro vector, except where otherwise stated.

Table 3.2: Predicted molecular weights for YFP-TM7SF2, YFP-DHCR7 and mCherry-LBR proteins.

Protein	Molecular Weight (kDA)	Tagged-Reporter Gene (KDa)	Predicted Weight
TM7SF2	46.5	27	73
DHCR7	54	27	81
LBR	70	28.8	98

3.5 Characterisation of Stable CHO-S Cells Expressing YFP-TM7SF2 and YFP-DHCR7

3.5.1 Generation of stable CHO-S YFP-TM7SF2 and YFP-DHCR7 expressing cells

Following confirmation from transient expression studies that YFP-DHCR7 and YFP-TM7SF2 were expressed in CHO-S cells, the next step was to generate stably expressing CHO-S cells of these lipid metabolism modifying targets. This approach would allow investigation of any reprogramming of the cell in terms of ER membrane expansion upon expressing the target YFP-TM7SF2 and YFP-DHCR7 fusion proteins. Quantification of the impact and influence of overexpression and impact on the yield of recombinant protein production could therefore be more accurately assessed than in transient systems. Constructs of YFP-TM7SF2 and YFP-DHCR7 in pcDNA3.1Hygro were linearised using FSP1 restriction digest enzyme for linearization of the DNA. CHO-S cells were seeded in replicates at different concentrations of $2x10^5$, $5x10^5$ and $1x10^6$ viable cells/ml in 6 well plates and kept in an incubator at 37°C for 24 h. 4 µg of linearized plasmid DNA for YFP-TM7SF2 and YFP-DHCR7 were added equally across the 6 well plates for each stable cell pool to be generated. 24 h post-transfection, Hygromycin B antibiotic was added as a selection drug for stable cells. Verification of linearisation of the DNA was carried out using agarose gel electrophoresis (Figure 3.7). Stable cells were cultured in shake flasks and experiments carried out in shake flasks. Confirmation of expression of the target proteins in the stable pools was undertaken using western blot analysis alongside further characterisation studies to determine the influence of overexpressing YFP-TM7SF2 and YFP-DFHCR7 on CHO-S cell phenotypes.

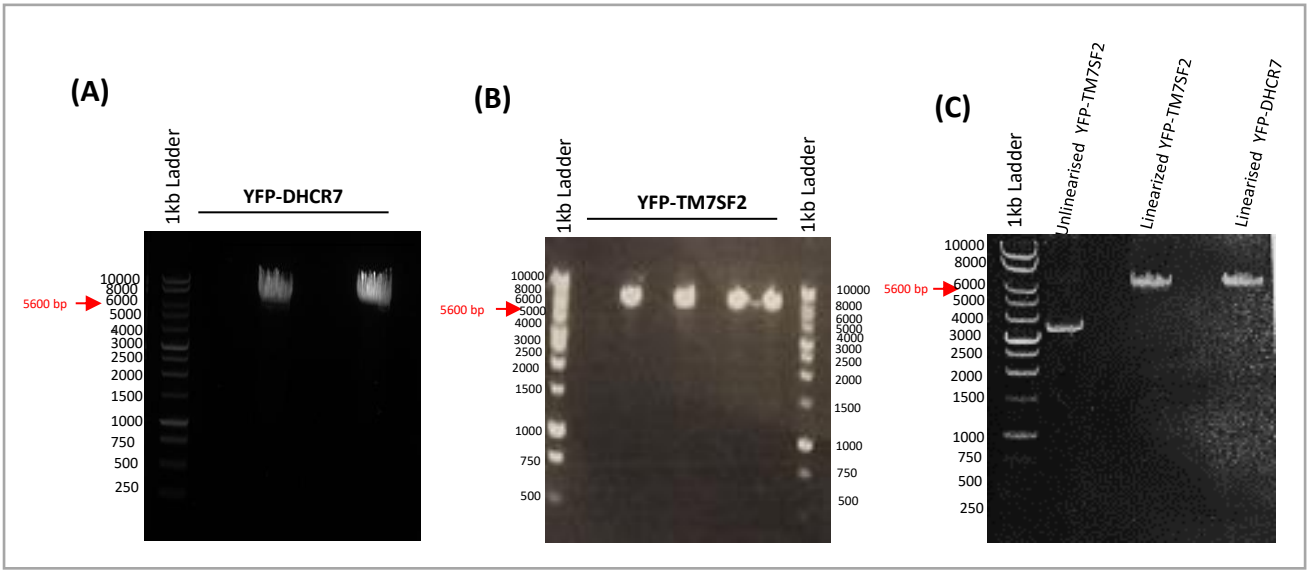

Figure 3.7 (A) 1% Agarose gel electrophoresis analysis of linearized construct YFP-DHCR7 in pcDNA3.1Hygro plasmid vector using Fsp1 restriction digest. (**B**) Agarose gel electrophoresis showing linearized construct of YFP-TM7SF2 in pcDNA3.1Hygro by Fsp1 restriction digest. (**C**) Agarose gel image showing the comparison of both the undigested plasmid DNA (representative YFP-TM7SF2) with the Fsp1 digested plasmid DNA for both YFP-DHCR7 and YFP-TM7SF2 in pcDNA3.1Hygro vector for generation of stable CHO-S cells for these proteins.

3.5.2 Validation Studies with Green Fluorescent Protein (GFP) as a Control

To assess the effect of overexpressing the target proteins in the engineered stable CHO-S cells, a control vector stable cell line was also constructed in parallel to analyse the level of stable protein expression of YFP-DHCR7 and YFP-TM7SF2. GFP was used as a control for quantitative and qualitative characterisation. Stable CHO-S expressing GFP was generated as earlier described (3.5.1). Preliminary cloning was undertaken to clone GFP from plasmid vector pcDNA5FRT, using Nhe1/HindIII into pcDNA3.1Hygro vector for generation of stable expression in CHO-S cells. GFP was released at 720 bp (Figure 3.8). Linearisation procedure was followed for the integration into CHO-S cells genome in order to generate stable cell lines.

This study uses GFP as reporter of protein expression for YFP-tagged DHCR7 and TM7SF2 as well as mcherry tagged LBR proteins.

The choice to establish a GFP control for an investigative role on the implication of overexpression of Yellow Fluorescent Protein (YFP)-tagged Transmembrane 7 Superfamily member 2 (TM7SF2) and (YFP)-tagged 7-dehydrocholesterol reductase (DHCR7) as well as mCherry-tagged Lamin B Receptor, on recombinant protein production was informed with the intention that a GFP control will serve two main purposes: Firstly, it would extent the perspective of this work beyond the scope of the original paper by Zwerger et al., (2010) where for instance a TM7SF2-specific antibody was used to detect the protein in U2OS cells. In CHO-S cells, a functional stably generated GFP is almost identical with its derivatives such as YFP at the protein level, differing only in a few amino acids and making it nearly impossible to differentiate between them with antibodies. GFP has become well established as a marker of gene expression in cell and molecular biology and is rapidly becoming one of the most (YFP)-tagged frequently employed molecular reporters (Campbell & Choy, 2001).

Both GFP and YFP are widely used by researchers because of their brightness, fast and simple chromophore maturation mechanism and excellent photostability and YFP is a slightly red-shifted variant of GFP, a shift that is caused by a π-π stacking interaction between the chromophore and a neighbouring hydroxyphenyl ring from a Tyrosine side chain (Shinoda et al., 2018). We understood, that a GFP control will not only detect YFP in gene monitoring but also maintain a neutral effect on protein expression levels. This is important more so at the molecular level and the use of GFP in monitoring gene expression and protein localisation has been well documented (Campbell & Choy, 2001). Although the Zwerger et al., (2010) group performed live cell imaging of U2OS cells co-transfected with the ER-marker SP-GFP (SP refers to spacer) and mCherry-LBR, it was not reported that this was carried out in YFP-TM7SF2 and YFP-DHCR7 cells. Therefore the approach taken towards this work was to use a different fluorescent protein combination which will confer a different but useful outlook to this project.

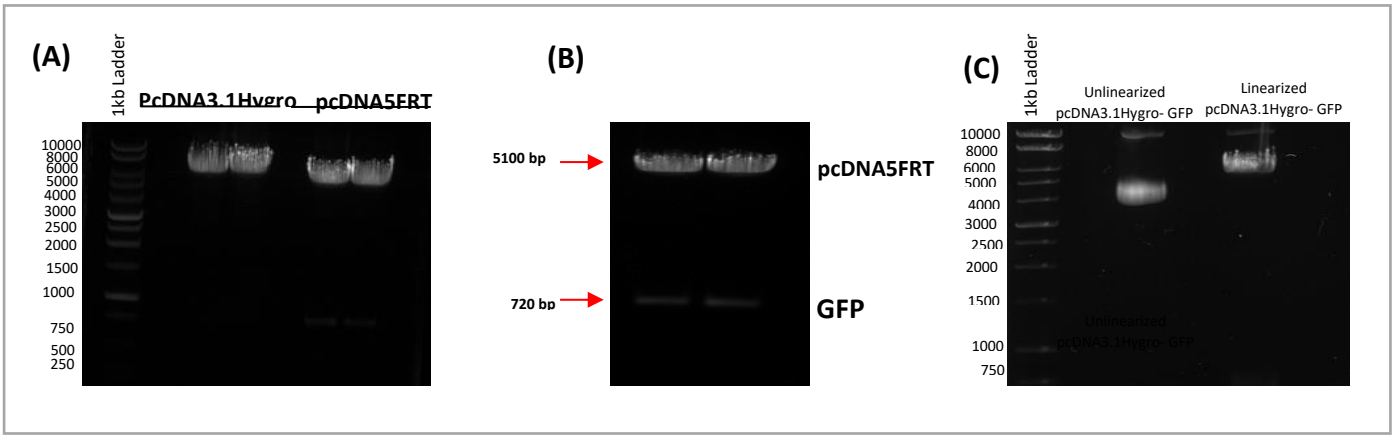

Figure 3.8 eGFP as a Control for Monitoring Impact of Lipid Modifying Proteins on CHO-S cells. eGFP was cloned from pcDNA5FRT plasmid vector using Nhe1 and HindIII restriction digest enzymes with expected fragment size 720 bp (**A**) Ligation procedure was used to clone eGFP into pcDNA3.1Hygro expression vector of 5600 bp size. (**B**) Agarose gel electrophoresis showing the release of eGFP from pcDNA5FRT vector. (**C**) After the cloning of eGFP into pcDNA3.1Hygro, it was linearised using Fsp1 restriction digest enzyme. Agarose gel electrophoresis was undertaken to analyse the linearised eGFP construct with unlinearised eGFP construct.

3.6 YFP-Reporter Expression Studies for the YFP-TM7SF2 Construct

To investigate the transient expression of YFP-TM7SF2 both qualitatively and quantitatively, YFP and GFP expression were used as a positive control for validation studies. To examine YFP-TM7SF2 expression, CHO-S cells were transiently transfected with plasmid DNA for this target following the transfection protocol outlined in the methods section (see section 2.9). The transfected cells were then harvested 24 h and 48 h post-transfection and analysed using western blot techniques (Figure 3.9). CHO cells were also transfected with an empty vector and a GFP expressing vector. Because the maximal yield of the fusion protein is highly dependent on the percentage of cells that are successfully transfected, exponential growth and viable cells were used for these studies with the percentage viability over 97%. CHO cells were then transfected using lipofectamine 2000 and Opti-MEM® media transfection reagent according to the protocol of the manufacturer and cells harvested at 24 and 48 h post-transfection. Lysates of these cells were collected and analysed. In order to enhance recombinant protein production, it was important to investigate the influence of overexpressing these lipid targets

on CHO cells (if any) and also their effects on secretion. Results show an increased expression of YFP-TM7SF2 lipid target at 73 kDa as time post-transfection increased.

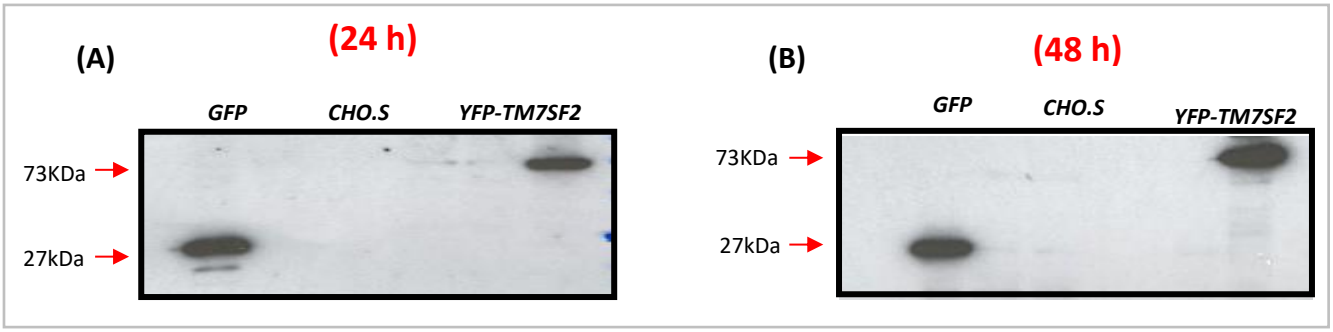

Figure 3.9 Time course investigation studies of transient YFP-TM7SF2, GFP and empty vector expression in CHO-S cells at 24 and 48 h post-transfection. YFP-TM7SF2 was transiently transfected into CHO cells along with GFP and an empty vector and cells harvested after 24 and 48 h. Western blot confirms expression of YFP-TM7SF2 at 73 KDa and GFP as 27 KDa, as a positive control while marginal increased expression was observed with increased time. However, there was no expression observed in the empty vector transfected cells as expected.

3.7 Transient Expression of YFP-DHCR7 in CHO-S Cells

Transient expression studies of YFP-DHCR7 was analysed on day 2 and day 3 post-transfection in CHO-S cells. The lipid target has a predicted molecular weight of 81 kDa (Table 3.1) and transfection was carried out in replicates. The essence of this study was to investigate and confirm the impact of overexpression of this lipid target on increased secretory capacity of CHO mammalian cells and its influence on expansion of CHO ER membrane for improved secretory protein production. CHO-S cells were transiently transfected with the plasmid DNA pcDNA3.1CAT constructs of YFP-DHCR7 and an empty vector as a negative control. The transfected cells were then harvested on day 2 and day 3 post transfection and analysed using western blot techniques (Figure 3.10). Lysates were harvested on the appropriate days after transfection and analysed accordingly. While no expression was seen across the two time points for the negative control transfected CHO-S cells, cells transfected with the YFP-DHCR7 construct showed increased expression with increased time post-transfection (Figure 3.10).

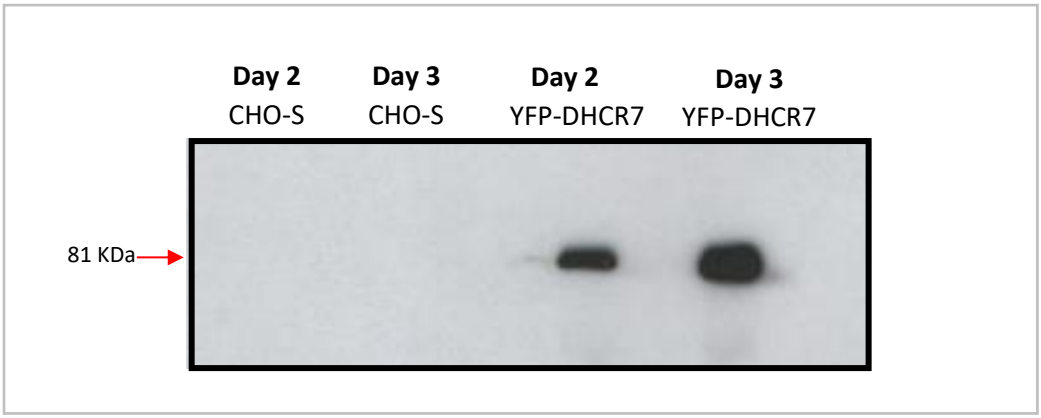

Figure 3.10 Western blot analysis of transient YFP-DHCR7 and empty control CHO-S cells on day 2 and day 3 post-transfection. Cell lysates were analysed for the transient expression of YFP-DHCR7 at the culture times (day 2 and day 3). Increased expression was observed at 81 KDa with increased time post-transfection. No expression was observed in CHO-S cells transfected with an empty plasmid at either time points as expected.

3.8 Transient YFP-TM7SF2 Expression at 24 h, 48 h and 72 h post transfection

Lipofectamine2000-mediated transfection was conducted with cells harvested on day 1, day 2 and day 3 post transfection to further confirm the expression of the target lipid-YFP fusion protein. CHO-S cells were sub-cultured at a seeding density of 2×10^5 cells/ml with viability >98% and transfected using 4 µg plasmid DNA and Lipofectamine. To determine the transfection efficiency, cells were transfected with GFP for validation using the same procedure and results analysed using western blot (Figure 3.11A). It was observed that both GFP and YFP-TM7SF2 increased in expression with increased post-transfection time. The GFP band was much more intense than the YFP-TM7SF2 protein band, indicating that the fusion protein was expressed at lower amounts than the GFP control as might be expected for a membrane associated and fusion protein.

Another transient transfection was also undertaken and transfected cells harvested on day 1, day 2 and day 3 post transfection and cell lysates collected. Western blot analysis showed YFP-TM7SF2 expression increased with post-transfection time point of harvested cells (Figure 3.11B).

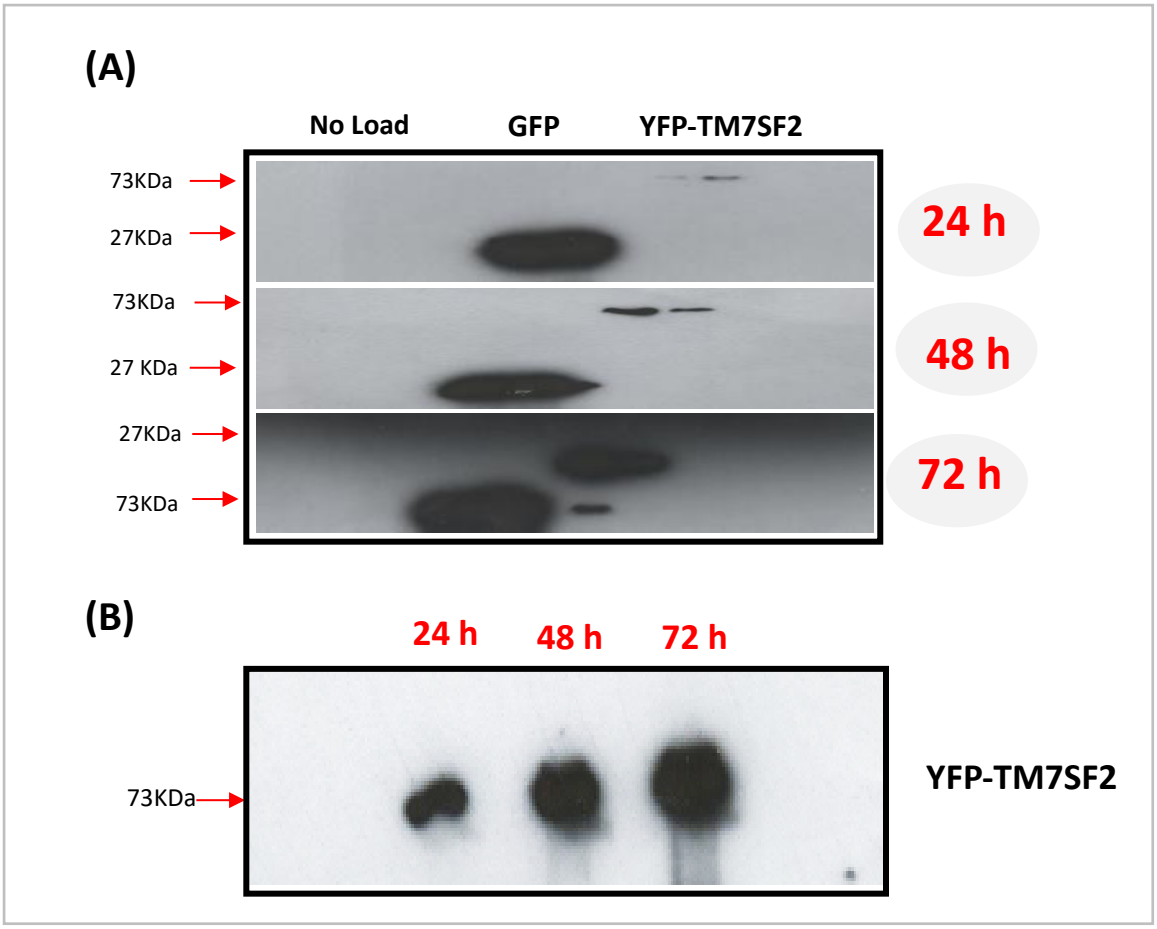

Figure 3.11 **(A)** Western blot analysis of transient expression of YFP-TM7SF2 and GFP in CHO-S cells on day 1, day 2 and day 3 post-transfection. Lysates of cells were harvested on day 1, day 2 and day3 post-transfection and analysed by western blot. GFP increased with time post-transfection and was observed at 27 kDa. YFP-TM7SF2 also showed increased expression at 73 kDa as post-transfection time increased. **(B)** Increased transient expression was observed with time post-transfection in a second transient experiment of YFP-TM7SF2 at 73 kDa as determined using western blot.

Additionally, as bands observed on western blot for transient CHO-S expression of YFP-TM7SF2 as well as GFP control showed varying intensities from the western blotting, the generation of quantitative numerical values became necessary to quantify the difference in YFP-TM7SF2 expression over a 3 day period against a GFP control. Densitometry quantification was therefore used to analyse data, with an image J application software, which involved the measurement of the optical density of the pixels on the photographic film (Figure 3.12). The relative index showed that CHO-S did transiently express YFP-TM7SF2 consistently between the day 1 and day 2 post transfection underpinning the capacity of CHO-S to produce recombinant proteins which is crucial

to the functional role of TM7SF2 in impacting cell growth and survival. However, for a future experiment it would be useful to extend this culture even longer and examine transient expression beyond day 3, as this may further inform on the impact on CHO-S cells, as to whether harsh or favourable.

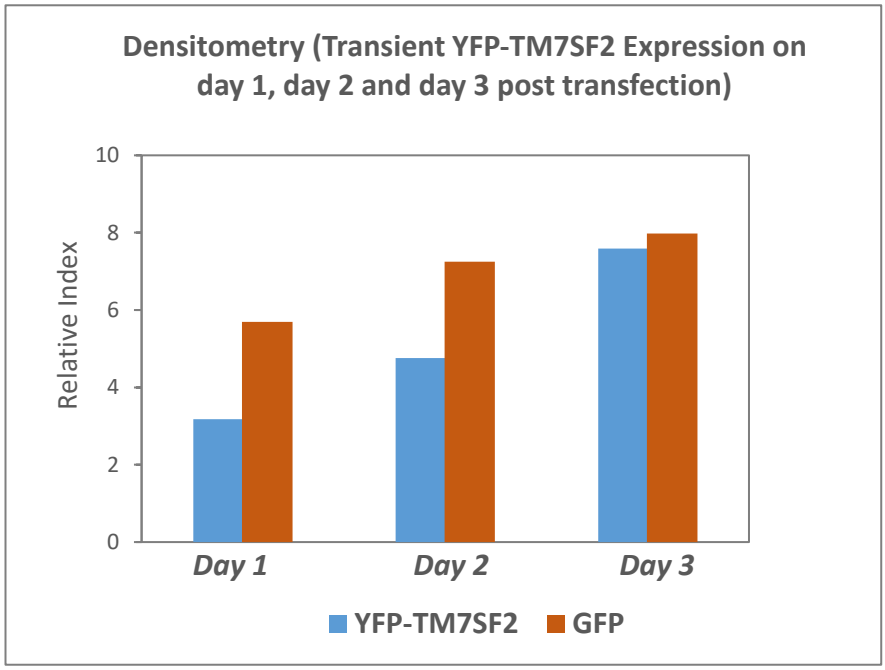

Figure 3.12 Densitometry analysis of the western blots images shown in Figure 3.11(A). Analysis was undertaken using the Image J freeware. Transient studies undertaken to analyse CHO expression levels of YFP-TM7SF2. Transfected cells were harvested Day 1, Day 2 and Day 3 post transfection as GFP control was analysed with YFP-TM7SF2. Graph shows a continued increased expression signal of YFP (TM7SF2) from Day 1 to Day 3.

3.9 Determining the Localisation of Exogenously Expressed YFP-TM7SF2 and YFP-DHCR7 during Transient Transfection of CHO Flip-In Cells

To further characterise YFP-TM7SF2 and YFP-DHCR7 expression and investigate localisation in relation to any influence on ER expansion, fluorescence microscopy was carried out on transiently transfected CHO Flip-In cells. These CHO cells were designed for rapid generation of stable cell lines that express a protein of interest from a Flip-In expression vector ensuring high-level expression of the gene of interest. This cell line was obtained from Smales' lab, University of Kent and only used for the transient studies reported here. As these lipid targets have been tagged with fluorescent proteins, investigation was undertaken to identify where in the CHO cells the exogenous proteins were localised and what influence overexpressing these proteins might have on the CHO cell. Fluorescent YFP for TM7SF2 and DHCR7 and mCherry-LBR (which however was not further studied) were investigated and DAPI staining used to show the nucleus of the cell.

Cells were grown on coverslips and transfected after 24 h with Lipofectamine 2000 and Opti-MEM® media transfection reagent. Plasmid DNA (4 µg) was introduced into $2x10^5$ cells/ml of highly viable CHO cells. For this study, the CHO Flip-In cells were used while a NO plasmid DNA was also transfected as a negative control. Upon harvest after 24 h, cells were fixed using formaldehyde following transfection protocol and images obtained on a DMR microscope. Cells were DAPI stained to identify the nucleus (Figure 3.13). Protein production using transient transfection is an attractive alternative method and has been used routinely in the production of post translationally modified proteins including antibodies, glycoproteins, and membrane proteins as transient transfection can be achieved in adherent as well as suspension cells (Sastry et al, 2015).

The essence of the investigation was to determine the extent to which transiently transfected CHO cells using fluorescent reporter proteins can efficiently result in greater protein expression as high transfection efficiency is significant in transient gene expression, hence the combination of the CHO Flip In and Lipofectamine transfection reagent was optimised in terms of DNA: TM7SF2, DHCR7 and LBR ratios to improve expression. While some signal expression of the fluorescent proteins was observed in the nucleus the majority was around the outside of the nuclear envelop and hence likely to be localised to ER as might be expected for these ER resident proteins. Some cells transfected with the lipid targets had no signal as they did not express any endogenous YFP signal

of lipid targets, DHCR7 and TM7SF2 and also mCherry signal of LBR, presumably due to transfection inefficiency.

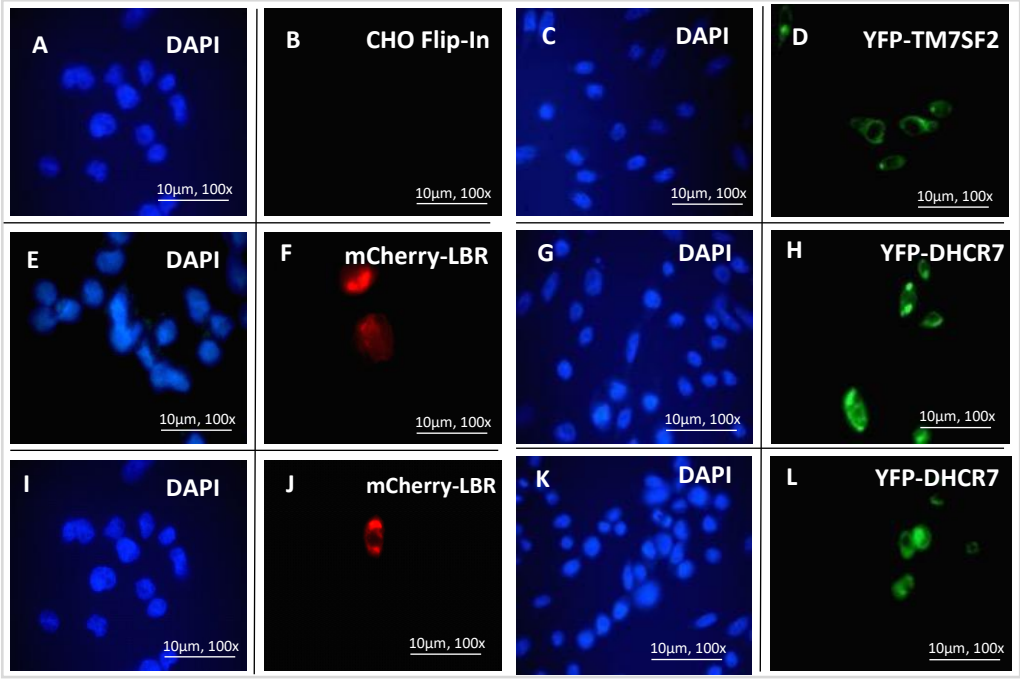

Figure 3.13 Transient expression studies of YFP-TM7SF2, YFP-DHCR7, mCherry-LBR in CHO Flip-In cells. Transfected CHO Flip-In cells were counter-stained with DAPI (blue) for the detection of nucleus (**A**, **C**, **E**, **G**, **I** and **K**). Untransfected CHO Flip-In cells represented a control feedback and showed no fluorescence signal of the transmembrane proteins (B) while YFP detection in cells transfected with DHCR7 and TM7SF2 (**D**, **H** and **L**) was observed and appear to localize to the nuclear rim as aggregates are seen to be formed in the cytoplasm. mCherry fluorescence signal was again detected in LBR (F and J) with signal observed to localise to the nuclear rim. Although these transmembrane proteins were detected in CHO Flip In cells, overall efficiency of transfection was however considered below 25% as percentage of cells successfully transfected was low as shown. Images were taken using the Leica® Fluorescent microscope 24 h post-transfection. For comparison, **A** and **B, C** and **D, E** and **F, G** and **H, I** and **J, K** and **L.** Scale bar: 10 µm.

3.10 Developing Cell Pools Expressing an Empty pcDNA3.1Hygro Vector as a Negative Control

In determining if over-expression of the lipid modifying fusion targets in CHO-S cell expression systems influence ER expansion and/or increased secretion of recombinant protein products, a negative control was also necessary. The Fsp1 restriction digest enzyme was used to linearize pcDNA3.1Hygro for efficient transfection into CHO-S cells. These cells integrate the empty pcDNA3.1Hygro vector without any heterogeneous protein. Agarose gel electrophoresis was used to confirm the size of plasmid vector pcDNA3.1Hygro. Varying amounts of the plasmid were transfected into cells and all showed pcDNA3.1Hygro with 5.6 kb size. Analysis of stable expression of CHO-S cells with the empty pcDNA3.1Hygro vector using western blot confirmed no signal was observed (Figure 3.14).

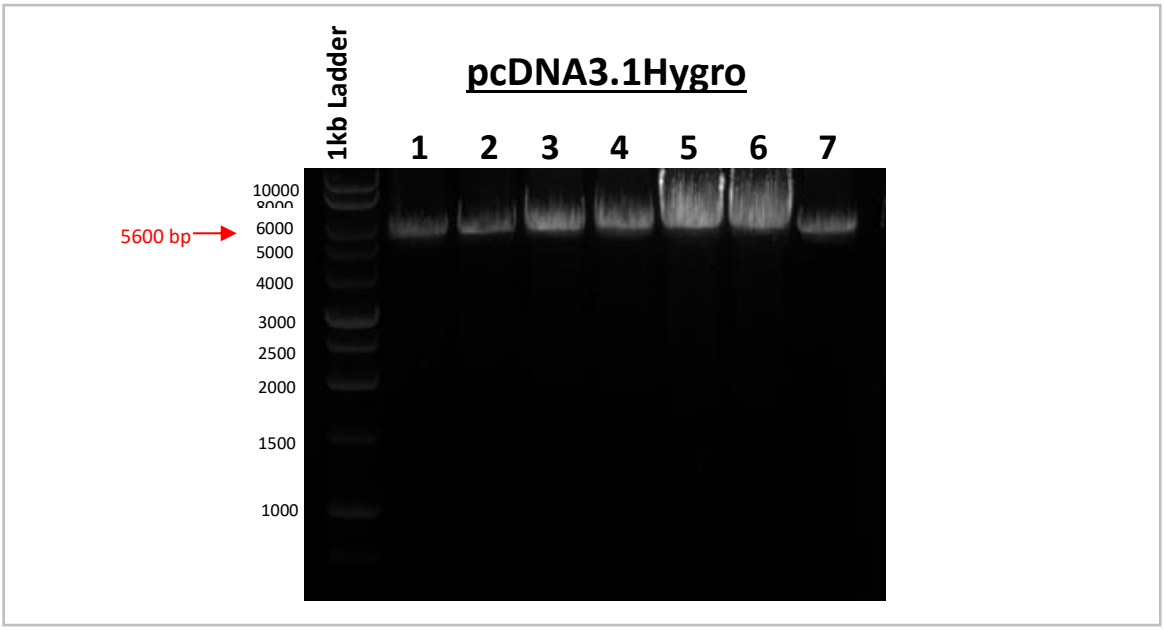

Figure 3.14 Agarose gel electrophoresis showing different loading amount of pcDNA3.1Hygro. 2 µg amount of samples were digested with different restriction enzymes as plasmid vectors to be ligated with inserts. Unequal loading amounts have been done with lanes 5 and 6 having the highest loading amount expressed 5600 bp. pcDNA3.1Hygro loaded on lanes 1 and 2 were cut using Nhe1/Xba1, lanes 3 and 4 using Nhe1/Apa1 while lanes 5 and 6 using Nhe1/BamH1. The implications of these was that these plasmid vectors, having being cut with corresponding restriction digestion enzymes would be later ligated accordingly for the generation of LBR, DHCR7 and TM7SF2 constructs of pcDNA3.1Hygro to the engineered in the mammalian cells for cell line development.

Once the stable cell pools were generated and verified to be expressing the target YFP fusion protein, regular passages were undertaken to culture cells. Different concentrations of the CHO-S stably expressing cells of YFP-TM7SF2 and YFP-DHCR7 were harvested and analysed by western blot (Figure 3.15). Increased expression of the lipid modifying targets were observed with increased cell concentrations.

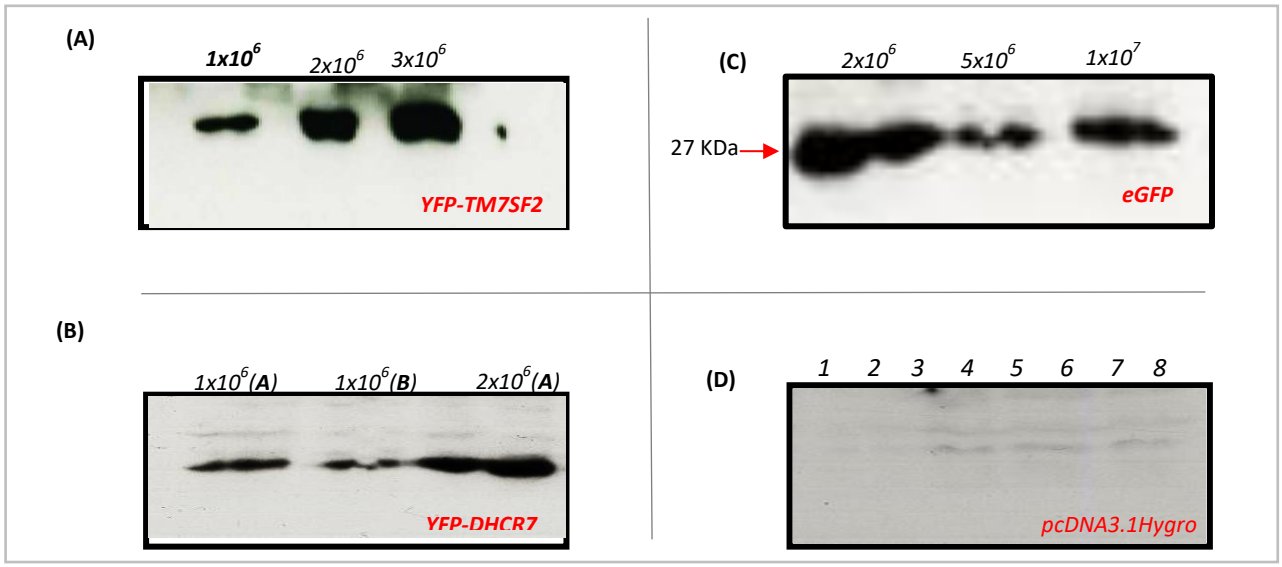

Figure 3.15 (A) Western blot confirmation of stable CHO-S cells expressing YFP-TM7SF2. Stable pools were harvested at different concentrations and expression levels vary accordingly. (**B**) Western blot confirmation of stable CHO-S cells expressing YFP-DHCR7. Expression shows the different pools of the same construct. Different passages of stable cells were analysed at different concentrations. YFP-DHCR7 show decreased expression as compared to YFP-TM7SF2 at the same cell concentration. (**C**) Western blot analysis of stable eGFP expression in pcDNA3.1Hygro vector in CHO-S cells. Stable cells of linearized eGFP construct cloned in pcDNA3.1Hygro plasmid vector were generated at different concentrations of $1x10^7$, $5x10^6$ and $2x10^6$ cells/ml in a 6-well experimental plate in duplicates. (**D**) Screening for verification shows constructs of lanes 1 and 2 as best expressing constructs and were carried on for further experiments. Western blot analysis of linearized pcDNA3.1Hygro at different concentrations to generate stable pools of pcDNA3.1Hygro empty vector as a negative control for monitoring CHO-S ER for expansion. Following this result, constructs from lanes 1 and 2 where carried further for experimental work.

3.11 Localisation of YFP-TM7SF2 and YFP-DHCR7 in Stable CHO-S Cells

TM7SF2 and DHCR7 are sterol reductases located at the ER and function at distinct steps of the cholesterol biosynthesis pathway (Bennati et al., 2006). Fluorescent microscopy was carried out to characterize these lipid biosynthesis targets in stably expressing CHO-S cells. GFP and pcDNA3.1Hygro were also investigated as controls. CHO-S cells were seeded on pre-coated cover slips to enable the suspension cells adhere to the surface of the cover slips. Cells with viability above 98% were seeded at $0.2x10^6$cells/mL in a 6 well plate and allowed to settle in an incubator for 24 h. Cells were then washed with phosphate buffered saline (PBS) and fixed with formaldehyde according to protocol. With the C-terminal sequence of YFP containing a localization signal, we monitored expression using GFP as a positive control. TM7SF2 and DHCR7 were expressed and localized around the nucleus, potentially at the ER as might be expected for these constructs. (Figure 3.16). The data confirm that TM7SF2 and DHCR7 are stably expressed in CHO-S although expression varied with different pools leading to the identification of two groups; named A and B. All images were acquired and size is indicated by a 10 µm bar.

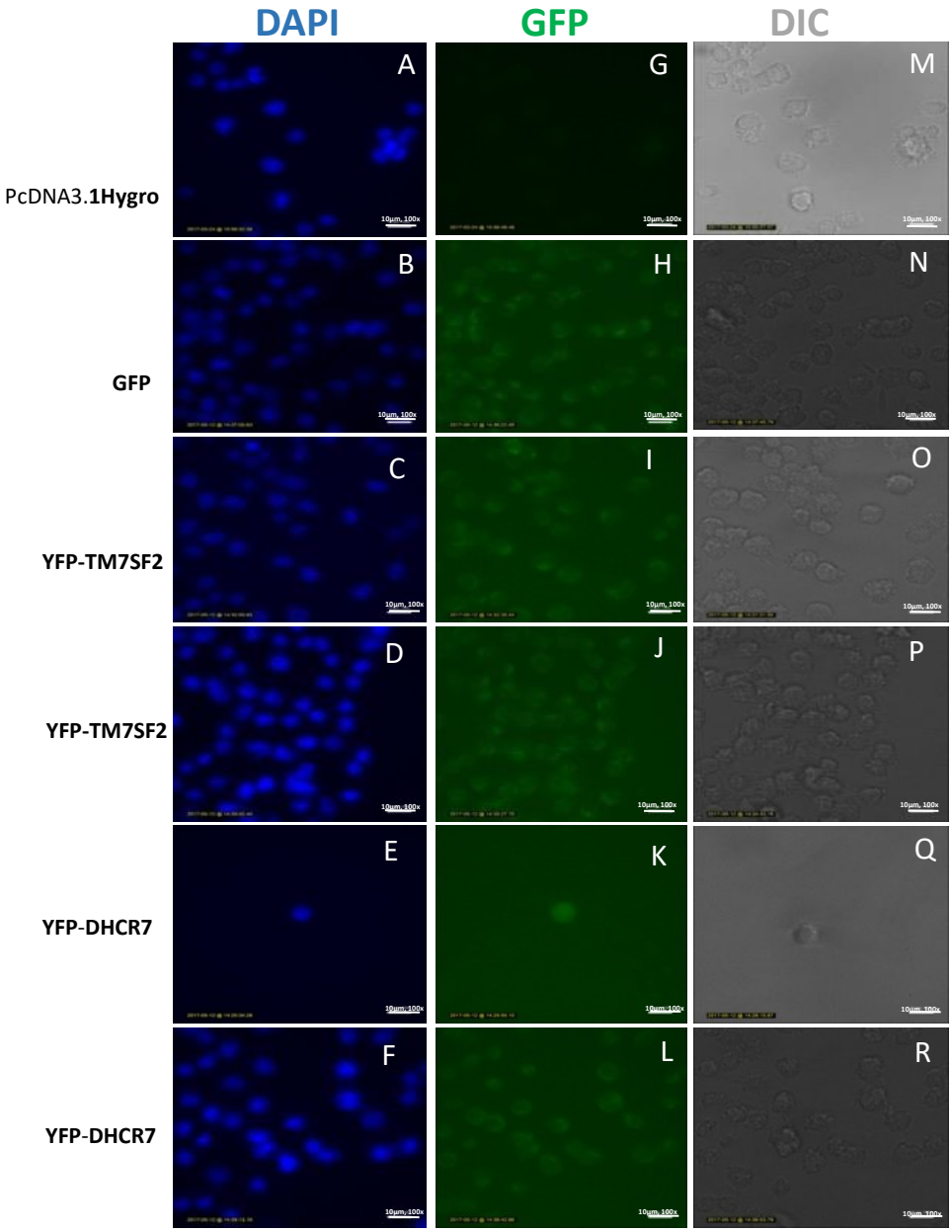

Figure 3.16 Fluorescent microscopy images of CHO-S cells stably expressing YFP-TM7SF2, YFP-DHCR7, GFP and pcDNA3.1Hygro. Following the seeding of cells on pre-coated coverslips at 37℃, cells were fixed with 4% paraformaldehyde. The nucleus of cells were DAPI-stained and fixed specimens were analysed. Expression patterns of YFP for TM7SF2 and DHCR7 were observed using GFP excitation wavelength between 395nm-475nm. While DAPI showed the cytoplasmic aggregates of the nucleus (**A-F**) as confirmed by the corresponding Differential Interference Contrast images (**M-R**), aggregates of TM7SF2 and DHCR7-transiently transfected cells showed some GFP fluorescence expressions (**G-L**) when compared with pcDNA3.1Hygro expressing CHO-S showed no GFP signal. Scale bar, 10 µm, magnification: 100x.

3.12 Generating Higher YFP-TM7SF2 and YFP-DHCR7 Expressing CHO-S Cells from Pools by Limited Dilution Cloning

In order to implement TM7SF2 and DHCR7 as engineering targets, it was pertinent to first establish the levels of exogenous expression of these proteins in CHO-S cells. Following characterisation studies of the stably expressing pools described above, it was evident that pools of stable TM7SF2 and DHCR7 cells had improved growth but also that the amount of expression was low. However, statistical analysis using T-Test showed difference higher with a probability value (p value) of 0.05 between both sets of triplicate samples suggesting that the difference between the corresponding control (GFP/pcDNA3.1Hygro) and test samples (YFP-TM7SF2) was insignificant.

Additional confocal microscopy analysis suggested that some cells had higher expression levels of YFP-TM7SF2 and YFP-DHCR7 than others within the pools (Figure 3.17). During the culturing of the engineered cells, it was observed that there was a clear disparity in the pattern of growth of the two kinds of pools of cells overexpressing YFP-TM7SF2 and YFP-DHCR7. Although, presentation appeared similar when viewed under the microscope, there remained a clear difference in expression levels as observed from western blot data. The reason for this is likely to be due to differences in the site of integration, amount of transcript produced and subsequent recombinant fusion protein produced.

To try isolate some of the higher expressing cells, a limited dilution cloning approach was undertaken. In obtaining single cell clones or monoclonal cells of YFP-TM7SF2**A**, YFP-TM7SF2**B**, YFP-DHCR7**A** and YFP-DHCR7**B**, we can eliminate variability of heterogeneous mixed population and focus on the high expressing cells. The two cultures of engineered cells YFP-TM7SF2 (**A** and **B**) and YFP-DHCR7 (**A** and **B**) were cultured and maintained under same experimental condition for subsequent investigative studies.

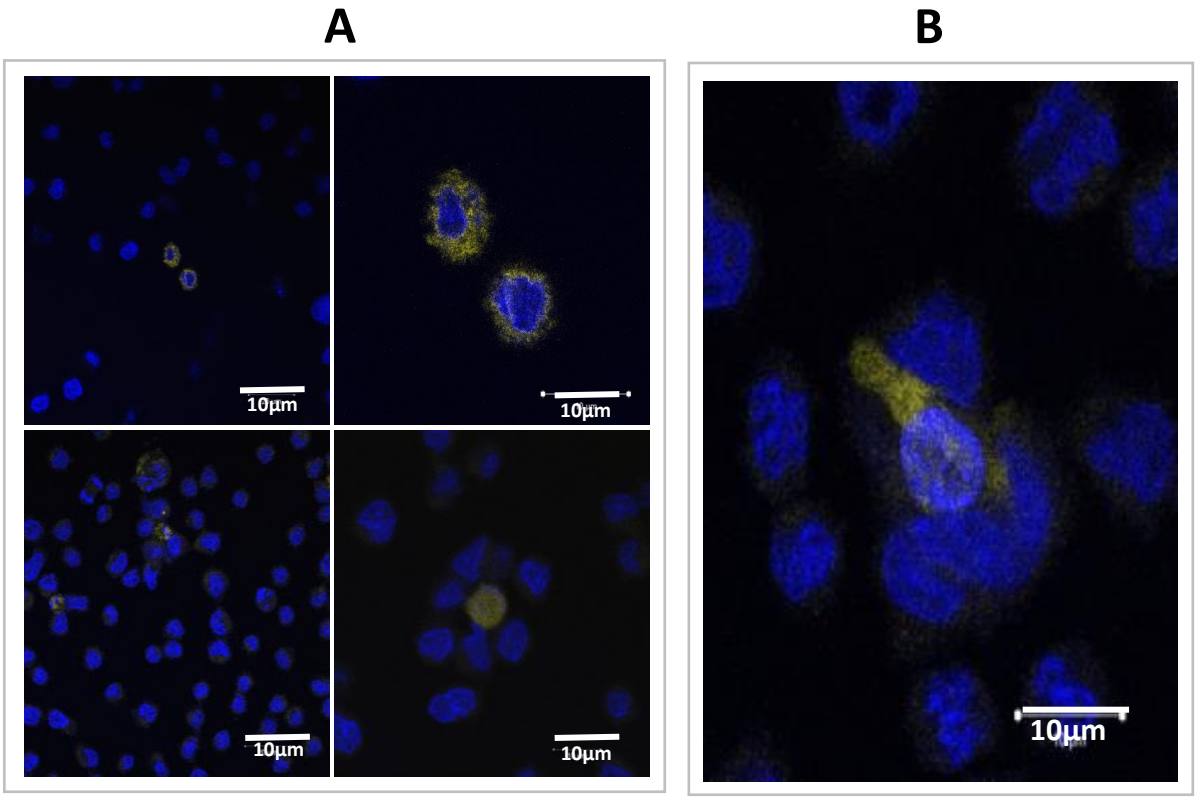

Figure 3.17 Confocal microscopy of stable YFP-TM7SF2A in CHO-S. (A) Representative images depicting the general localisation expression of YFP-TM7SF2A in CHO.S cell pools. Not all cells are seen expressing the protein. (**B**) Images of YFP-TM7SF2B showing different signal expressions in cells as in (A). Scale bar: 10 µm.

3.13 Growth Characterisation of CHO Cell Pools Stably Expressing YFP-TM7SF2 and YFP-DHCR7

The alteration of key components of lipid biosynthesis could impact on cell growth and therefore it was necessary to examine the impact of overexpressing the lipid modifying targets on cellular responses which are important for cell growth, viability, and recombinant protein yields and quality. Therefore, the question of whether stables CHO-S cells overexpressing YFP-TM7SF2 and YFP-DHCR7 impacts upon CHO-S growth and viability was investigated and the results are shown (Figures 3.18 and 3.19). Since the aim was to investigate the cell behaviour over culture, cells were seeded at a low concentration initially. In one study, stable cells of the empty vector control and YFP-TM7SF2 were seeded at $0.2x10^6$ viable cells/ml in a 20 ml shake flask in replicates and 0.2 ml of cells were counted over a period of 10 days. The viability of the culture was also assessed to determine whether the lipid bio-engineering impacted the growth and survival of the CHO-S cells. The results show that

over a 10-day period, YFP-TM7SF2 showed increased growth compared with the CHO-S control reaching a higher viable cell number. However statistical analysis showed no significant difference between the means of the two trials (CHO-S and YFP-TM7SF2). In another experiment, the growth pattern of YFP-TM7SF2 and YFP-DHCR7 were examined with eGFP and pcDNA3.1Hygro as positive and negative controls. All experiments were carried out in replicates and graphs represent the mean of three independent experiments.

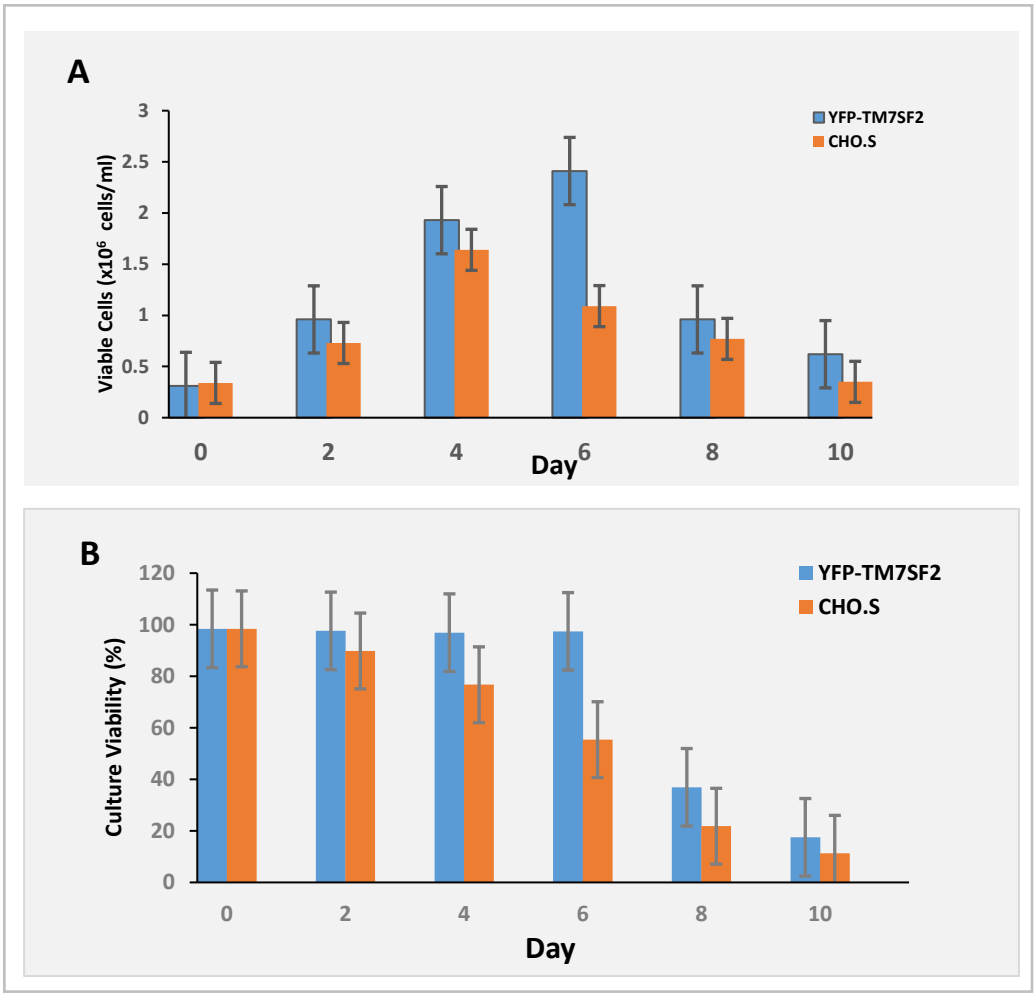

Figure 3.18 Growth profiles of CHO cells stably expressing YFP-TM7SF2 generated with Hygromycin B. **(A)** Cells were seeded at 0.2x10^6 viable cell/ml and cultured for 10 days. Viable cell counts were undertaken using 0.2 ml cells. Error bars represent the standard error of the mean from technical replicates (where n=3). Stable cells grow steady but decline on day 7. **(B)** Lipid engineered TM7SF2 over-expressing CHO.S cells show a significant increase in growth as compared to CHO-S and does not appear to be toxic to cells. Quantification of three independent experiments using t-test with p-value (<0.05), when comparing CHO-S control against YFP-TM7SF2 values shows no statistical significance.

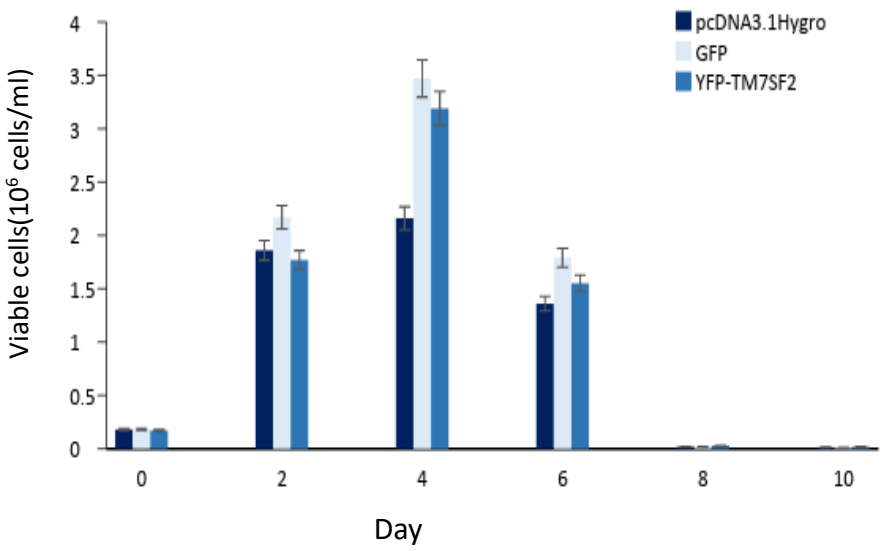

Figure 3.19 Stable CHO-S expressing TM7SF2 cells were cultured at the same time as stable pools expressing GFP and pcDNA3.1Hgro as controls. Cells were seeded at $0.2x10^6$ viable cell/ml in a 20 ml shake flask and cultured for a 10-day period. Cell counts were undertaken using 0.2 ml cells with 0.8 ml to a 1 ml sample volume. Error bars represent the standard error of the mean from technical replicates (where n=3). No statistical significance at $p<0.05$ when comparing YFP-TM7SF2 to the controls GFP and pcDNA3.1Hygro.

Two pools of YFP-TM7SF2 and YFP-DHCR7 stably expressing CHO-S cells were generated with each showing different culture viability and viable cell counts. Each pool was labelled as YFPTM7SF2**A**, YFP-TM7SF2**B**, YFP-DHCR7**A** and YFP-DHCR7**B**. During cell culture, cell pools of YFP-TM7SF2**B** attained a higher viable cell number as compared with YFP-TM7SF2**A.** Meanwhile, YFP-DHCR7**A** pools obtained a higher maximum viable cell number when compared to YFP-DHCR7**B** pools. To study their growth pattern further, 10-day growth profiles of YFP-TM7SF2**A**, YFP-TM7SF2**B**, YFP-DHCR7**A** and YFP-DHCR7**B** in replicate, with GFP and pcDNA3.1Hygro stable CHO-S cells, were setup. Cells were seeded at $0.2x10^6$ cells/ml and counts taken on day 0. Results show data generated on days 0, 3, 4, 5, 6, 7 and 10 (Figure 3.20A). TM7SF2**B** pools showed more rapid growth when compared to TM7SF2**A as** longevity span day 3 to day 6 suggesting an extended survival in culture when compared to theTM7SF2**A** pools. By day 7, both the controls and the lipid engineered cells began tending towards a decline in growth, presumably due to an absence of

essential nutrients. Pools of DHCR7**A** showed a similar growth pattern when compared to DHCR7**B** cell pool although, DHCR7**A** pools showed a higher viable cell number from day 0 until day 5 where DHCR7**B** pools were observed to have increased viable cell numbers. However the growth rate of TM7SF2 pools and DHCR7 pools do not show statistical significance at $p<0.05$ when compared to controls GFP and pcDNA3.1Hygro.

Figure 3.20**B** shows a growth profile of the average growth cell data from three independent experiments. Stable pcDNA3.1Hygro expressing CHO-S showed a lower maximum viable cell number when compared with the GFP control and pools of TM7SF2 and DHCR7. It is interesting to note, that pools of YFP-TM7SF2 and YFP-DHCR7 CHO-S cells do not negatively impact on cell growth and viability but appear to positively impact as viability and growth pattern increased in the lipid engineered CHO-S cells.

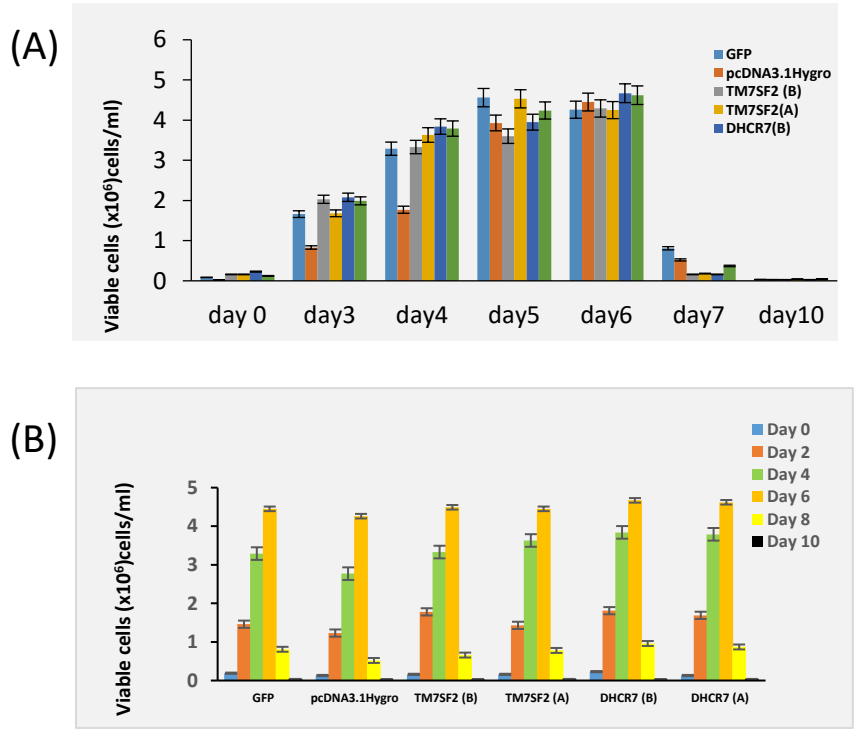

Figure 3.20 Graphs showing growth of stably expressing TM7SF2**A**, TM7SF2**B,** DHCR7**A** and DHCR7**B** CHO-S pools. **(A)** Comparison of TM7SF2**A** and **B** pools indicate there was a higher maximum viable cell number for TM7SF2B over TM7SF2A. DHCR7**A** shows a similar growth pattern to DHCR7**B** pools although a higher viable cell number was observed on day 7 for the B pool. Viable cell counts on day 0, 3, 4, 5, 6, 7 and 10 are shown. Error bar represents standard error of biological triplicates of samples, where n = 3. (**B**) Effect of overexpressing TM7SF2 and DHCR7 in CHO-S cells. Graph shows typical growth pattern of engineered DHCR7 and TM7SF2 over-expressing CHO-S cells with GFP and pcDNA3.1Hygro do not appear to be toxic to cells.

3.14 Flow Cytometry Analysis of CHO-S Cells Stably Expressing YFP-TM7SF2

To further elucidate and characterise the engineered cell pools, we analysed pools of YFP-TM7SF2 by flow cytometry. The experimental technique was carried out as it has the ability to detect functional and identify correctly folded YFP whereby it measures forward scattering. The heterogeneity in the populations and the expression of the fluorescence of single cells of stable CHO.S cells expressing YFP-TM7SF2**A** and YFP-TM7SF2**B** were analysed and compared the CHO.S growing cells from culture samples. While each pool population behaves in a heterogeneous way with potentially dead cells not generally excluded from the analysis, the amount of folded and functional YFP present in the cells may be detected at the excitation of 514 nm and emission peak at 527 nm using flow cytometer. Cells were washed once in phosphate buffered saline and 10,000 events were counted for each sample. Data generated show a shift to the right on the histogram for YFP-TM7SF2**A** and YFP-TM7SF2**B** due to the yellow fluorescence intensity being emitted by each cell per each count. Following analysis from this data, clones from each pools were generated and further analysed.

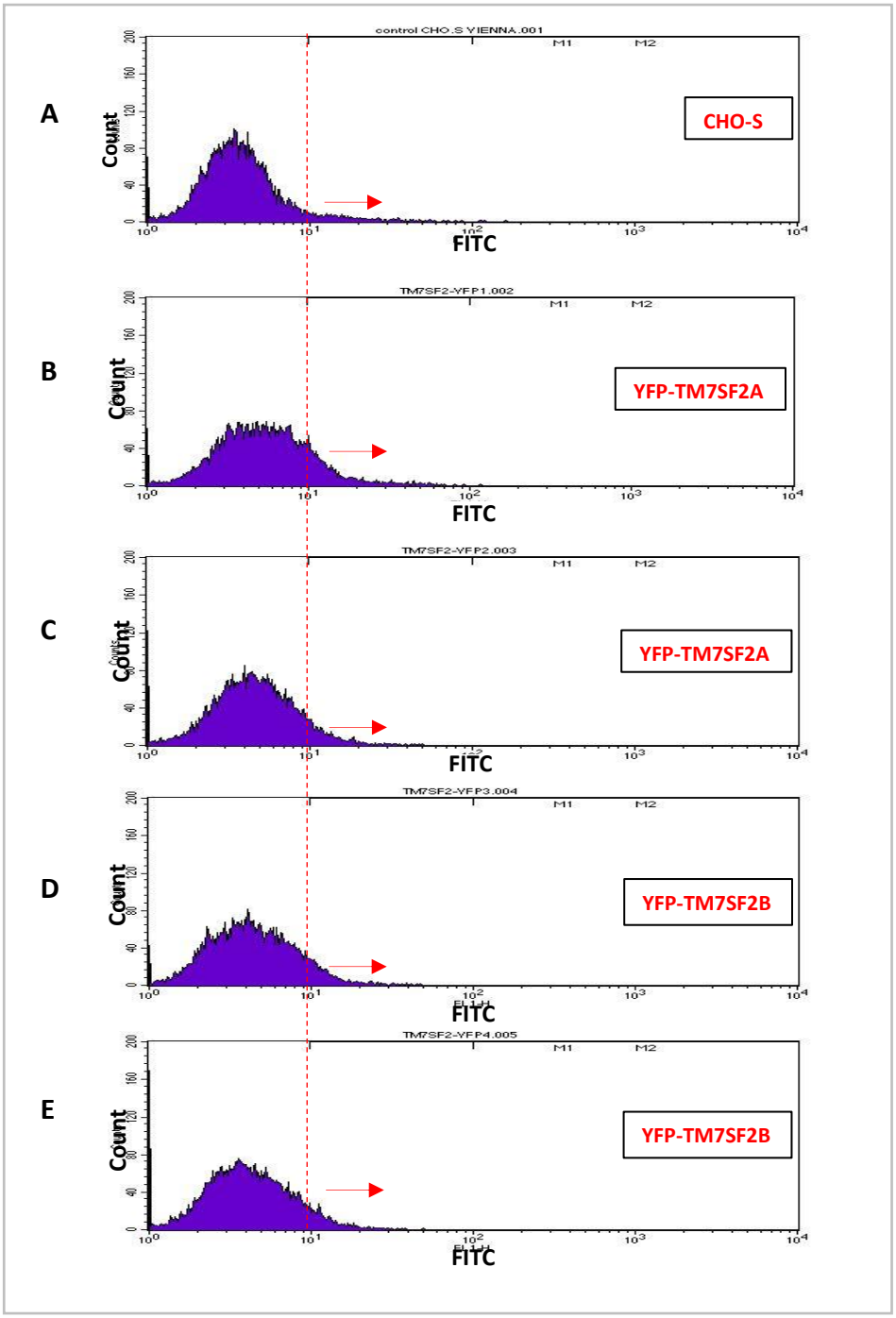

Figure 3.21 Histogram comparing of yellow fluorescence intensity emitted by representatives of heterogeneous populations of YFP-TM7SF2 pools (**A** and **B**) as compared with the CHOS host. Stable YFP-TM7SF2 cells pools show a shift to the right as YFP is emitted in the cell population. The histogram is plotted by counts of each event independently which represents the signal intensity which was detected using fluorescein isothiocyanate (**FITC**) forward-scatter, side-scatter, and each wavelength of fluorescence emission. Analysis of the data was gated at the $\mathbf{10^1}$ mark where positive events are observed. Data suggests the gated region consists of cells of interest with YFP fluorescence emission signal from YFP-TM7SF2 pools.

3.15 Conclusions and Summary Statements from this Chapter

This chapter focused on the cloning and characterisation of TM7SF2 and DHCR7 overexpressed in CHO.S cells and therefore reports on the manipulation strategies used to improve the capacity of CHO lipid biosynthesis machinery by altering key components. Our preliminary synthetic biology approaches involved identifying the subcellular localisation of YFP-TM7SF2 and YFP-DHCR7 as target models and analysed interpretations of possible implications. Elnasri et al., (2018) had reported on a detailed peculiar connection between the DHCR7 gene and TM7SF2 gene despite its association with 20 other different genes. It was reported that transiently and stably overexpressed TM7SF2 and DHCR7 in particular have led to expansion of the ER in CHO cells (Zwerger et al., 2010). Using the principle of sub-clonal genetic engineering, we showed how TM7SF2 and DHCR7 lipid targets were cloned into expression vectors for transient and stable investigation studies for overexpression.

With the introduction of an affinity YFP-tag for reporter of expression studies, Zwerger et al., (2010) in their report had expressed a wild-type and C-terminally truncated TM7SF2 and DHCR7 and N-terminally truncated LBR where they observed a compaction of the nucleus with development of NAVs in U2OS cells and this was the case with untagged TM7SF2 wild-type and TM7SF2 indicating impact was independent of the fluorescent tag. A direction taken in this work, was to analyse how CHO-S cells may be influenced for improved protein production. Hence**,** the sequences for these tagged genes were released from EYFP-C1 plasmid vector expressed in U2OS cells using restriction digest enzymes following a sub-cloning approach and using the same restriction enzymes to cut and ligate into the expression vector pcDNA3.1CAT and pcDNA3.1Hygro.

Confirmation of fragment and vector sizes was carried out using agarose gel electrophoresis. YFP-DHCR7 was released from plasmid vector EYFP-C1 using Nhe1/Apa1, releasing the fragment at a predicted size of 2.1 kb from the 4.7 kb vector after which sub-cloning of this was done into the pcDNA3.1CAT and pcDNA3.1Hygro vectors (Figures 3.1 and 3.2). The sub-cloning of LBR, another target protein which was released as a 2.3 kb sequence from EYFP-C1 and thereafter cloned into pcDNA3.1CAT and pcDNA3.1Hygro plasmid vectors using the same restriction enzymes. Research on the LBR protein was discontinued and focus was on YFP-TM7SF2 and YFP-DHCR7 only as targets to improve the CHO.S secretory pathway machinery by expansion of ER.

Expression of the constructs was initially assessed using transient expression. Advances in transient mammalian expression systems enable high yields (>100 mg/liter) that now allow for effective recombinant antibody production at a reasonable cost (Vazquez-lombardi et al., 2017).

In one approach, we transiently transfected pcDNA3.1CAT constructs of YFP-TM7SF2 and YFP-DHCR7 where we monitored the localisation of the protein. Fluorescent microscopy was used to detect the presence of YFP-TM7SF2 and YFP-DHCR7 in the CHO.S cells (Figure 3.16). While the transfection efficiently was less than 30%, YFP signal was observed and confirmed the expression of the target proteins. Western blot analysis was also used to confirm expression of the target proteins. Different time points post-transfection were harvested and analysed using this approach. Results showed there was an increase in the expression of the proteins with increased post-transfection time. The calculated sizes were used to confirm expression. GFP was also transiently transfected as a control and observed as a 27 kDa band while YFP-TM7SF2 and YFP-DHCR7 were observed as 73 kDa and 81 kDa bands respectively. Although it was interesting to test product gene expression by transient approach system, a more desirable involves the generation of stable cell lines over-expressing these targets.

Quantitative evaluation was done to determine whether there was a significant level of protein expression in engineered cells when compared to controls. Densitometry results provided further evidence that CHO-S cells showed increased expression of YFP-TM7SF2 from day 1 to day 3 and although the expression observed on western blot given the exposure time analysed, the densitometry assay showed a gradual and consistent expression level as confirmed with the GFP control. On day 3, CHO-S cells were transiently expressing YFP-TM7SF2 protein nearly as much as they expressed GFP which suggests that the lipid machinery of the CHO platform was capable of proper protein folding and the proven ability of CHO cells to produce complex recombinant proteins and to perform human like post-translational modifications underlines the advantages connected with these host cells (Thoring et al., 2017) .

We have confirmed the stable CHO.S expression of the target proteins using hygromycin B selection, and also of eGFP and pcDNA3.1Hygro as controls. The strategies carried out to generate these stable cell lines have been detailed in this chapter. Stable cells were selected with Hygromycin B antibiotics. Indeed, the use of antibiotic selection methods may itself effect

transgenic protein expression (Kaufman et al., 2008). Biochemical and cytolocalisation studies suggested that the lipid targets are associated with the ER. However, not all CHO.S cells expressed these proteins and there was a variety of expression amounts observed across cells. Variance in their expression levels was confirmed by fluorescent microscopy analysis where pools of stable CHO.S cells expressing YFP-TM7SF2 and YFP-DHCR7 showed different growth patterns in the growth profile analysis carried out. It was therefore decided to investigate the higher expressing YFP-TM7SF2 and YFP-DHCR7 stable CHO.S by limited cloning dilution.

The studies here show that over-expressing YFP-TM7SF2 or YFP-DHCR7 stably in CHO.S over a 10-day cell growth period does not have toxicity to the CHO-S cells. Growth profiles were carried out with GFP and empty pcDNA3.1Hygro vector expressing CHO.S as controls. The different pools of stably expressing YFP-TM7SF2 and YFP-DHCR7 did not impede growth of CHO.S while empty pcDNA3.1Hygro expressing CHO.S cells showed a reduced growth pattern compared to the CHOS host alone. The over-expression of the two target proteins did appear to confer a growth advantage on the CHO-S cells. Therefore, the next focus was to consider the influence of this over-expression and impact on growth on production capacity of recombinant proteins. The next chapters therefore describe investigating whether these engineered cells can deliver enhanced expression of recombinant secretory proteins.

Chapter 4

Molecular Characterisation and Functional Consequences of the Transient and Stable Expressions of Transmembrane7 Superfamily member 2 (TM7SF2) on the Recombinant Protein Expression from CHO-S Cells

4.1 Introduction

This chapter describes the impact of the investigations on the engineered CHO-S cells developed and described in Chapter 3 of this thesis to determine if the overexpression of YFP-TM7SF2 enhances the ability of CHOS cells to produce target bio therapeutic recombinant proteins. As described in the introduction chapter of this thesis, recent years have witnessed the emergence of new engineered, non-nature bio therapeutic proteins, such as fusion proteins (e.g. Etanercept). These so called next-generation biologics can be significantly more difficult to express (DTE) compared to natural protein formats as generally host cells cannot correctly fold and process the recombinant polypeptide (Johari et al., 2015).

A number of gene manipulation strategies have been published with the aim of increasing the productivity of such DTE exogenous protein from a host cell line (Chalmers, 2016). For example, a previous work published on a novel CHO cell line which was developed by researchers contained two stably overexpressed genes thought to be capable of improving the efficiency of protein folding (Cain et al. 2013). In order to investigate the impact of overexpression of lipid target YFP-TM7SF2 on engineered mammalian CHO-S cells and their recombinant protein secretory capacity, CHO-S cells expressing YFP-TM7SF2 were transfected to transiently or stably express target recombinant proteins for investigation. Stable CHO-S expressing GFP and empty vector pcDNA3.1Hygro cell pools were also generated and used as controls to validate the impact of YFP tagged TM7SF2 expression levels on CHO-S cell phenotypes and behaviour.

Phenotypic studies included evaluation of engineered cells cellular secretory capacity to secrete recombinant protein products, investigated by transiently transfecting Erythropoietin (EPO) and etanercept, two major bio therapeutic recombinant proteins used in the clinic and manufactured using CHO cells by the biotechnology industry. Protein expression was characterised using gel electrophoresis and western blot analysis. The biological and phenotypic activities of the cells were

also analysed by characterisation of growth profiles and fluorescence microscopy analysis. mRNA transcript analysis was also attempted to investigate the correlation between the stable CHO-S expressing YFP-TM7SF2 protein and mRNA expression levels. The results and implications of these findings are discussed in this chapter.

It is noted that the work in this chapter uses in particular transient gene expression (TGE) systems to investigate the impact of overexpression of TM7SF2 on transient expression of recombinant bio therapeutic proteins. Transient expression is an attractive alternative for rapid production of research-grade protein during the early stages of drug development due to the substantial time and resources associated with stable cell line generation (Johari et al., 2015). As the preliminary characterisation of stable CHO-S over expressing YFP-TM7SF2 engineered cells using microscopy showed a wide variation in expression levels of YFP-TM7SF2 in CHO-S cell pools (Figure 3.14), limited dilution single cell cloning was undertaken to generate more clonal cell lines from the population of YFP-TM7SF2 stable CHO-S expression cells. Qualitative and semi-quantitative analysis was undertaken on these, including western blot, fluorescence imaging, growth characterization and immune staining localisation to investigate any impact on cell behaviour and the CHO-S ER in particular.

As described above, investigations into the molecular implications of over-expression of engineered cells on Erythropoietin (EPO) production levels has been detailed here. In addition, the chapter also investigates the fusion protein etanercept. This is protein that fuses the ligand binding domain of a receptor to neutralize or destroy the targeted protein or cells with an antibody Fc heavy chain constant region (see introduction chapter for more detail). Examples of targeted protein drugs are the antibody drug (Infliximab) and decoy receptor drug (etanercept) that sequester the tumor necrosis factor alpha (TNFα). Tumor necrosis factor alpha has been implicated in the pathogenesis of many human diseases such as graft-versus-host disease (GVHD), Crohn's disease, and several autoimmune diseases (Chiang et al., 2014).

The overall aim of this chapter was therefore to determine if there is/are any significant impact(s) on increased recombinant protein production in TM7SF2 engineered CHO-S cells with reference to control cell pools or lines and determine what influence this engineering exerts on CHO-S cell growth, secretory capacity and ER expansion. The implications of the findings are discussed

4.2 Results

4.2.1 Consequences of YFP-TM7SF2 over expression on growth characteristics of CHO-S cells

TM7SF2, like most of the enzymes involved in the cholesterol biosynthesis pathway, localises to ER membranes, and its expression is regulated by the sterol regulatory element binding protein (SREBP) in response to cellular cholesterol levels (Nikolakaki et al., 2017). In fact, the master regulator of the TM7SF2 is SREBP (Bennati et al., 2006). Further, reports have previously shown that expression of YFP tagged versions of TM7SF2 results in expansion of the ER membrane although the mechanism by which this occurs is unknown. We hypothesised therefore, that overexpression of TM7SF2 in CHO-S cells would influence the CHO-S ER membrane expansion and overall influence increase in secretory capacity of the cell and impact on cell growth characteristics.

A summary of the work, discussed below, shows that two stably expressing pools of YFP-TM7SF2 show different recombinant protein yield patterns and productivity levels. However, the significance of the expression is relative to controls. It has previously been reported that the overexpression of fully functional TM7SF2 can critically affect the ER and nuclear envelope morphology (Zwerger et al., 2010). Stable Pools of YFP-TM7SF2 (A and B), GFP expressing and empty pcDNA3.1Hygro in CHO-S cells were cultured in the presence of hygromycin B. The hygromycin-resistant CHO-S stable cells were cultured with several passages prior to experimental use.

The initial studies were to understand any impact of overexpression of YFP-TM7SF2 on cell growth and to confirm that the protein was indeed being expressed. To better understand the impact of overexpression of TM7SF2 on CHO-S, a 10-day growth profile study was carried out on YFP-TM7SF2 pools as well as the control (GFP expressing and empty vector containing) cell lines. Cells were sub-cultured to a starting concentration of $0.2x10^6$ viable cells/ml and cell number counted on day 0. This is as a result of the inaccuracy in numbers when cells were confluent and overgrown, hence the low starting concentration. The experiment was carried out in triplicate and average data analysed. The resulting growth data is shown in Figure 4.1. There was a very similar growth profile observed for all of the cells investigated with the two TM7SF2 pools showing similar

growth characteristics to the negative control and GFP expressing cells (Figure 4.1A). This pattern of growth was observed in repetitive cultures and therefore both pools had similarities in cell growth pattern as shown. Of the pools, TM7SF2B reached a higher maximum viable cell number by day 6 when compared to TM7SF2A, although TM7SF2A had a higher viable cell number on day 4. All cell pools showed a similar death pattern upon diminished nutrients. Thus, expression of the YFP-TM7SF2 protein does not appear to have a major negative impact on CHO-S cell growth.

The growth dynamics of the TM7SF2 pools suggest a similarity in the phenotypic and metabolic characteristics and therefore tend to behave in a typical way. However, the presence of the overexpressing lipid target appears associated with increased growth and reduced apoptosis as confirmed by comparison to the control. It was observed that TM7SF2 overexpressing cells grew faster and had prolonged viability compared to the control and by day 4 in culture, TM7SF2 pools was nearly double the amount of cells compared to the pcDNA3.1Hygro-expressing cells.

To further validate the data on increased growth of TM7SF2 engineered cells with reference to the control, and relate this to YFP-TM7SF2 expression, investigations into the expression and localisation of TM7SF2 was done. By confocal imaging, confirmation of expression levels of stable YFP-TM7SF2 in CHO-S cells was carried out, with GFP as a control. Cells were seeded at $5x10^5$ viable cells/ml on a pre-coated poly D-lysine cover slips with viability generally above 97%. After 24 h, cells were then washed with PBS and fixed using 4% PFA in PBS for 15 min (Figure 4.1B).

The data confirms expression of the target YFP-TM7SF2 in the cell pools but that there was differential expression between cells. This was also true for the GFP control. It is not possible from these data to confirm that expression of the TM7SF2 is localised to the ER. In essence, the rationale for overexpressing TM7SF2 as a target of driving ER expansion will not be achievable if certain phenotypic characteristics which are beneficial, are not first considered. Factors such as resistance to apoptosis, cell growth, quality and yield of recombinant protein products and also extended viability are of essence in the validation of the engineered cells.

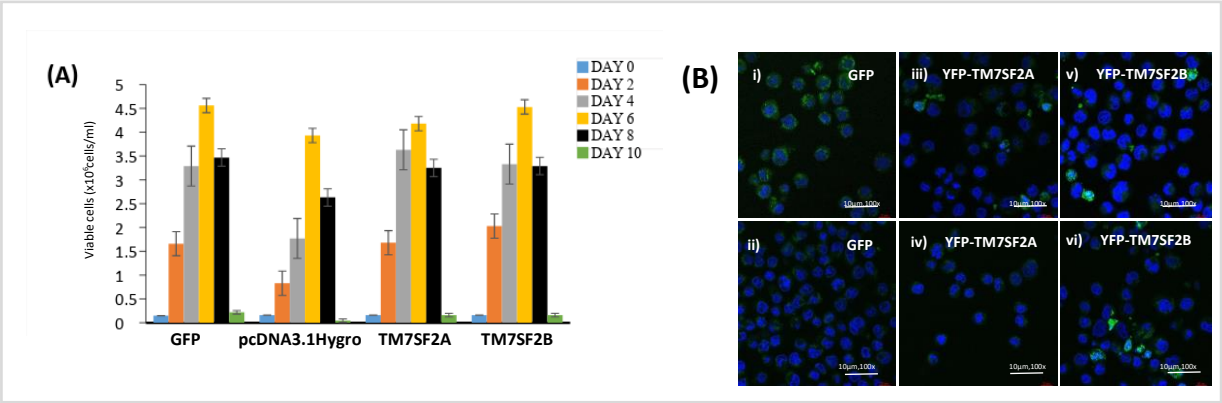

Figure 4.1 Analysis of stable YFP-TM7SF2 pools and a control GFP pool overexpression in CHO-S cells. (**A**) A 10-day growth profile of the two pools of YFP-TM7SF2 with GFP and pcDNA3.1Hygro controls stably expressed in CHO-S. Cells were seeded at $0.2x10^6$ viable cells/ml in a 20 ml media volume of a 125 ml shake flask in triplicates. TM7SF2B reached a higher maximum viable cell number than TM7SF2A on day 2 and day 6, although TM7SF2A had a higher viable cell number on day 4. Overexpressing TM7SF2 for both pools do not appear to inhibit CHO-S growth. Error bars represent standard error between three biological samples where n = 3. (**B**) Microscopy imaging comparing CHO-S expressing GFP and pools of YFP-TM7SF2. Pools of different passage numbers were randomly selected and analysed. YFP-TM7SF2A and B show sparsely-expressed YFP when compared to stable GFP-expressing CHO-S. YFP-TM7SF2B appears to have more cells expressing the target and this may relate to the growth pattern as shown in (A). Original images were collected with a 100x objective. Scale bar: 10µm.

4.2.2 Clones of CHO-S cells expressing different amounts of YFP-TM7SF2 differ in phenotypic characteristics

The specific attributes of large-scale mammalian cell cultures (for example, high level of spatial heterogeneity) demand a modelling framework that captures both the biology and the hydrodynamics of the system and also their interactions (Farzan & Ierapetritou, 2018). Mammalian cell culture systems are intrinsically heterogeneous at all scales from the genetic to bioreactor level (Davies et al., 2013). Having identified variation in expression levels of YFP-TM7SF2 engineered CHO-S cells, there was a need to distinguish the high-expressing clones from the pool which consists predominantly of low expressers as determined by fluorescence microscopy (see Figure 4.1B). Clonal variation for recombinant CHO cell lines does not originate only from functional heterogeneity in the host cell line, as genetic heterogeneity is also introduced during the generation of recombinant cell lines (Hansen et al., 2017).

The cell cycle is a complex regulatory network that influences not only growth and division, but also other relevant cellular events (for example; death and productivity) (García Münzer et al., 2015) hence, it is an important aspect of biological systems that impacts either directly or indirectly on the ordered series of events relating to cellular growth, death and productivity. Therefore there is the need for a better understanding and knowledge of the cell cycle timing, transitions and associated production profiles which can aid the development (modelling, control and optimisation) of industrially-relevant systems (Karra et al., 2010). In this case, differences in expression may be due to the site of integration into the genome (more or less active transcriptionally), mRNA amounts/turnover or stability, limitations in different cells abilities to synthesise the material and impact on cell fitness.

The approach used to isolate higher YFP-TM7SF2 expressers from the low, was the limited dilution cloning method. The methods utilised for this purpose are described in section 2.8.5. The presence of a single cell in wells, or potential clones, in each well of a 96-well plate was identified by using a microscope to examine cells of individual wells. Clones derived from potential single colonies were transferred to a 24 well plate once they had started to grow into colonies and the media amount doubled and cells grown at 37°C for a further week. After a week, the growing colonies from a single clone were further transferred to a 12 well plate and later into a 6-well plate.

In the development of the monoclonal cell lines of engineered YFP-TM7SF2 in CHO-S cells, clones were cultured over a period of time in the shake flask and the relative expression amounts identified in individual clones by western analysis. A Bradford assay was carried out to calculate the amount of protein required for equal loading on SDS-PAGE analysis. eGFP primary antibody, known to bind YFP, was used to probe for YFP-TM7SF2 expression while anti-mouse IgG whole molecule, peroxidase produced in rabbit from SIGMA was prepared at 1:5000 and used for secondary antibody and detection. A total of 11 clones of CHO-S overexpressing YFP-TM7SF2 from TM7SF2B pool which showed the higher expression of both pools were investigated (see Figure 4.2A). The size of the band observed was consistent with that for YFP-tagged TM7SF2 and was not due to YFP alone.

Of the clones investigated, six clones (C3, C4, C7, C8, C1O and C11) of the total of eleven clones were considered to belonging to higher expressers/producers of YFP-TM7SF2 generated from TM7SF2B clones (numbered C1-C11). Upon confirmation of YFP-TM7SF2 expression and the relative amounts by western blotting, growth profiling was also analysed for the clones to identify the relative growth characteristics in relation to YFP-TM7SF2 expression (Figure 4.2B). To confirm that expression of the different amounts of YFP-TM7SF2 as determined by western blotting in Figure 4.2A did not negatively impact on CHO-S cell growth, batch-culture growth curves were setup and followed. The resulting data from days 3 to 7 are shown in Figure 4.2B. On day 6, clones 4, 10 and 11 achieved the highest viable cell number when compared to the pcDNA3.1Hygro negative control engineered cells. This pattern is in agreement with the blot representation as shown in Figure 4.2A. On day 6, all 6 clones were either at, or close to the maximum viable cell number but this dramatically reduced on day 7, presumably due to nutrient deprivation. This was observed in control too.

Capturing the dynamic behaviour of the system sometimes requires considering multiple phases for cellular growth and death, during which cells react differently to environmental stimuli, Xing et al. 2010. Since it is a known fact that variability exists in steady state protein expression levels among the variants, we hypothesised that there might be feedback implications in the viability rates hence the need to assess clonal cells' viability over time-course. The viability profile of clones further validates the stability of TM7SF2 engineered clones of CHO-S cells. This positive impact in a batch culture of a 10-day period eliminates the query of whether there may be an impedance in growth of stable clones (see Figure 4.2C).

The representative plot shows the average of triplicate samples for each clone as we monitored the viability of culture over the time course. One would expect, that individual clones would show a particular pattern of growth as a result of variance from distinct phenotypic characteristics. With a starting viability of about 97%, clones showed an overall rather impressive cell density and culture viability when compared to the control. The viability of the engineered clones remained high and above 80% until day 8. From this characterisation of YFP-TM7SF2 expression and cell growth, the remainder of the study focused on the 6 of the 11 clones that appeared to exhibit the highest expression levels and potentially an ability to achieve increased maximum viable cell numbers.

Thus, a key difference between the clones and the control was their response to growth. While pcDNA3.1Hygro showed a fast and steady decline in viability from day 0 to day 7, TM7SF2 clones grew slower but with robust viability as growth was maintained to almost a constant state during the exponential phase between day 3 and day 6. At this stage the comparison of clones with the control has been at the level of the growth characteristics.

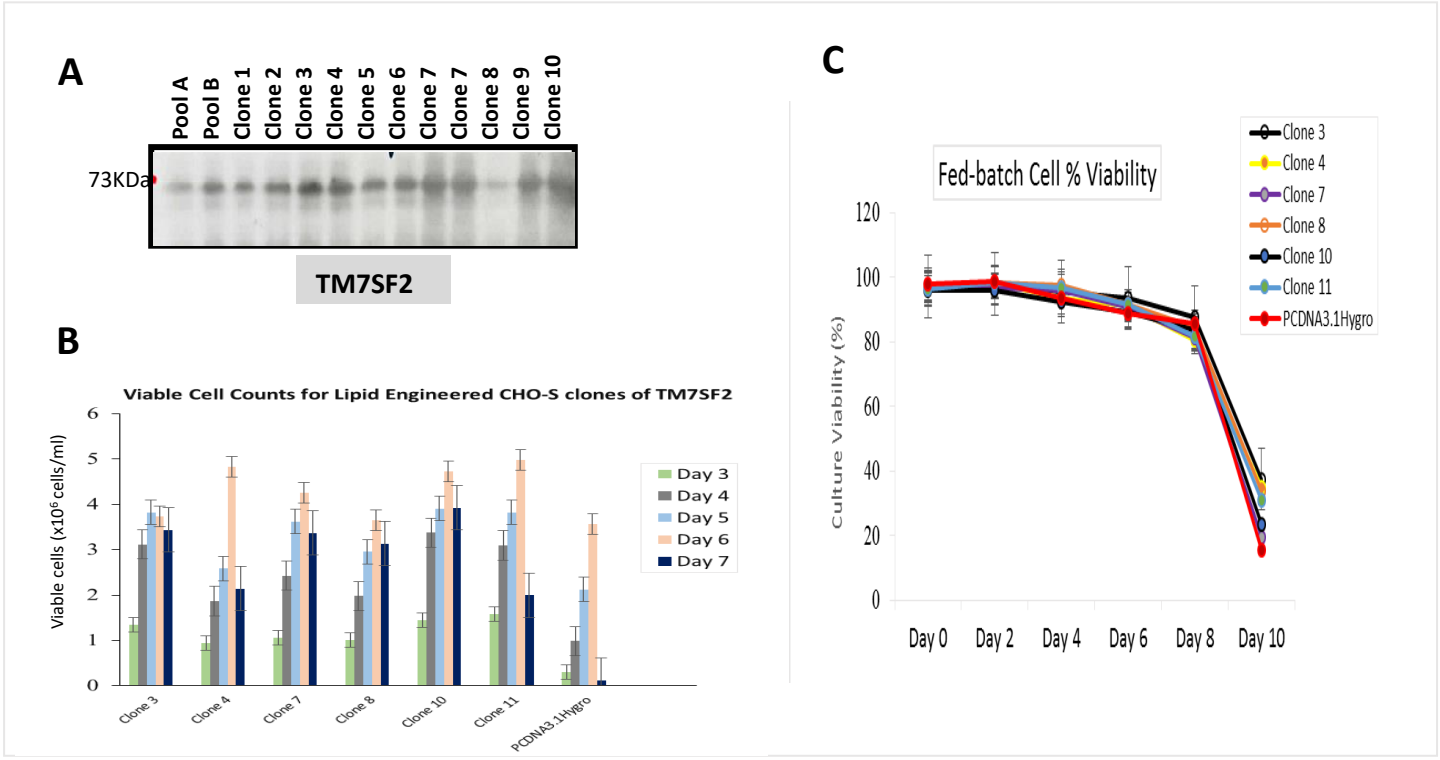

Figure 4.2 CHO-S YFP-TM7S2 expressing engineered clones exhibit variation in YFP-TM7SF2 expression and growth profiles and in Culture viability (**A**) Western blot analysis probed for YFP showing 11 individual clones generated from both pools of YFP-TM7SF2A and TM7SF2B. 20 ug of total protein from prepared samples was loaded onto SDS-PAGE. Clones show a varied YFP signal of YFP-TM7SF2 protein molecular weight of 73 kDa. Clones 3, 4, 7, 8, 10 and 11 were identified as higher expressers when compared to the other clones and pools. **(B)** Growth profiles of 6 of the 11 clones generated for YFP-TM7SF2. Clones were sub-cultured and seeded at $0.2x10^6$ viable cells/ml and grown for 10 days. A cross-section of 5 days is shown here. At day 3, all 6 clones show increased growth when compared to negative control pcDNA3.1Hygro. There was variation between clones in terms of the maximum viable cell number obtained with clone11 achieving the highest. Error bars represent standard error of replicate biological samples where n = 3. **(C)** CHO-S expressing TM7SF2 were cultured for a 7-day period for six high expresser clones (Clone 3, Clone 4, Clone 7, Clone 8, Clone 10 and Clone 11) as well as pcDNA3.1Hygro control. Clones were seeded at $0.2x10^6$ viable cells/ml and in replicates. Cell viability was observed to be above 80% as all clones showed cells of post exponential phase by day 7. Error bars represent standard error of replicate biological samples where n = 3.

4.2.3 Investigation of the cell morphology and localisation of YFP-TM7SF2 fluorescent protein in CHO-S cells stably expressing the protein

To try and assess if the YFP-TM7SF2 protein was localised to the ER, and hence could potentially have a positive impact on the expansion of the ER membrane and influence the secretory pathway capacity, it was important to investigate the cellular localisation of the expressed protein. It has been reported that the human sterol reductase, TM7SF2 is located at the endoplasmic reticulum (ER) and functions at distinct steps of the cholesterol biosynthesis pathway (Nikolakaki et al., 2017). Confocal microscopy was carried out to investigate the localisation of YFP-TM7SF2 which had been reported to be present, along with some other secretory pathway proteins, in the ER (Bellezza et al., 2015).

Stably-generated YFP-TM7SF2 clones in CHO-S were therefore analysed for localisation studies where the presence of YFP would indicate the subcellular localisation of YFP-TM7SF2. The results (Figure 4.3) revealed the six different clones showed different expression signals in CHO-S nucleus, potentially ER and cytoplasm. YFP was detected at an emission signal of 527 nm when its excitation peak was 514 nm. Comparison of subcellular localisation showed YFP being expressed at the outer region of the nuclear envelope where the outer nuclear membrane compartmentation of ER is localised (Figure 4.3). While YFP signal was observed across all clonal cells, the level of signal and presentation of nucleus observed somewhat differed across the clones.

Microscopic evidence confirmed that each clone showed peculiarity both in expression levels and also in cellular morphology. Panel A, for instance, which assessed clone 3 showed cells to be presented in a layer-like formation with smaller cells attaching to bigger cells while the signal level appear reduced when compared to other clones. Clone 4 cells showed a rather different condensed presentation as clones appear in groups, YFP signal is observed to be fully expressed as overlay of DAPI and YFP images of panel B cells showed YFP expression covered in the intercellular bridges in a synchronistic form. The same differential cellular morphology was observed in clones of panel C to F. Variation in presentation pattern may be related to different growth dynamics. For example, YFP-TM7SF2 Clone7 cells appear in a cluster-like presentation and cell nucleus appearing relatively larger than YFP-TM7SF2 Clone 11 cells when imaged at same magnification. YFP-TM7SF2 Clone 3

cells show similarity in cell morphology to YFP-TM7SF2 Clone 10 cells but expression signals differ between both clones.

The YFP-TM7SF2 clone 4 (Figure 4.3B) showed a pattern that would be consistent with ER localisation. In the production of an expansive ER membrane frame network system in CHO-S cells, the cellular levels of this biological target is key in driving the alteration of normal cellular processes of lipid biosynthesis with YFP signalling confirming the presence of TM7SF2 over expression reported to be functional in the outer nuclear membrane channel of the cells. Meanwhile, from the data presented (Figure 4.3), there are no evidences to confirm the site of YFP signal expression from this data.

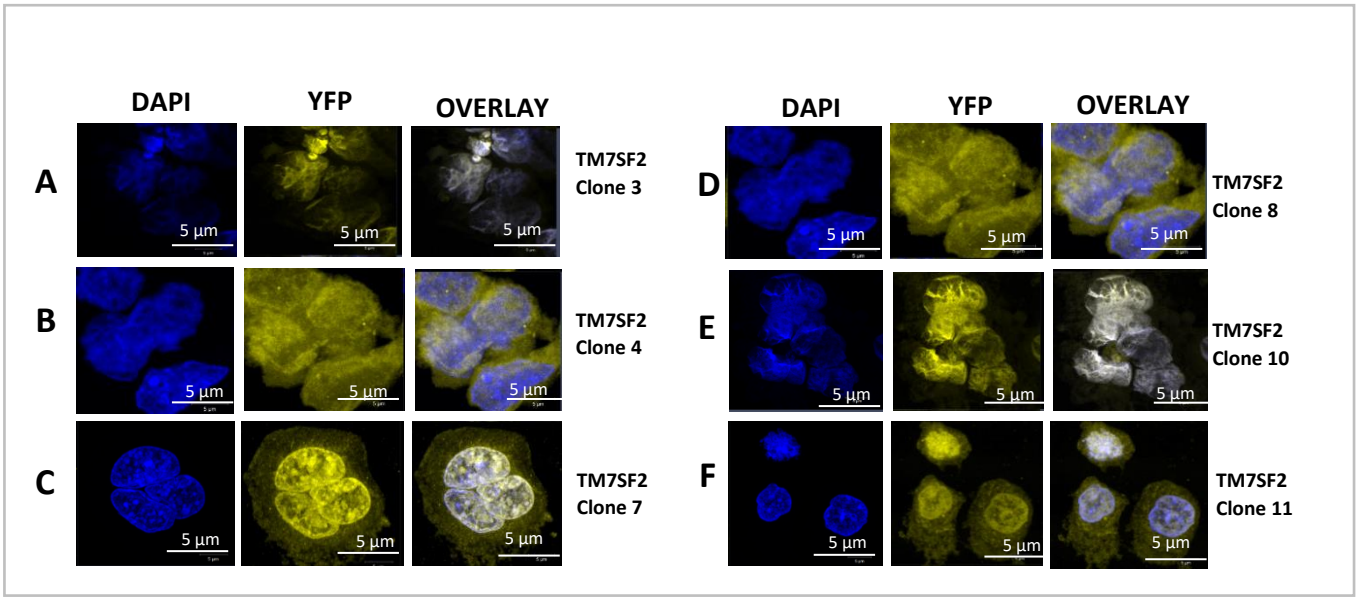

Figure 4.3 Confocal microscopy of clones generated for CHO-S overexpressing YFP-TM7SF2. Stable cells overexpressing YFP-TM7SF2 were seeded at 0.2 x 10^6 viable cells/ml and fixed using 4% PFA. Upon washes with Tween, nucleus was DAPI-stained and YFP signal detected using confocal imaging. Images represent cellular presentation of ER-localised TM7SF2 where expansion has been predicted for improved capacity secretory pathway. Each clone showed peculiar cellular morphology as YFP signal is observed to be relative to individual clones. (**A**) Clone 3 presents in a layered cellular formation with smaller cells clumping to bigger cells and presents in a conformation similar to clone 10. YFP signal varies across clones although all clones showed significant YFP expression level. (**B**) Clone 4 cells (which presents in a condensed cellular conformation appear to have larger cell size. (**C**) Clone 7 cells however showed conformation similar to clone 11 with a higher YFP signal. Images are shown relative to 5 µm size bar. Expression levels appear to vary with each clone and some clones show similarity in presentation such as clones 3 and 10.

4.2.4 Further Profiling of ER targeted YFP-TM7SF2 by microscopy to confirm subcellular localisation in engineered CHO-S cells

The ER network membrane is the site for much of lipid biosynthesis, hence is an important site for controlling expansion and control of secretory pathway capacity. Correct protein folding and modifications of most secretory and membrane proteins depends on the availability of molecular chaperones and enzymes located in the ER such as protein disulfide isomerase (PDI), calnexin (CANX), calreticulin (CALR), glucose-regulated protein (GRP78/BiP) and glucose-regulated protein (GRP94) (Bode et al. 2012).

Further investigation into whether engineering of CHO-S cells to express YFP-TM7SF2 results in localisation to the ER and if any influence on the appearance of the CH0-S ER was carried out by examining the isolated clones for immunolocalisation studies whereby the localisation of YFP-TM7SF2 with calnexin, a molecular chaperone which helps nascent proteins during folding and assembly and remains retained in the ER, was investigated. The ER was therefore marked using calnexin, as a reporter of compartmentation where YFP-TM7SF2 co-localises and to further analyse phenotypic characteristics for ER expansion. In order to define the ER, anti-calnexin antibody was used to visualise the ER and compared to the YFP signal from the YFP-TM7SF2 of clones to probe the connection between YFP-tagged TM7SF2 and the ER resident secretory protein calnexin.

In order to do this, engineered and control CHO-S cells of culture viability over 96% were seeded at $0.2x10^6$ viable cells/ml on a poly D-Lysine coated cover slip placed in 24-well plates. 24 h later, cells were washed briefly in PBS before being fixed in 4% PFM for 15 min at 37°C. Following several washes, BSA blocked cells were incubated with anti-calnexin antibody, prepared in BSA/PBS and slides were incubated overnight at 37°C in a dark humidified chamber. For the immunofluorescence *in-situ* hybridisation 50 µl drops of 1:100 TRITC-mouse was introduced onto slides in a light sensitive, darkened area. After 2 h reaction time, antibody was washed using Tween-PBS while cell nucleus were DAPI stained for 1 min before washing off with PBS. DAPI (4'6-diamidino-2-phenylindole) is a blue-fluorescent DNA stain that exhibits ~20-fold enhancement of fluorescence upon binding to the A-T region of dsDNA. It is commonly used as a nuclear counterstain for the visualisation of nuclear DNA in both living and fixed cells where it acts as a DNA-specific probe

thereby forming non-fluorescent intercalative complexes with double-stranded nucleic acids. DAPI staining was used to confirm and identify the presence of the nucleus of CHO-S cells.

When YFP-TM7SF2 expression was compared to calnexin localisation, the aim was to investigate whether the two were co-localised. The presence of DAPI confirmed the position of the nucleus allowing the monitoring of highlighted YFP of TM7SF2 protein in the ER membrane compartment. The co-localisation observed for YFP-TMSF2 and calnexin further confirmed that TM7SF2 is targeted to the ER secretory pathway and accumulation of this transmembrane protein may impact on ER secretory capacity. The confocal microscopy also confirmed that TM7SF2 clones had variation in expression levels as clusters of cells express YFP. Clones 3 for instance appear to produce more TM7SF2 proteins than clone 5. Clones 6 and 7 appear to have similar expression levels while presentation of clone 8 is somewhat different than as expected (Figure 4.4).

This is in agreement with the previously reported localisation studies that the TM7SF2 gene resides in the ER (Roberti et al., 2002). Thus, the YFP tag does not appear to effect localisation. Results generated from microscopy show four images of representative clones at different excitation wavelengths for respective parameters. DAPI, Calnexin, YFP and the merge of all three. All images are shown at the same magnification. Calnexin was observed to spread around the nucleus into the cytosolic space which defines the ER subcellular compartment of the cell (see Figure 4.4).

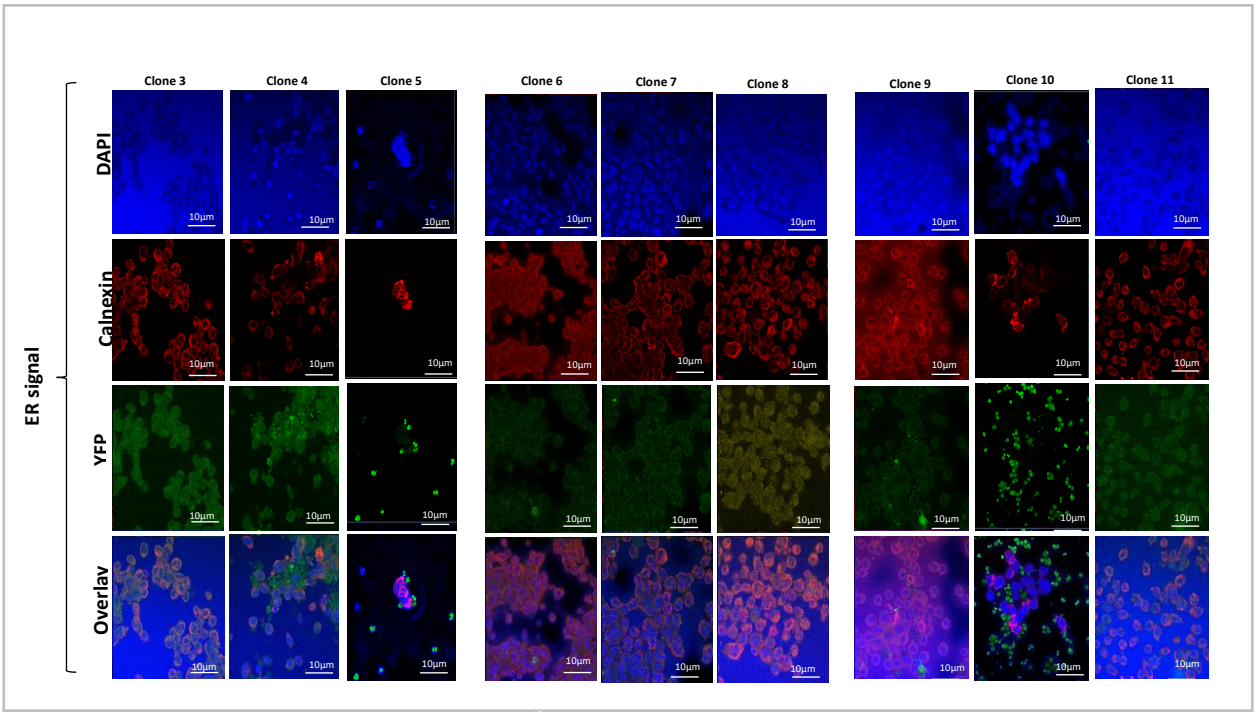

Figure 4.4 Immunofluorescence microscopy analysis to investigate ER localisation of YFP-TM7SF2 in different engineered CHO-S clones by co-localisation with the ER resident protein calnexin. Fixed CHO-S cells section were stained with primary anti-calnexin and a secondary anti-rabbit TRIT-C and DAPI to monitor and determine any cellular compartmentation of stably overexpressing YFP-TM7SF2 in ER membrane. Clones show the expression signals for calnexin and YFP of YFP-tagged TM7SF2. Clones show different expression levels of YFP through the GFP channel. Here, an examination of the clones was done without the GFP and pcDNA3.1Hygro expressing control cell lines, which would have been beneficial for comparative analysis to determine whether there was an influence or changes in the ER membrane structure on over-expression of YFP-TM7SF2. Co-expression of calnexin and YFP-TM7SF2 indicate the targeting of TM7SF2 to the ER. Original images were collected with a 100 x objective. Scale bar: 10 µm.

4.2.5 The impact of YFP-TM7SF2 overexpression in CHO-S cells on Transient EPO expression

Since the ultimate objective of this study was to improve the recombinant protein productivity of CHO-S cells, investigations into whether secretory productivity was impacted from the YFP-TM7SF2 genetically modified CHO-S cells were undertaken. Genetic modification of host cells can be a promising strategy to eliminate or reduce the bottlenecks in recombinant protein expression (Zhao et al., 2012), although few such systems have yet been used industrially for the large manufacturing of recombinant bio therapeutic proteins. Although protein secretion in mammalian cells is a complicated process, the genetic engineering strategies based on the overexpression of individual components of the secretory pathways might not always improve product secretion (Rahimpour et al., 2016). By altering TM7SF2 expression, we hypothesise that cholesterol biosynthesis may be altered, leading to ER expansion as previous reported and thus increase secretory capacity and secreted recombinant protein products yield.

However, EPO is considered difficult to express and is a highly glycosylated protein. Many studies have been conducted to try and increase the production of recombinant human EPO (Trummer et al., 2006) which is widely used for clinical purposes in the treatment of anaemia that results from chronic renal failure (Walsh 2014). In the studies here, we investigated the impact generated on transient recombinant EPO secretion in the engineered YFP-TM7SF2 expressing CHO-S cells compared to GFP expressing and pcDNA3.1Hygro control cells. Both extracellular and intracellular relative amounts of this protein were determined using western blotting. Two pools of stable CHO-S overexpressing YFP-TM7SF2 (A and B) were transfected with 4 µg EPO plasmid DNA as well as the control cell lines and cell supernatants processed and analysed using western blot to determine EPO secretion levels. In additional studies, clones of YFP-TM7SF2 engineered CHO-S cells were transfected with EPO and recombinant yields determined by western blot.

4.2.5.1 Effect of YFP-TM7SF2 expression in CHO-S cells on recombinant transient EPO expression

To determine if expression of YFP-TM7SF2 had any impact (positive or negative) on EPO secretion, we conducted a transient transfection of recombinant EPO on both pools of TM7SF2 and the control cell line (GFP). In this study, EPO plasmid DNA was transfected using lipofectamine 2000 and Opti-MEM® a day after stable cells were seeded at $0.2x10^6$ viable cells/ml in a 6-well plate containing 2 ml of cell media. Transfection was undertaken on the same cell pools and lines at different passage numbers. For instance, in Figure 4.5A, transfections were carried out in replicate with TM7SF2 pools A and B and GFP stables of CHO-S cells after passage numbers 5 and 9 whilst for pcDNA3.1Hygro this was of passage number 9. On day 2 after transfection, cells were collected and supernatants analysed. Equal volume of samples were loaded onto SDS-PAGE gels.

The recombinant EPO contained a His-Tag and an anti-his-tag antibody was used to detect EPO expression at approximately 37 kDa. Generally, as described below, when analysed by western blotting there was an increase in the amount of transient EPO detected in both the YFP-TM7SF2 engineered CHO-S pools when compared to the GFP and pcDNA3.1Hygro control cells (see figure 4.5A). In order to further investigate the impact of over-expression of YFP-TM7SF2 on EPO expression, a further study was done where transfections of TM7SF2 pools A and B with controls were carried out in replicates and 200 µl cell media collected on day 1, day 2 and day 3 post transfection and the samples analysed for EPO expression.

To analyse the samples from this experiment, 15 µl of prepared supernatant was loaded onto SDS-PAGE gels, protein samples transferred onto a nitrocellulose membrane for 1 h and then western blotting, using a conjugated anti-EPO (anti-his tag HRP) antibody, undertaken. The antibody was diluted to 1/1000 before use and the western protocol followed as described in section 2.12. By day 1, no EPO expression was detected in the control cell samples (pcDNA3.1Hygro, GFP) and very weak bands at the size expected observed from the YFP-TM7SF2A and YFP-TM7SF2B cell pools (Figure 4.5B). By day 2 post transfection, EPO was detected in control samples and the YFP-TM7SF2 although there was little difference in observed expression levels, unlike that in Figure 4.5A. By day 3, the YFP-TM7SF2B pool (that previously was shown to have higher YFP-TM7SF2 expression than pool B) did have increased EPO expression compared to controls although the YFP-TM7SF2A pool did not show an increase compared to the controls (Figure 4.5B).

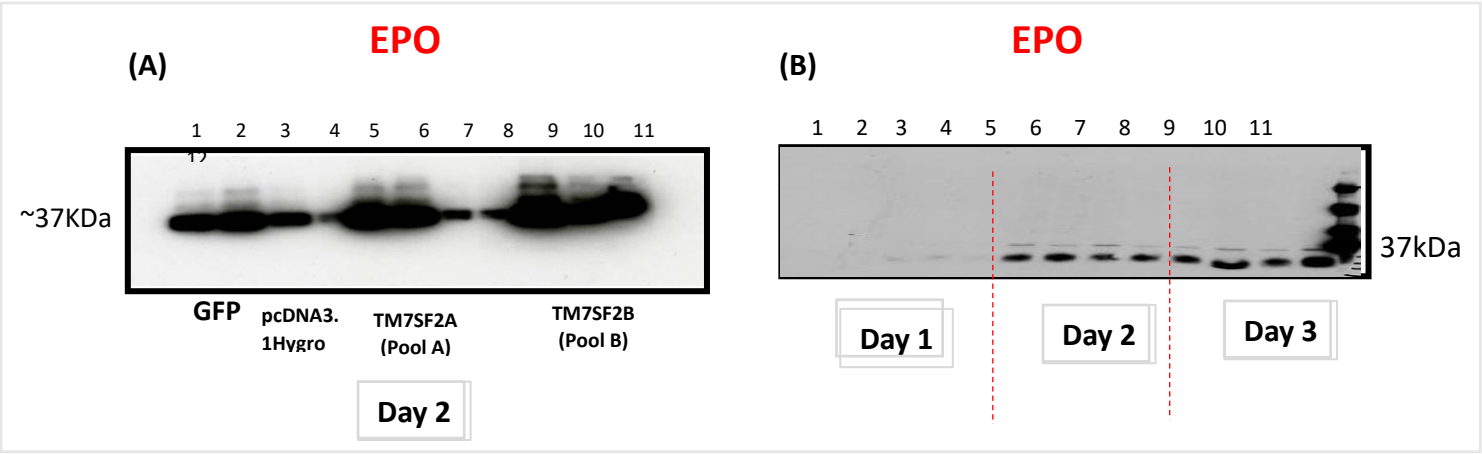

Figure 4.5 Western blot analysis reveals increased EPO expression in CHO-S YFP-TM7SF2B pools compared to control cells. Transient EPO transfection was carried out to investigate the secretion amount of EPO in CHO-S overexpressing YFP-TM7SF2 pools A and B along with GFP control. 4 µg EPO plasmid DNA was transfected into cells of different passage times. Experiments were completed in 6-well experimental plates where CHO-S cells were seeded equally at 0.2 x 10^6 viable cells/ml to a 2 ml total cell media per well. **(A)** Representative blot shows increased EPO expression on day 2 in YFP-TM7SF2A and B expressing CHO-S cell pools when compared to GFP control. Analysis of the different passage time points of construct showed similar expression signals. **(B)** Blot shows experimental study of transient EPO transfection in CHO-S constructs of YFP-TM7SF2A and YFP-TM7SF2B as well as GFP and pcDNA3.1Hygro controls. 200 µl cell media was collected per well of replicate samples for all constructs and analysed. EPO was detected by using a conjugated anti-His tag HRP antibody at about 37 kDa. An increased expression signal was observed for TM7SF2B unlike A, on day 3. It is not clear why controls showed more expression than TM7SF2A and B on day 2. Samples were loaded respectively as follows; Lanes 1, 5, 9 = pcDNA3.1Hygro. Lanes 2, 6, 10 =GFP control. Lanes 3, 7, 11 = YFP-TM7SF2A while Lanes 4, 8, 12 = YFP-TM7SF2B.

To further investigate whether there was a positive impact of overexpression of YFP-TM7SF2 pools on CHO-S secretory capacity, EPO band intensities from the western result (Figure 4.5 A and B) were analysed using quantitative numerical values using densitometry to quantify the difference in EPO production where comparison was done with GFP and pcDNA3.1Hygro expressing control cells analysed on day 2 post transfection. Upon calculation of a percentage and fold-change increase relative to the control samples at each time-point, there was a considerable increase in the YFP-TM7SF2 B pool EPO expression than the A pool as shown (Figure 4.6A). A similar pattern was observed

in another study where post transfection time was extended over a 3 day period. YFP-TM7SF2B pool again showed to have expressed increased EPO levels unlike pool A (Figure 4.6B) by day 3 although was almost same till on day 2. It appears therefore that the B pool of YFP-TM7SF2 was expressing EPO more than the A pool hence, conferring a positive impact on the engineered CHO-S secretory machinery.

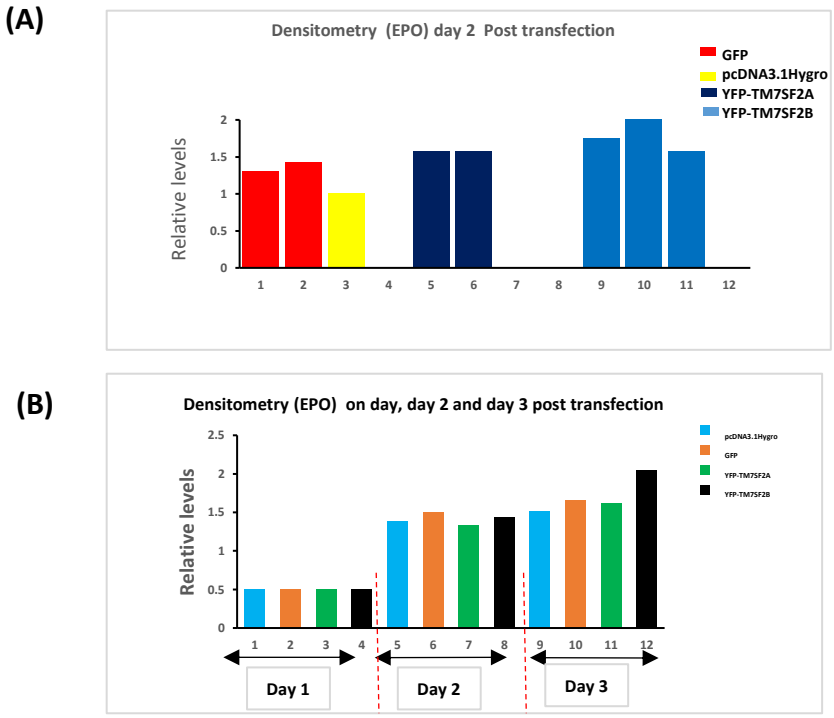

Figure 4.6 Densitometry analysis of the western blots images shown in Figure 4.5. Analysis was undertaken using the Image J freeware. The density of each band was determined and then normalised to the GFP and pcDNA31Hygro controls for quantitative analysis of expression levels of pools of lipid engineered CHO-S. (**A**) TM7SF2A pool cells expressed EPO on day 2 post transfection at a decreased level than TM7SF2B pools, while both pools showed higher EPO secretion levels (**B**) Quantification of EPO expressed at day 1, day 2 and day 3 post transfection. Again YFP-TM7SF2B showed increased quantity of EPO expression when compared with YFP-TM7SF2A pools on day 3.

4.2.5.2 YFP-TM7SF2 engineered CHO-S Pool B cells deliver higher transient EPO productivity over a batch culture than control cells

CHO-S cells overexpressing YFP-TMSF2 pools A and B were grown in batch culture in 6-well plates where cells were seeded at 0.2 x 10^6 viable cells/ml in triplicates. Viable cell counts were undertaken throughout culture (Section 2.7.3). The YFP-TM7SF2B pool showed increased EPO expression level on day 3 (lane 2) compared to the control cells, unlike YFP-TM7SF2A pools which did not appear to have increased EPO expression over that observed in the control samples. When analysed along with the controls, GFP (lane 4) and pcDNA3.1Hygro (lane 5), YFP-TM7SF2B pools showed much higher expression levels of EPO in supernatants compared to the controls whereas the YFP-TM7SF2A pool did not appear to show any increased EPO secretion. This experiment was carried out along with YFP-DHCR7 engineered CHO-S cell pools (Chapter 5), as shown on lanes 3 and 8 for all time points and this is discussed in detail in chapter 5. When comparing expression on day 3 with day 8, both YFP-TM7SF2B and 2A pools showed increased expression signal when compared to controls (Figure 4.6).

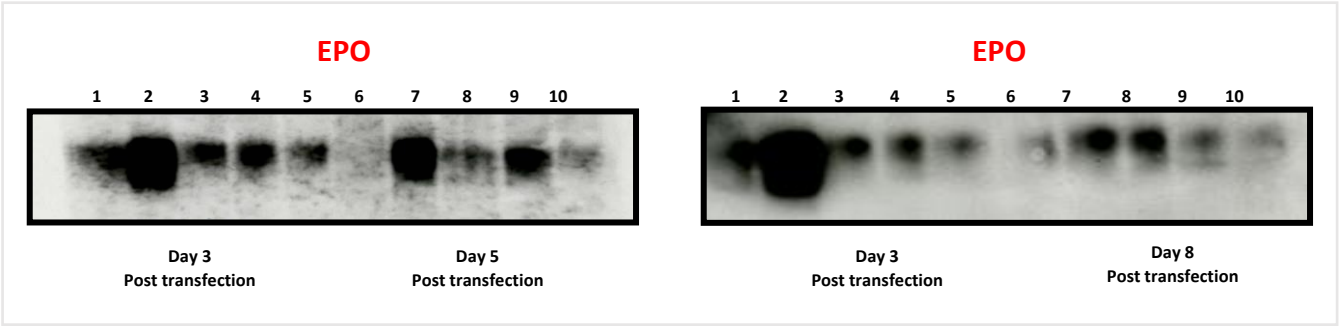

Figure 4.7 Western blot analysis of transient secretory EPO expression on day 3, day 5 and day 8 post-transfection in YFP-TM7SF2, YFP-DHCR7, GFP and empty vector control expressing CHO-S pools. EPO was detected using anti-his HRP antibody and represented blots were analysed at same exposure time of 2 min. 20 µl supernatant samples were analysed for EPO expression in YFP-TM7SF2 pool A (lanes 1 and 6) and pool B (lanes 2 and 7), YFP-DHCR7 pool A (lanes 3 and 8), GFP expressing (lanes 4 and 9) and pcDNA3.1Hygro empty vector control (lanes 5 and 10) CHO-S cell samples.

The western blots of Figure 4.7 were quantified using densitometry where further evidence showed that YFP-TM7SF2 B pool did produce more EPO than YFP-TM7SF2 A pool from day 3 post transfection to day 8 post transfection with an increase of over 1.00-fold on the average. This confirmation agrees with findings reported in section 4.2.5.1 where the YFP-TM7SF2 pools had been transiently transfected with EPO, in another study, along with GFP and pcDNA3.1Hygro expressing CHO-S cells. While the submissions are interesting to know, it would be ideal to extend the culture, longer than day 3 and examine EPO productivity beyond as product quality tend to depreciate in harsh culture conditions.

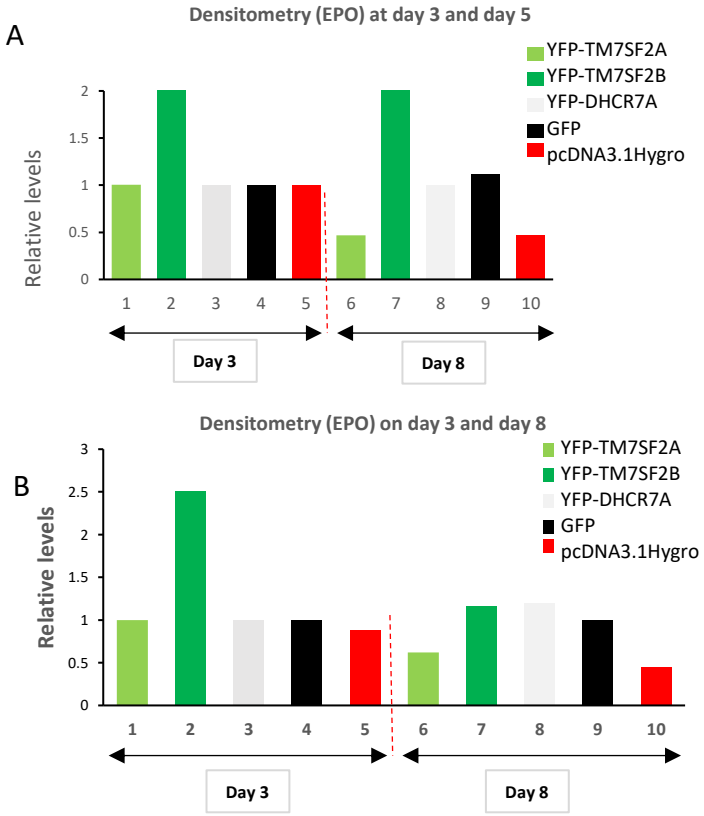

Figure 4.8 Densitometry analysis of the western blots presented in Figure 4.7 Supernatants were loaded equally per each sample presented. Quantitative analysis of transient EPO expression confirms YFP-TM7SF2 B pool doubled in expression levels over the controls and YFP-TM7SF2 A pool on day 3 and day 5. By day 8, where there might have been a decline due to nutrient deprivation, although YFP-TM7SF2 B pool showed a drastic decline, EPO expression was near twice as much of YFP-TM7SF2A and pcDNA3.1Hygro controls. Densitometry confirms, there was a positive impact of over-expression of YFP-TM7SF2 B pool on CHO-S ER secretory system. Numbers 1 to 5 (A and B) represent YFP-TM7SF2A, YFP-TM7SF2B, YFP-DHCR7A, GFP and pcDNA3.1Hygro on day 3. Numbers 6 to 10 (A) represent YFP-TM7SF2A, YFP-TM7SF2B, YFP-DHCR7A, GFP and pcDNA3.1Hygro on day 5 while numbers 6 to 10 (B) YFP-TM7SF2A, YFP-TM7SF2B, YFP-DHCR7A, GFP and pcDNA3.1Hygro on day 8.

4.2.5.3 Effect of YFP-TM7SF2 expression in a range of CHO-S engineered clones on transient EPO secretion

The studies described earlier in this chapter established that six of the eleven generated clones of CHO-S overexpressing YFP-TM7SF2 showed increased YFP signal when compared to pools and these are being identified as the high expressers of YFP-TM7SF2 (section 4.2.2). To further investigate the impact of the impact of expression of YFP-TM7SF2 on secreted recombinant protein production, transient EPO transfection was carried out on all eleven clones. Cells were seeded at $0.2x10^6$ viable cells/ml and EPO DNA transfected using NovaCHOice™ transfection reagent and booster as previously described (section 4.2.5.1). Western analysis was used to detect EPO expression in all eleven clones (Figure 4.7), although no GFP and pcDNA3.1Hygro controls are presented in Figure 4.7. As earlier reported, we observed that six of the eleven generated clones showed increased EPO expression of which the 6 clones identified as high producers had increased EPO expression compared to controls. Clones 3, 4, 7, 8, 10 and 11 appear to have increased expression of YFP-TM7SF2 in CHO-S. EPO was produced more in all 6 clones and also clone 2, which was not observed as a higher YFP-TM7SF2 producer. This experiment focused on investigating whether EPO secretion levels in each clone had any relationship with amount of lysates expressed as shown in Figure 4.2A.

Note that this study was carried out without a loading control, which accounts for the validation of the outcome of findings on western blot due to common errors in loading. Hence when this study was undertaken, a useful direction would have been to load a control, for the interpretation of western blots as to whether there was a change in abundance of YFP-TM7SF2 clonal cell lines being expressed per well. Meanwhile, it is important that the protein levels of loading controls remain constant (relative to the total protein content) under the test conditions (Johnson 2018). In other words, in future, it would be good to express a protein loading control such as Beta-actin with the cells presented on western blots. Beta-actin is a common loading protein and is usually present in cells at high concentrations. Others include Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Tubulins (Alpha tubulin and Beta tubulin), Lamin B1 amongst others.

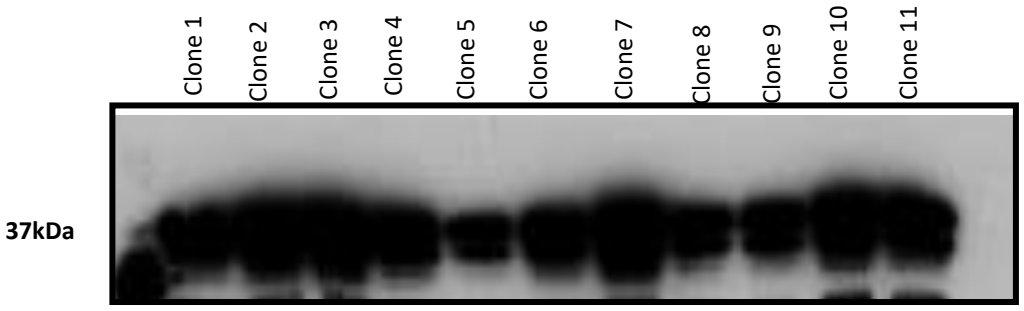

Figure 4.9 Western blot analysis of EPO expressed in CHO-S clones engineered to express YFP-TM7SF2. Upon isolating 11 clones overexpressing YFP-TM7SF2, 6 were selected as high producers. EPO was expressed in all 11 clones and supernatants assayed using conjugated anti-histag antibody. Interestingly, all 6 earlier selected clones as high YFP-TM7SF2 expressers showed enhanced EPO secretion compared to other clones (Clone 3, Clone 4, Clone 7, Clone 8, Clone 10 and Clone 11) whilst Clone 2 also exhibited enhanced EPO expression compared to other clones. Experiment

4.2.6 The impact of lipid engineered TM7SF2 over-expressing CHO-S cells on recombinant and transient etanercept expression.

To further investigate whether engineering of the CHO-S host with YFP-TM7SF2 had a general impact on the transient secretory capacity of the cell to generate recombinant proteins, and to compare with the transient EPO expression studies that showed increased secretion in the lipid engineered cells when compared with controls, a second bio therapeutic molecule was transient expressed. In this case the molecule was etanercept, an important target drug molecule manufactured by the biopharmaceutical industry. Etanercept, although originally developed for rheumatoid arthritis and Crohn's disease, has also demonstrated beneficial activity in other inflammatory diseases (Genovese et al., 2005). Transient expression studies were therefore undertaken to investigate the effect of engineering the CHO-S with YFP-TM7SF2 on secretory etanercept production. The strategy involved

transfecting an etanercept plasmid into stably expressing YFP-TM7SF2 cells and analysing the amount of secreted product in the supernatant collected at different times across a batch culture.

4.2.6.1 Effect of YFP-TM7SF2 expression in CHO-S cells on recombinant transient etanercept expression

To determine the effect of YFP-TM7SF2 expression on recombinant etanercept secretion, transient transfection studies were carried out as described in section 4.2.5.1 in triplicates in 6 well-plates and 200 µl of cell culture media collected on day 1, day 2 and day 3 post-transfection. Supernatant was collected, centrifuged and appropriate SDS-PAGE Laemmli sample loading buffer of 4x was used to prepare samples. Equal volume loading of samples at 15 µl were loaded onto the SDS-PAGE gel for electrophoresis after which protein samples were transferred onto a nitrocellulose membrane probed for the presence of etanercept using anti-heavy chain antibody overnight and a secondary anti-rabbit IgG peroxidase antibody for detection.

For each harvest, the secreted amounts of etanercept from the YFP-TM7SF2 pool A and YFP-TM7SF2 Pool B CHO-S engineered cells were investigated alongside the GFP and pcDNA3.1Hygro control cell pools. After western blot analysis it was observed that on day 1 post-transfection, there was weak expression from all pools although the YFP-TM7SF2 pools were less than (Pool A), or approximately the same as (Pool B), the GFP control (Figure 4.10A). However, by day 2 post-transfection etanercept amounts were elevated in the YFP-TM7SF2 pool B compared to the control pools whilst the pool A cells had similar amounts to the empty vector control (Figure 4.10A).

When day 3 expression was investigated, it was observed that all constructs expressed etanercept at different expression levels, with pool B and the GFP control showing similar amounts and the pool A and empty vector control having less, but similar amounts. The YFP-TM7SF2 pool B cells showed a general trend of increased etanercept expression compared to the other pools from day 1 to day 3 post-transfection (Figure 4.10 A).

Again, we investigated clones of TM7SF2 Pool B with pcDNA3.1Hygro as a control. For this experiment, stable cells were seeded in 6 well-plates and in replicates with a starting concentration

of 0.2x10^6 cells/ml in 2 ml media per well. A transient transfection experiment was set up as described in section 4.2.5.1. On day 2 post-transfection, supernatant was collected and prepared using SDS-PAGE Laemmli sample buffer. Samples were equally loaded on the SDS-PAGE gel for electrophoresis and thereafter prepared for blotting. Anti-heavy chain antibody was used as primary antibody which was allowed to react to a secondary anti-rabbit IgG peroxidase after day 1 (section 2.4.3) and proteins were later assayed using western blotting. Findings from data showed that clones showed different expression levels of etanercept with clone 2, clone 3, clone 8 and clone11 as top expressers when compared to pcDNA3.1Hygro.

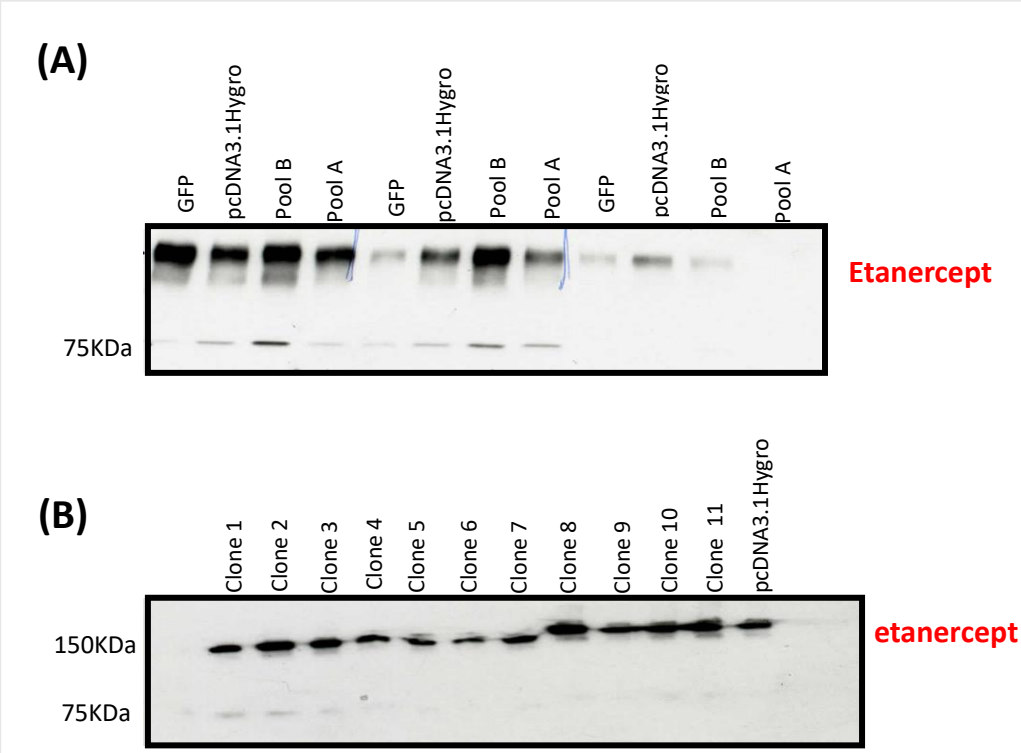

Figure 4.10 Western blot analysis of the ability of YFP-TM7SF2 engineered CHO-S cell pools in **(A)** and clones in **(B)** to express and secrete the recombinant bio therapeutic protein etanercept compared to a GFP engineered and empty vector engineered CHO-S pool. **(A)** YFP-TM7SF2 Pool B generally shows increased expression from day 1 to day 3 post-transfection compared to other pools. **(B)** Eleven generated YFP-TM7SF2 engineered CHO-S clones were assayed for their ability to secrete etanercept and compared to the amounts from an empty vector pcDNA3.1Hygro CHO-S control. Different expression levels were observed from the clones with clones 2, 3, 8, 10, 11 showing increased etanercept expression.

Following western blot analysis, there was the need to quantify the amount of etanercept being expressed by the engineered cells relative to control cells. Densitometry was used to determine the level of expression of etanercept in YFP-TM7SF2 pools on day 1, day 2 and day 3. Data showed that on day 1, there was a fairly similar level of expression between control cells and lipid engineered cells. However by day 2, there had been a drastic increase in the secretion amount allowing YFP-TM7SF2 B pool to express Densitometry confirms the data from western blot as there was a gradual increase across all cell lines on day 3 and even at that, YFP-TM7SF2 B pool maintained an enhanced etanercept secretion level, transiently (Figure 4.11).

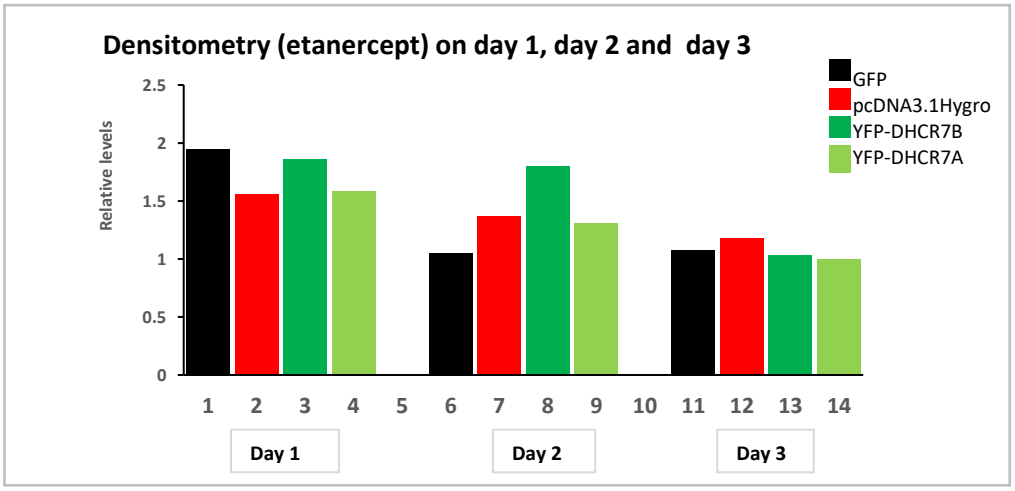

Figure 4.11 Densitometry analysis of the western blot shown in Figure 4.10. Levels of etanercept expressed in lipid engineered cells and control cells. Pools of TM7SF2 were transfected with etanercept and harvested on day 1, day 2 and day 3 post transfection. Experiment undertaken with GFP and pcDNA3.1Hygro control cells. Densitometry data suggest an increase in etanercept expression on day 1 in YFP-TM7SF2 B pool and pattern was still maintained on day 2 as compared to YFP-TM7SF2 A pool.

4.2.7 Effect of Expression of YFP-TM7SF2, EPO and etanercept transient transfection on CHO-S cell growth and viability during batch culture

An important aspect to this study was to determine the effect, if any, of YFP-TM7SF2 and transient EPO and etanercept expression on CHO-S growth over a batch-culture time course (section 4.2.2). It was hypothesized that expression of YFP-TM7SF2 would improve cell growth and product yield resulting in an overall enhancement in cell productivity. On days 0, 3, 5 and 8 post transfection, samples were taken to assess viable cell numbers and culture viability for both EPO and etanercept transfected cells. Data obtained (Figures 4.12 and 4.13) show that generally, the viable cell numbers increased steadily during the time of experimental study and culture viability of pools transfected with EPO and etanercept had a similar pattern of growth as both YFP-TM7SF2 CHO-S expressing pools had a similar culture viability compared to controls. This thus confirms that the expression of the YFP-TM7SF2 did not negatively impact viable cell division and culture viability when expressing a recombinant bio therapeutic load.

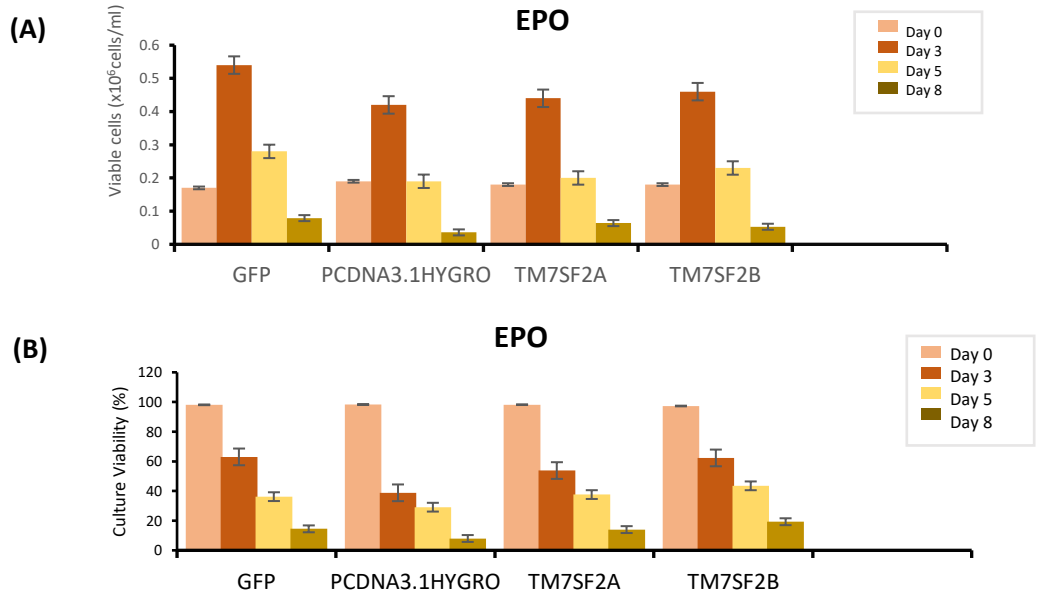

Figure 4.12 Characterisation of batch cell index versus culture viability of CHO-S engineered pools transiently transfected with EPO over 8 days of culture. Cell counts were done during the time course of study as cell numbers remarkably changed. By day 3, cells had doubled in cell size as TM7SF2 pools maintained a higher growth pattern than negative control. Counts were taken on days 0, 3, 5 and 8 post transfection. Assay for viability of stable TM7SF2 pools with controls showed a similar pattern in growth from day 0 to day 8 of culture. On day 8, YFP-TM7SF2 engineered cell pools showed higher viability over negative control. With p value < 0.05 determined by students T-Test when comparing YFP-TM7SF2pools to the controls GFP and pcDNA3.1Hygro, there was no statistical significance at $p<0.05$. Error bars represent standard error of the mean of triplicate biological samples (n =3).

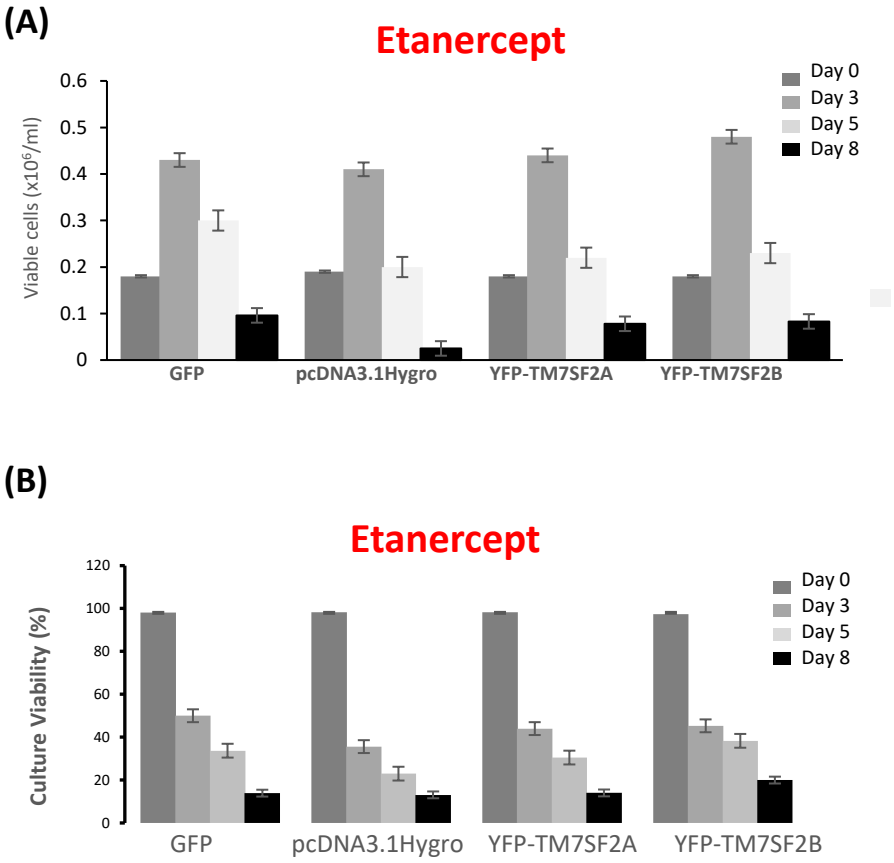

Figure 4.13 Characterisation of batch cell index versus culture viability of CHO-S engineered pools transiently transfected with etanercept over 8 days of culture. **(A)** Cell counts were done during the time course. By day 3, cells had doubled in cell size as TM7SF2 pools maintained a higher growth pattern than negative control a similar observation seen when EPO was transiently expressed. Counts were taken on days 0, 3, 5 and 8 post transfection. Error bars represent standard error of the mean for three biological samples (n =3). **(B)** A similar viability pattern was observed for both pools of lipid engineered cells and the controls. Cells maintained a steady decline in growth 8 days post transfection. TM7SF2 pools maintained a higher yield than negative control (pcDNA3.1Hygro. No significant inference at $p<0.05$ as determined by students' T-Test when comparing YFP-TM7SF2 pools to the controls GFP and pcDNA3.1Hygro where n = 3.

4.2.8 Investigation of YFP-TM7SF2 mRNA and protein amounts in engineered CHO-S cells

In order to determine if protein expression levels of CHO-S engineered to express YFP-TM7SF2 correlated with mRNA yield, engineered clones and pools cell pellets were harvested and RNA isolated from the cells in the exponential growth phase. Cells were lysed according to QIAprep^{R} Miniprep protocol. Following purification, the RNA extract was transferred into a sterile micro centrifuge tube and stored at -80°C. In order to determine if mRNA transcript of YFP-TM7SF2 was present, pre-designed primers for GFP in the laboratory were used that would also be appropriate for detecting YFP. Analysis was performed in a 96-well reaction plate. For confirmation of the GFP primers, a PCR analysis was carried out using a GFP template which is a plasmid vector GFP and the presence of an amplicon of the expected size verified by running an agarose gel along with a no DNA negative template control (Figure 4.14).

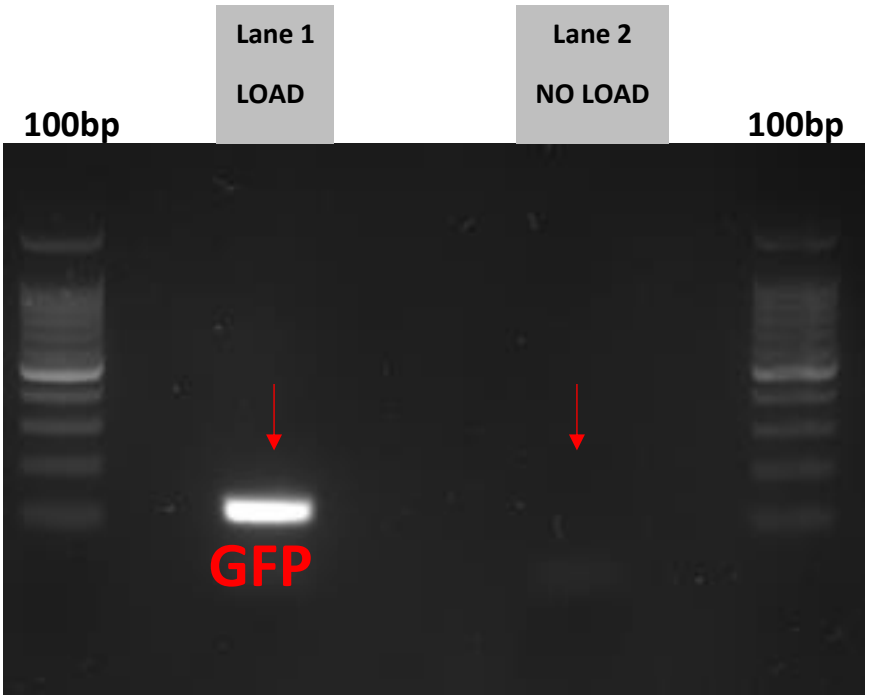

Figure 4.14 Agarose gel electrophoresis analysis showing PCR amplification product for GFP, DNA positive control template quality check. A band of the expected amplicon size of 100 bp was observed at the expected size for the positive control (Lane 1) and no band with the negative control (no DNA, Lane 2). Separate master mixes (25 µl prepare sample) were prepared for each master mix and housekeeping gene primer pairs were prepared on ice under sterile conditions. In lane 1, the GFP positive control master mix had sterile nuclease-free water (8.5 µl), SYGR Green intercalating dye (12.5 µl), GFP Forward primer 10 µm (0.75 µl), GFP Reverse primer 10 µm (0.75 µl), Reverse transcriptase (0.5 µl) and GFP (2 µl), as template. For the master mix of 25 µl prepared in lane 2, all contents as prepared for lane 1 were added except without a GFP template.

Following confirmation that the GFP primers gave a band of the expected size with a positive template, extracted RNA samples from the YFP-TM7SF2 cells to be analysed by qRT-PCR were first DNAse treated and then the PCR reaction undertaken In order to verify that mRNA transcript was present and a band of the expected size was generated, the reaction products from the PCR reaction for the clones and pools were analysed along with samples from known amounts of a positive template (Figure 4.15).

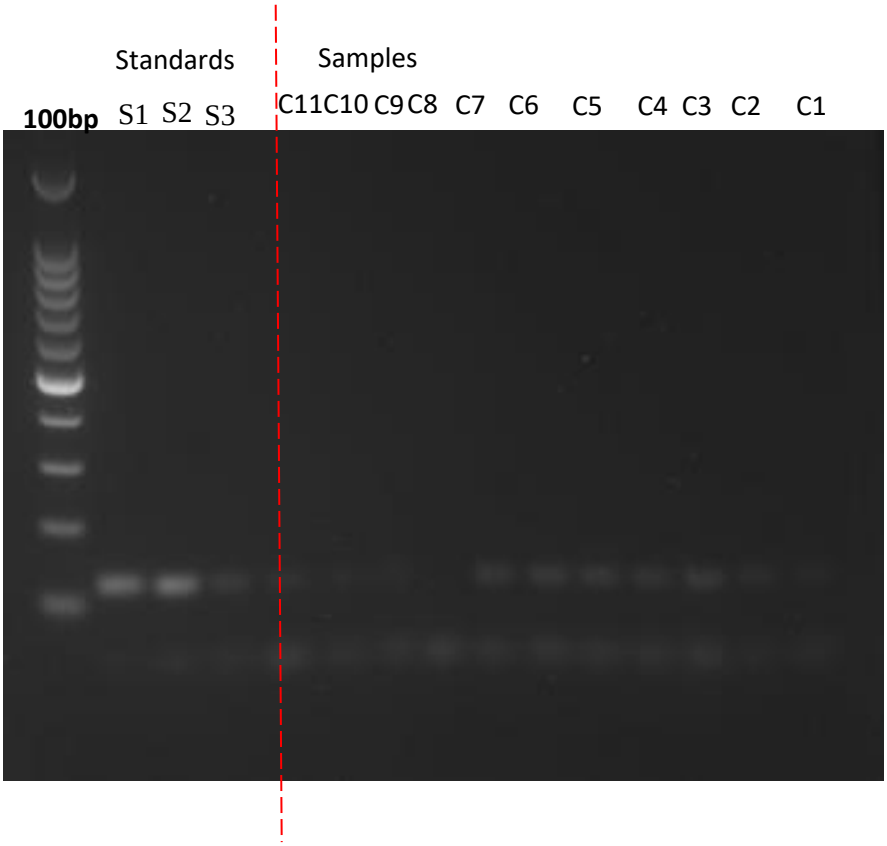

Figure 4.15 Agarose gel electrophoresis analysis of RT-PCR analysis for presence of YFP transcript in mRNA samples generated from YFP-TM7SF2 expressing CHO-S cell pools and samples. Lanes 1, 2 and 3 represent bands from a positive GFP containing plasmid with differing known copy numbers whilst lanes 5 to 13 contain samples from different pools and clones Bands of the expected size were observed, however these were also observed in the absence of the reverse transcriptase enzyme suggesting the bands originate from genomic DNA. Whilst this does not allow any conclusions about mRNA amounts to be made, it confirms the presence of the YFP gene sequence integrated into the genome. Standards were generated from standard curve (10^1 to 10^6) onto qPCR reaction plate as sample set RNAs which had previously been reverse transcribed.

The transcript analysis data in figure 4.15 and that from a PCR without any reverse transcriptase (no RT, data not shown including the PCR data from the non-engineered cells) showed bands of the expected size. The fact these were observed in the no RT reaction shows DNA template must be present to allow amplification without mRNA being reverse transcribed into cDNA. Whilst this was disappointing and did not allow any conclusions around the amount of mRNA in different clones and pools to be evaluated, it did confirm that YFP was present in the genome and successful integration into the CHO-S as the band was not observed in samples from host, non-engineered CHO-S cells.

4.3 Conclusions and Summary Statements from this Chapter

The overall aim of this study was to gain an understanding of the impact of engineering CHO cells on recombinant protein production and investigations in this chapter have been summarised broadly into two. The consequence of TM7SF2 and DHCR7 over-expression on CHO cells' growth and productivity and the investigations into the localisation of TM7SF2 and DHCR7 as ER resident proteins with confirmation studies using calnexin. By limited dilution cloning approach, a total of eleven clones from TM7SF2 pool B and six clones of total eleven identified as top producers/expressers of TM7SF2 by western blot techniques hence focus for the rest of the study was on these six.

Generally, the ability of CHO cells to reach high cell densities in suspension conditions, in chemically-defined media free of serum, is a key principle that underpins the process of recombinant protein production from mammalian cells growing in culture (Whitfield 2014). When these targets were engineered in CHO cells, the propensity was for cells to reach a maximum level in growing culture. There is a need for a clear understanding of the effect of cell culture parameters obtainable from engineered cells with its influence on recombinant protein production. By qualitative analysis, our data shows that proliferation rate of experimental cultures was higher when analysed with GFP and pcDNA3.1Hygro expressing control cells with increase in rate of growth and turnout of EPO and etanercept secretion level. The effectiveness of EPO and etanercept delivery from TM7SF2 and DHCR7 have shown that EPO and etanercept production increased transiently with increased exposure time.

Further investigations showed the heterogeneity of clones with variations in expression capacity, an observation reported also by Pedrazzini et al., (2003), that the high abundance of transmembrane proteins in the ER can induce extensive membrane growth, whereas low expression does not alter ER morphology. With the identification of cells, we carried out localization studies where we identified distinct expression levels of each clone. When we carried out western blotting, the observation confirmed the increased TM7SF2 pool B expression over TM7SF2 pool A and interestingly related with the growth profiles analysed for the parent pool and resulting generated clones. When we expressed EPO in the clones, the expression level varied significantly and was not unconnected to their TM7SF2 expression levels. The cells over-expressing TM7SF2 produced more biomolecule products than

controls and the B pool of TM7SF2 remained the more producer/expresser of EPO and etanercept when both pools were analysed. This findings was consistent across different experiments and further investigations should be done to optimise the approach.

An investigation into the impact of transient EPO and etanercept expression on CHO-S cells' viable cell numbers and viability showed there might have been a positive influence by engineering the cellular circuit of CHO-S secretory system by over-expressing YFP-tagged TM7SF2 and YFP-tagged DHCR7 sterol reductases of the cholesterol biosynthesis pathway. When EPO and etanercept were expressed over a time course, both pools of YFP-TM7SF2 showed increased EPO expression (Figure 4.6) and as post transfection time increased, it was observed that EPO expression level increased.

It is important to note that the B pool of YFP-TM7SF2 maintained a higher level of expression which suggests that these cells have a higher cell division so as to maintain complete coverage of cholesterol biosynthesis. Considering the importance and the efficient regulation of cholesterol metabolism and transport, it is likely that these finely tuned mechanisms will become altered during the high velocity of cell division and membrane synthesis needed for carcinogenesis (Cruz et al., 2013). As expression levels of EPO and etanercept appeared to have increased in lipid-engineered CHO-S as presented on western blots, there was a need for a further analysis as to how much of these recombinant proteins were expressed with reference to GFP and pcDNA3.1Hygro control CHO-S cells, hence densitometry analysis was undertaken for Protein (EPO and etanercept) quantification as measured by band intensity using Image J. Note that all constructs of proteins were quantified by Bradford assay to allow for equal loading on SDS PAGE gel.

Densitometry data generated for Western blots are commonly used to compare protein abundance between samples (Butler et al., 2019) by measuring intensities of specific bands, corresponding to the proteins of interest using commercially available software. For example, when EPO bands presented on western blots (Figure 4.9) were analysed using densitometry, the relative amounts of each protein ratio was quantified with lipid-engineered cells compared to controls. It was confirmed that B pool of YFP-TM7SF2 over-expressing cells produced more transient EPO over a time course before a decline (day 3 to day 8) than the A pool, suggesting there was a positive impact of overexpressing YFP-TM7SF2B on CHO-S secretory system. However, the increase in EPO and etanercept observed on western blots and as confirmed by densitometry was not statistically significant at a probability of

$p<0.05$. Some other assays could also be used to quantify EPO and Etanercept, which could require the use of traditional methods such as the measurement of Ultraviolet (UV) absorbance at 280 nm, Bicinchoninic acid (BCA) and Bradford assays, as well as alternative methods like Lowry, Biuret or novel assays developed by commercial suppliers whereas individual protein quantitation methods include enzyme-linked immunosorbent assay (ELISA) assay, western blot analysis, and more recently, mass spectrometry, among others (Johnson 2018).

To probe the localisation of TM7SF2, Calnexin, an ER chaperone protein served as an ER marker for verification studies of YFP-TM7SF2 as both calnexin and TM7SF2 co-localise to the ER. Morphological presentation showed YFP-signal of TM7SF2-engineered cells in the inner nuclear membrane along with calnexin. Although the size of ER was not monitored as at the time of this report, the identification of calnexin in the ER, where TM7SF2 is reported in the literature to be localised further supports the involvement of the transmembrane protein in ER secretory processing.

However some other methods have addressed the ER monitoring by using the ER tracker dye (for example, the bodipy green or bodipy red ER which binds to potassium channels in the ER by targeting glibenclamide) where the ER is stained for easy detection and size analysis. We compared our results with previous findings on TM7SF2 overexpression studies in other cell lines and our findings may not be peculiar to CHO-S as studies done on TM7SF2 using other cell lines also confirmed that the presence of wild type TM7SF2 might also alter membrane properties, e.g. membrane fluidity or permeability. (Herrmann et al., 2009). Attempts to correlate mRNA with protein levels was not successful. Overall, our study shows promising data that overexpressing TM7SF2 can positively impact on secreted recombinant protein production from CHO-S cells.

Chapter 5

Molecular Characterisation and Functional Consequence(s) of Expression of YFP-tagged 7-dehydrocholesterol reductase (DHCR7) in CHO-S Cells

5.1 Introduction and Objectives

As described earlier in this thesis, over half the bio therapeutic protein products on the market are made in mammalian cells (Walsh, 2014). Although mammalian cell culture is generally more complicated and expensive than microbial cultivation, the former is often preferred for the production of bio therapeutics because mammalian cells are capable of performing human-like post-translation modifications of proteins, especially glycosylation (Walsh & Jefferis, 2006). Indeed, mammalian cells are currently the main hosts for commercial production of many therapeutic proteins, particularly monoclonal antibodies and antibody like molecules (Lee et al., 2009). The majority of these products are made in CHO cells, where the ER plays a major role in the folding and assembly of these products and hence the focus in this chapter was on CHO-S cells and the ER secretory system when expressing YFP-DHCR7.

In this chapter, stable over-expression of YFP-DHCR7 was undertaken and the impact investigated on CHO-S cell recombinant protein secretory yields. Such engineering of CHO cells for increased survival/growth/viability, have been attempted to try and increase the capacity of mammalian cells and by far, the greatest demand for recombinant mammalian proteins is for therapeutic development and applications (Bandaranayake & Almo, 2014). To try and reprogramme the CHO-S cell ER for improved secretion of protein yields, we manipulated YFP-DHCR7, a terminal enzyme in the Kandutsch-Russell pathway of cholesterol synthesis reported to occur in the ER. When the lipid engineered CHO-S cells overexpressing YFP-DHCR7 were generated, two pools were isolated. Subsequently, by using a limited dilution cloning approach, we identified a number of relatively high YFP-DHCR7 expressers compared to the rest of the cells isolated and these were further characterised.

In order to assess the level of recombinant protein production from YFP-DHCR7 engineered CHO-S cells in relation to controls, model protein-based bio therapeutics were used for the basis of establishing the impact on the CHO-S cell secretory machinery, and in turn, any impact on cell growth and viability investigated. To elucidate the impact on secretory productivity, two model biomolecules were investigated, Erythropoietin (EPO) and etanercept, which were transiently transfected into CHO-S cells, as discussed in Chapter 4, and the yields from YFP-DHCR7 engineered cells compared with control cells (stable CHO-S expressing GFP and an empty pcDNA3.1Hygro vector engineered CHO-S control cell pool) using western blotting techniques. In addition, cell growth, cell morphology and localisation of YFP-DHCR7 were studied. DHCR7 localisation was assessed by fluorescent microscopy studies and co-localisation of YFP-DHCR7 with calnexin (an ER marker) for characterisation of YFP-DHCR7 compartmentalisation.

5.2 Results

5.2.1 The Effect of YFP-DHCR7 Expression on CHO-S Cells' ER Secretory System

DHCR7 is a human sterol reductase, located at the ER and functions at distinct steps of the cholesterol biosynthesis pathway (Bennati et al., 2006) (Porter, 2008). It has been reported that both DHCR7 and TM7SF2 (discussed in Chapter 4) are two structurally related sterol reductases and that a comparable phenotype in susceptible cells lines is observed when a forced expression of DHCR7 or TM7SF2 is placed on the cell (Herrmann & Zwerger 2010). Due to the reported similarity in phenotypic expression of DHCR7 and TM7SF2, we further investigated the effects of over-expression of YFP-DHCR7 on the CHO-S host cell and ER expansion and secretory system. By analysing YFP-DHCR7, the possibility of enhancing recombinant protein production in CHO-S cells by stable over-expression was explored.

The impact of YFP-DHCR7 expression on CHO-S cells was compared with that observed when GFP was expressed and to cells engineered to express an empty vector pcDNA3.1Hygro control, both stably expressed in CHO-S. The empty vector control means that all cells under assessment have been through the same selection process and have the same metabolic load other than the GFP control and YFP-DHCR7 having the additional load of an additional exogenous protein (the GFP where YFP-of DHCR7 is to account for this). Several investigation strategies confirm there was a positive impact of YFP-DHCR7 overexpression on the accumulation of recombinant protein expression levels as detailed below. Stable hygromycin-selected CHO-S expressing YFP-DHCR7 cells were generated and cultured and subsequently two distinct pools were isolated with different proliferation rates and secretory productivity levels. We further characterised these two distinct pools both qualitatively and quantitatively. A 10-day growth profile analysis was initially carried out to examine the cell proliferation rate of CHO-S cells over-expressing YFP-DHCR7 in comparison to the empty vector and GFP expressing CHO-S controls.

For the growth curve studies, cells were seeded at $0.2x10^6$ viable cells/ml in 125 ml shaker flasks in triplicate and cells counted from Day 0 and then every day to determine the total cell number, viable cell number and culture viability. The resulting data is presented as the average of the replicate cultures (Figure 5.1A). The two YFP-DHCR7 expressing pools, designated YFP-DHCR7A and YFP-DHCR7B, showed similar growth patterns to each other and the two controls. Interestingly, both pools

showed an initial faster growth rate than the pcDNA3.1Hygro control and on days 2 and 4 had approximately twice the number of viable cells compared to the empty vector control although only a small difference was observed compared to the GFP control cell numbers than the pcDNA3.1Hygro control on day 8 of culture.

To confirm expression of the YFP-DHCR7 molecule in the cell pools isolated, qualitative analysis by fluorescent microscopy to detect YFP expression as a reporter for DHCR7 was also undertaken (Figure 5.1B). YFP-DHCR7 lipid engineered stable cells from a culture viability of approximately 96% were seeded on D-lysine pre-coated cover slips in 24-well plates at $5x10^5$ viable cells/ml with 600 µl cell media at 37°C. Cells were washed 24 h later using PBS and subsequently fixed with 4% PFM. Following a BSA block and several TBST washes (section 2.3.4), images of fixed cells were taken using Leica® Fluorescent microscope (Figure 5.1B). These data showed YFP expression in both engineered cell pools and a variety of expression levels between cells as would be expected in a cell pool. However, these data only confirm expression of YFP, not of YFP-DHCR7 as the YFP could be cleaved from the fusion molecule and hence western blot analysis was also undertaken as described in the next section.

5.2.2 Screening of YFP-DHCR7 Engineered CHO-S Cells Shows they Exhibit Different Amounts of YFP-DHCR7 Expression

During the generation of stable CHO-S over-expressing YFP-DHCR7 pools, two pools were isolated. Because mammalian host cells have been reported to vary considerably in their intrinsic ability to manufacture a given recombinant protein independent of transgene copy numbers (Char Aniya et al., 2009), we investigated clonal populations of the heterogeneous YFP-DHCR7 pool A, generated using a limited dilution cloning approach. This allowed the isolation of seven clones which were further characterised. Clones were generally cultured in a 125 ml shaker flask in 20 ml cell culture volume and regularly sub-cultured in the presence of hygromycin selection and investigated using western analysis. Western analysis would also confirm that the full, intact YFP-DHCR7 molecule was expressed or if any fragmentation or degradation of the molecule was observed.

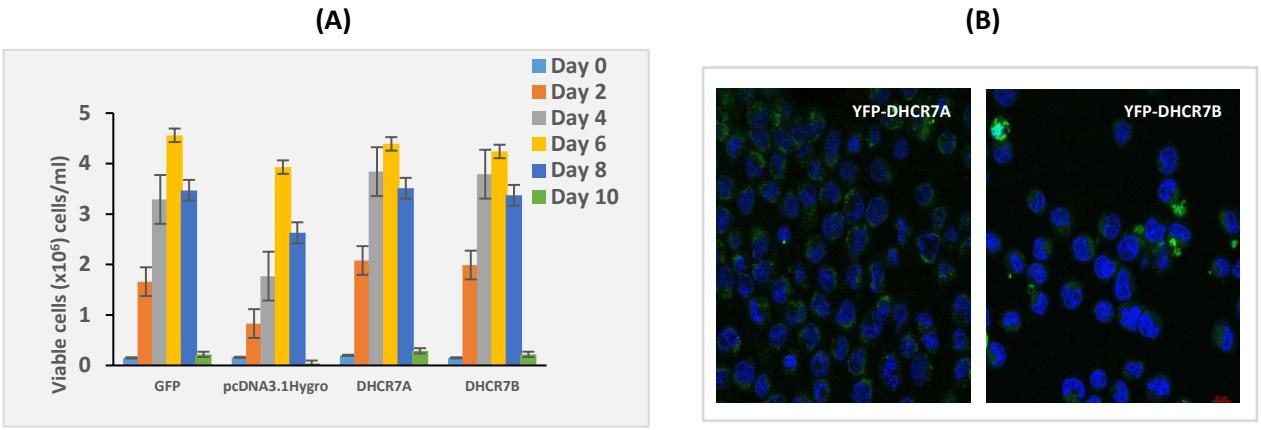

Figure 5.1 Characterisation of CHO-S stably expressing YFP-DHCR7A and YFP-DHCR7B pools. (A) A 10-day growth profile viable cell numbers of YFP-DHCR7A and YFP-DHCR7B pools alongside the GFP expressing and empty vector CHO-S controls. Error bars represent standard error between three biological cultures (n = 3). (B) Fluorescence microscopy studies showing expression of YFP in the CHO-S pools engineered to express YFP-DHCR7. Both DHCR7A and DHCR7B pools show stable YFP signal detected, although different expression levels are apparent in individual cells. Representative images show YFP-DHCR7A pool generally expressing more YFP-DHCR7 than in the YFP-DHCR7B pool.

For western blotting analysis, YFP-DHCR7A and YFP-DHCR7B engineered CHO-S cells were harvested at $1x10^6$ viable cells/ml and lysed using freshly prepared lysis buffer. A Bradford assay was subsequently used to calculate a 20 µg protein amount to allow for equal loading on SDS-PAGE gels. To probe for YFP, anti-eGFP primary antibody was used with a secondary anti-mouse IgG whole molecule, peroxidase labelled antibody for detection. When CHO-S YFP-DHCR7A and YFP-DHCR7B pools were analysed along with the GFP and pcDNA3.1Hygro controls, a band of the expected size for the YFP-DHCR7 molecule was observed in both pools (at 81 kDa, see Figure 5.2). Further, the YFP-DHCR7A pool appeared to have increased signal, and hence expression, when compared to the YFP-DHCR7B pool, in agreement with the fluorescent microscopy studies reported in Figure 5.1B. A band corresponding to GFP at 27 kDa was observed in the GFP control pools and as expected there were no bands detected in the pcDNA3.1Hygro samples.

Of the seven generated clones from pool A, labelled clones 1 to 7, three were identified as high expressers of DHCR7; Clones 4, 6 and 7 (Figure 5.2B) and further evaluated. However, a recurrent observation was that the expression tendency of DHCR7 was generally poor when compared to TM7SF2. Initially, it was thought that this might be due to improper stable cell line generation of these constructs. However, attempts to re-generate the stable cells expressing YFP-DHCR7 still showed low signal

strength. Interestingly, these YFP-DHCR7 constructs continued to show increased maximum viable cell concentrations .Thus it is likely that cells expressing higher amounts of the YFP-DHCR7 do not survive the selection process.

Attempts were made to improve YFP signal in case the presumably membrane associated YFP-DHCR7 was not being fully lysed or released from the membrane, by subjecting the cells to sonication, which involves using sound energy to cause agitation of the particles in samples in sonication water bath, and shearing of the lysate material using a needle shear to break open the cell wall. Neither of these resulted in a stronger signal being observed. The appearance of non-producing populations of cells, can lead to an overall decrease in production. Cellular burden represents a significant problem in the scaling of fermentation processes from proof-of-concept to long-term cultures, as the load of heterologous gene expression and depletion of the cell intracellular resources cause unpredictable cellular physiological changes that can lead to decreased growth and lower production yields (Bena et al., 2018). Nevertheless, stably expressing YFP-DHCR7 cells were isolated and hence further evaluation in the variations of key phenotypic traits, including investigating their proliferation rate, peak viable cell diameter, cell size and cell biomass content and morphology, were investigated. Note that all constructs of proteins were quantified by Bradford assay to allow for equal loading on SDS PAGE gel,

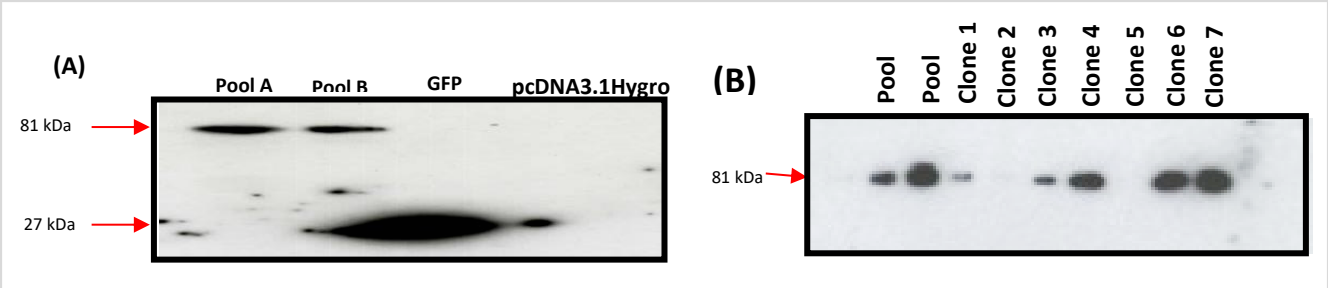

Figure 5.2 Western blot analysis of YFP-DHCR7 expression in CHO-S clones. **(A)** Pools of YFP-DHCR7 (A and B) alongside the GFP and pcDNA3.1Hygro controls were analysed for expression. 20 µ g amount of protein was loaded for each sample on an SDS-PAGE. YFP signal for YFP-DHCR7 was observed at 81 kDa while GFP positive control is observed at 27 kDa. The blot represents a 5 min exposure time. **(B)** Analysis of YFP-DHCR7 expression in the parent pool A and B and clones isolated from these that show a varying YFP-DHCR7 band/signal. Clones 4, 6 and 7) were considered as higher expressers when compared to the other clones and further examined. Represented blot shows exposure time at 5 min.

5.3.0 The impact of engineering YFP-DHCR7 expression in CHO-S cells on transient secretory EPO expression

One of the main goals of this study was to improve recombinant secretory protein productivity of CHO-S cells. Since protein productivity is directly proportional to viable biomass, viability and culture longevity of the producer cells (Kumar et al., 2007), we hypothesised that the impact of expressing YFP-DHCR7 on the CHO-S secretory system would result in an expansion of ER membrane network, which would in turn result in increased amounts of secretory recombinant protein production. This was investigated using recombinant Erythropoietin (EPO), a therapeutic protein manufactured in CHO cells and efficient mammalian erythropoietin (EPO)-expression systems are required for therapeutic applications (Kim et al., 2004). As previously described in Chapter 4, EPO is a commonly used model protein in development of CHO-based bioprocesses (Surabattula et al., 2011) and metabolic engineering of CHO cells for improved protein production (Kim et al., 2011). As discussed in Chapter 4 for the engineering of CHO-S cells with YFP-TM7SF2, where the impact of engineering CHO-S with YFP-DHCR7 on transient recombinant EPO secretion was compared to the GFP and pcDNA3.1Hygro control cells. Extracellular amounts of EPO were therefore determined using western blotting. The two pools of stable CHO-S expressing YFP-DHCR7 (A and B) were transfected with 4 µg EPO as were the controls and cell culture supernatants processed and analysed using western blot to determine EPO secretion levels. Clones of the YFP-DHCR7 engineered CHO-S cells were also transfected with EPO and EPO expression determined by western blot.

5.3.1 Clones of YFP- DHCR7 engineered CHO-S cells express different transient EPO amounts

To characterise the ability of YFP-DHCR7 engineered CHO-S to produce EPO, samples were collected from the cell culture supernatant day 2 post-transfection and prepared for SDS-PAGE and western analysis with 4X laemmli sample buffer. 20 µl volume of samples were then loaded equally across the SDS-PAGE gel for electrophoresis. Following nitrocellulose membrane transfer, recombinant EPO was probed using conjugated anti-his tag HRP antibody (the EPO was tagged at the C-terminal with a his-tag) for western analysis with the EPO band being detected at approximately 37 kDa (Figure 5.3). Clones 4, 6 and 7 were observed to express higher amounts of recombinant EPO than the two pools expressing YFP-DHCR7 and the GFP control CHO-S cell pool (Figure 5.3). These three clones had been

shown to be higher expressers of YFP-DHCR7 in CHO-S. Clone 3 did not show an elevated EPO signal from supernatant samples compared to the GFP control, suggesting there may be other limitations in the clone on secretory productivity.

The fact that not all YFP-DHFR7 clones exhibited the same phenotype with respect to EPO expression is perhaps not surprising. Cultivation of recombinant cells is performed in different ways, depending on the goal of the experiment (Ley et al., 2015). Previous reports have suggested that clonally-derived cell lines can display erratic and uncontrollable behaviour in culture such as variation in specific growth rate (Barnes et al., 2006) and differences in productivity characteristics (Heller-Harrison et al., 2009). This is because there are other factors that determine the ability of a cell to make recombinant protein than ER and secretory capacity (for example.; transcriptional and translational limitations) and hence whilst in clone 4, clone 6 and clone 7 secretory capacity improvements may account for increased yields compared to the control, in clone 3 other cellular processes may be limiting productivity.

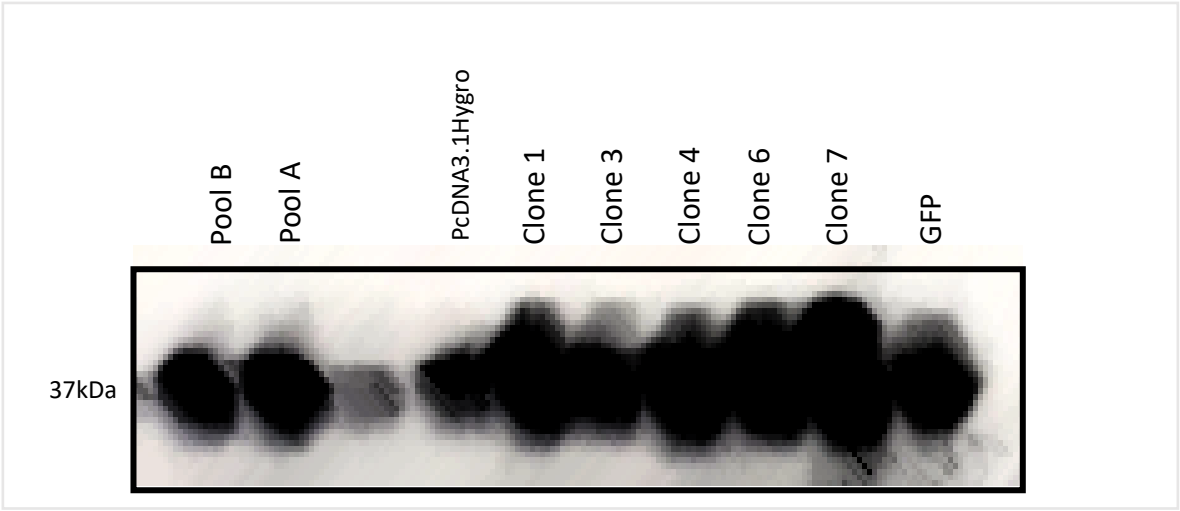

Figure 5.3 Western blot analysis of the ability of pools and clones of CHO-S expressing YFP-DHCR7 to secrete EPO. Cells were seeded at $0.2x10^6$ viable cells/ml in 2 ml of cell media of a 6-well plate. Transient EPO transfection was carried out with 4 µg EPO plasmid DNA using NovaCHOice™ transfection reagent and booster. Equal amounts of prepared supernatant with laemilli sample buffer were run on SDS-PAGE gel two days after transfection. Analysis of both pools and clones showed elevated EPO levels in clone 4, clone 6 and clone 7 when compared to GFP and pcDNA3.1Hygro control pools.

Additionally, EPO band intensities from the western result were converted into quantitative numerical values using densitometry in order to quantify the difference in EPO production between CHO-S expressing controls and engineered CHO-S expressing DHCR7 pools including their generated clones. Using Image J software, analysis was done to determine the optical density of the relative levels of bands on pixels of the photographic film (Figure 5.4). The densitometry data suggested that there had been an increase in EPO expression in pool A over pool B and that clonal cells overexpressing DHCR7 appeared to be expressing more amount of EPO than pcDNA3.1Hygro as clones 4, 6 and 7 did show increased EPO levels up to 0.5 fold of pcDNA3.1Hygro EPO secretion amount.

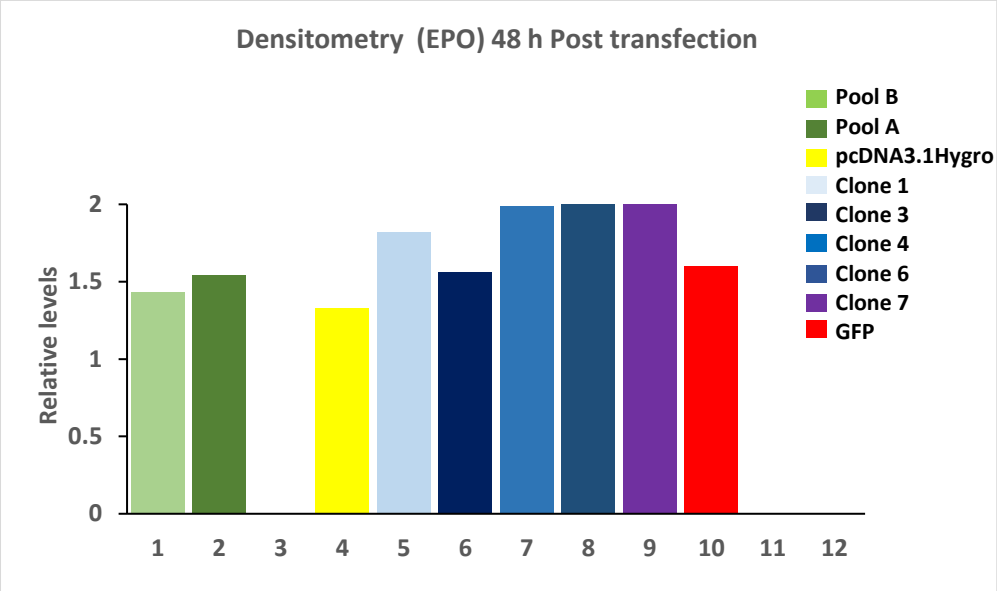

Figure 5.4 Densitometry analysis of EPO expression levels on western blot image in Figure 5.3. Relative levels of the two pools of DHCR7 were determined alongside clonal cells generated from pool B as well as GFP/pcDNA3.1Hygro expressing control cells, using the freeware tool Image J.

5.3.2 Comparison of the transient secretory EPO productivity ability between YFP-DHCR7 and YFP-TM7SF2 engineered CHO-S cell Pools

Almost all of the enzymes required for cholesterol synthesis reside in the ER. DHCR7 and TM7SF2 are human sterol intermediates and function at distinct steps in the cholesterol biosynthesis pathway. However when these two related sterol reductases were over-expressed, they were reported to have induced identical phenotype, including membrane dilation and nuclear compaction when expressed both as truncated and full length variants in U2OS cells (Hermann & Zwerger, 2010). The influence of expression of the YFP tagged version of these proteins in engineered CHO-S in terms of ability to influence secreted EPO amounts was directly compared. Transient transfection of EPO was carried out on stable CHO-S cells expressing YFP-DHCR7 (A pool) and two pools of YFP-TM7SF2 alongside the GFP and pcDNA3.1Hygro controls.

The YFP-TM7SF2 pool B cells gave the highest amounts of EPO as determined by western blot (see Figure 5.5) whilst the pool A samples were marginally higher visually than the controls (Figure 4.6). The YFP-DHCR7 samples were generally similar to the controls, although by day 8 of culture, these were higher than the controls (Figure 5.5). The YFP-TM7SF2 pools therefore appear to give a greater increase in transient EPO expression than the YFP-DHCR7 pool, possibly due to the fact that YFP-TM7SF2 expression, as determined by YFP amounts, is higher than YFP-DHCR7 expression in the pools (Figure 4.7). A greater increase is observed in the YFP-DHCR7 clones than the pools (see Figure 5.3) and hence these clones may have been more appropriate to investigate than the pools for such a comparison.

In assessing the level of recombinant protein expression, it is crucial to undertake a comparative study between YFP-TM7SF2 clonal cells and YFP-DHCR7 clonal cells on their influence on protein production for a significant correlation on their respective capacities. While expression data has shown by western blots and densitometry, confirm the levels of EPO expressed in pools and clones to CHO-S expressing GFP and pcDNA3.1Hygro controls (Figure 5.5), we did not directly compare the respective clones with controls for investigation on their functional expression levels. It will be of great benefit to investigate the responsiveness of these clones for transient EPO and etanercept production. Since these clonal cells reveal high proliferation rates under appropriate culture conditions, it is conceivable therefore, that fully reprogrammed cells outgrow partially reprogrammed cells in the course of culture expansion

(Willmann et al., 2013). Hence, a western blot analysis comparing clones of CHO-S over-expressing YFP-TM7SF2 and YFP-DHCR7 to GFP and pcDNA3.1Hygro would be more significant.

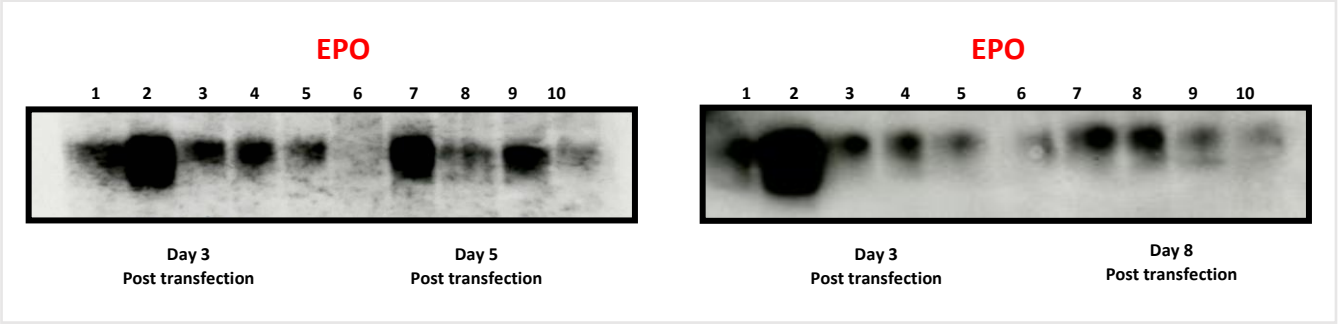

Figure 5.5 Western blot analysis of transient secretory EPO expression on day 3, day 5 and day 8 post-transfection in YFP-TM7SF2, YFP-DHCR7, GFP and empty vector control expressing CHO-S pools. EPO was detected using anti-his HRP antibody and represented blots were analysed at same exposure time of 2 min. 20 µl supernatant samples were analysed for EPO expression in YFP-TM7SF2 pool A (lanes 1 and 6) and pool B (lanes 2 and 7), YFP-DHCR7 pool A (lanes 3 and 8), GFP expressing (lanes 4 and 9) and pcDNA3.1Hygro empty vector control (lanes 5 and 10) CHO-S cell samples.

5.3.3 Transient EPO expression from different clones of CHO-S expressing YFP-DHCR7

In addition to analysing EPO expression in YFP-DHCR7 pools, the highest expressing YFP-DHCR7 clones were investigated by using a western blot to look at the relative transient secreted EPO amounts from these (Figure 5.6). This experiment was carried out on four of the six identified high expressers of DHCR7 (A) pool and cells harvested day 2 post EPO transfection. Western analysis was used to detect EPO expression in all four clones. It was observed that all clones showed elevated EPO levels when compared to the empty vector pcDNA3.1Hygro control cell pool (Figure 5.6). While EPO was observed at the expected size of 37 kDa, the amount of expression observed among the four clones varied. There was generally a relationship between the amount of YFP-DHCR7 expression detected in the clones by western blot (Figure 5.2 and Figure 5.3) and the amount of EPO secretion observed, suggesting that the expression of YFP-DHCR7 was influencing secreted EPO amounts. The presented western blot image is without a loading control. Loading controls are used to confirm that protein loading is the same across the gel (as errors are common during loading) and these controls are usually proteins that

exhibit high-level, constitutive expression in the cell and should be of different molecular weight to the protein of interest so as to be able to distinguish between bands. Usually, housekeeping genes are often chosen for use this purpose. A loading control would serve as a confirmation to the observed findings that DHCR7 clones expressed more EPO amounts than pcDNA3.1Hygro. In the future, this should be undertaken. Examples of loading controls include Alpha tubulin, Beta tubulin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Lamin B1, Transferrin, Vinculin amongst several others.

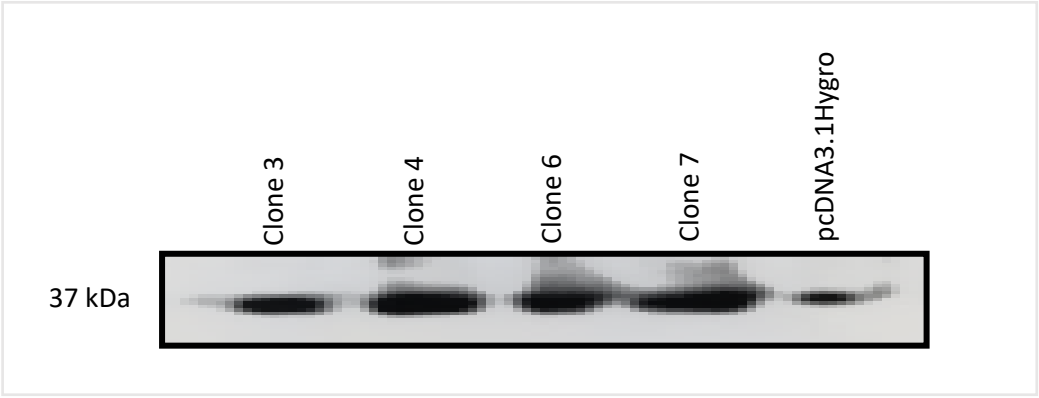

Figure 5.6 Western blot analysis of secreted EPO from CHO-S clones expressing YFP-DHCR7 and the empty vector control (pcDNA3.1Hygro). Four clones identified as high producers of CHO-S expressing YFP-DHCR7 were analysed with pcDNA3.1Hygro. His tagged EPO was detected at 37 kDa in supernatant using conjugated anti-his HRP tag antibody. Represented clones showed elevated EPO expression levels as compared with pcDNA3.1Hygro.

Following data from western blot, there seemed to be a relationship between these band intensities and culture productivity. The densitometry analysis confirmed the results of the initial study with relative levels of clones reaching about 0.5 fold of pcDNA3.1Hygro, suggesting that the clones continued to express EPO transiently on day 2 post transfection (Figure 5.7). While the clones continue to show increased EPO expression levels over control cells, it is important to note, that this observation was for 2 days post EPO transfection and it is not certain, whether this is applicable for a longer period. When EPO was transiently expressed on day 8 (Figure 4.8) however, findings were observed to be reproducible suggesting a positive impact may be observed on the long run during culture productivities. Densitometry was carried out using image J freeware.

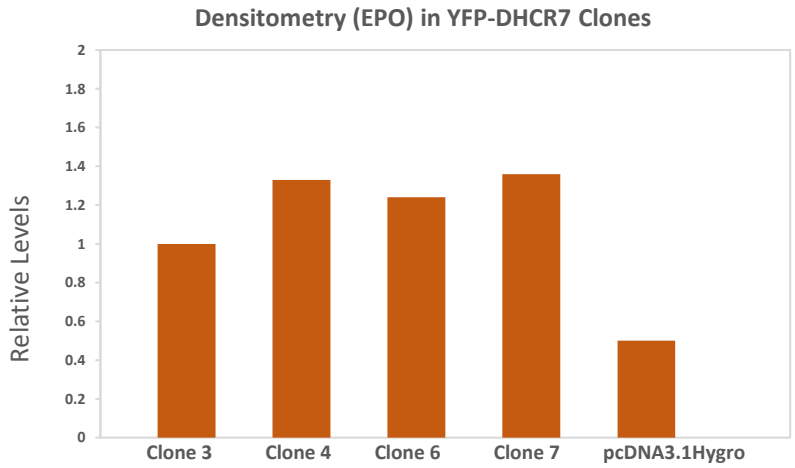

Figure 5.7 Densitometry analysis of the western blots images shown in Figure 5.6. The density of each band was determined and then normalised to pcDNA31Hygro control for quantitative analysis of EPO expression levels of YFP-DHCR7 clones. YFP-DHCR7 Pool A cells expressed EPO at about 0.5 fold above pcDNA3.1Hygro expressing CHO-S cells confirming that there was a positive impact of over-expression of YFP-DHCR7 on CHO-S ER membrane.

5.4.0 The impact of YFP-DHCR7 expression in CHO-S cells on transient etanercept expression

The previous studies in this chapter have shown that YFP-DHCR7 expression appears to impact on secreted EPO expression. In order to determine if this was an EPO specific impact, or a more widely applicable impact, the transient secretory expression of a second bio therapeutic protein was analysed in these and the control cells. For this purpose etanercept, which remains a very successful therapeutic protein in terms of commercial success with annual sale of 9.3 billion US dollars (Willrich et al., 2015) was transiently transfected into the different CHO-S engineered cells. We investigated the influence of both pools and clones of YFP-DHCR7 expression on etanercept. The approach involved transfecting etanercept in stable cells and analysing the extracellular culture supernatant at different times of a batch culture time-course for anti-TNF expression signal. As stated in chapter 4, etanercept, has been demonstrated to have beneficial activity in a number of inflammatory diseases although it was originally developed for rheumatoid arthritis and Crohn disease (Genovese et al., 2005).

5.4.1 Clones of YFP-DHCR7 engineered CHO-S cells express different amounts of etanercept under transient conditions

To examine the impact of YFP-DHCR7 expression on etanercept secretion, we carried out transient transfection in replicates in 6 well plates, where three clones (clone 4, clone 6 and clone 7) which were considered higher expressers of YFP-DHCR7 were investigated. These clones were previously shown to give enhanced EPO secretion compared to control cells. Upon transfection, 200 µl cell media was collected 2 days post transfection. After western transfer, probing of etanercept was achieved using a heavy chain 1° antibody overnight and a re-probe with 2° anti-rabbit IgG peroxidase antibody. While between the clones there was different etanercept secretion (Figure 5.8), YFP-DHCR7 Clones 3 and 7 showed higher etanercept amounts in their culture supernatant than other clones. Although western blot image (Figure 5.8) was shown with the intention to compare the relationship of high-expresser clones using etanercept, there are no GFP and pcDNA3.1Hygro controls presented along with the image.

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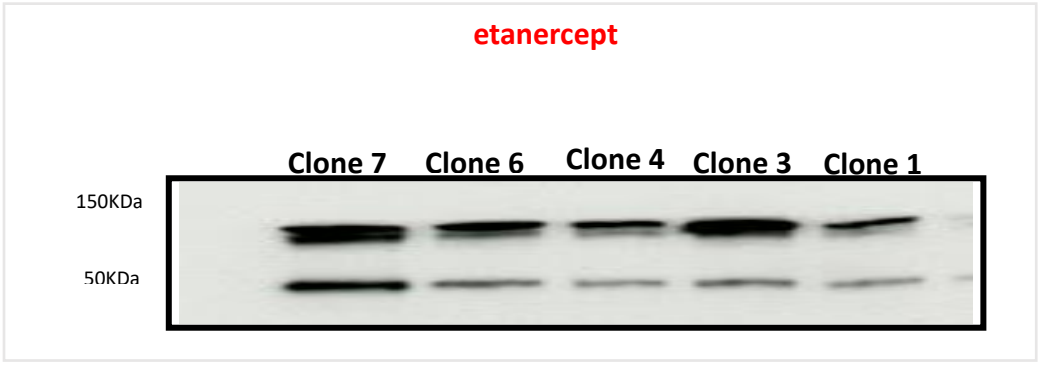


Figure 5.8 Western blot analysis of etanercept expressed in CHO-S clones expressing YFP-DHCR7. Five clones of CHO-S expressing DHCR7 were analysed for transient etanercept expression detected at 150 kDa in supernatant using anti-heavy chain 1° antibody with anti-rabbit 2° antibody. Represented clones showed varied etanercept amounts.

5.5.0 Investigating the cellular localisation of YFP-DHCR7 in stably engineered CHO-S cells by comparison to an ER resident protein, calnexin

DHCR7 is endogenously located in the ER membrane. The ER is an important site of protein synthesis and post-translational modification and for proteins to be transported from the ER to other sites within the cell and even out of the cell, biologically active proteins follow a well-defined route known as the secretory pathway (Bouchekhima et al., 2009). The forward route, starts with the ER, followed by the Golgi complex and the trans-Golgi network, before proteins reach the plasma membrane (PM) which surrounds the cell, and subsequently the extra cellular space (Anelli & Sitia, 2007).

Here it was investigated whether expression of YFP-DHCR7 in CHO-S still targeted DHCR7 to the ER. The hypothesis of this work is that the quality control mechanism of DHCR7 in the lipid biosynthesis pathway enhances the ER secretion production capacity by providing a greater ER capacity, thus balancing inward and outward flux of recombinant protein load, hence preventing accumulation of toxic misfolded proteins. Thus, the ER subcellular localisation of YFP-DHCR7 in the cell was investigated by determining its localisation status relative to the ER resident protein calnexin of the calnexin/calreticulin machinery, which monitors the folding status by interaction with sugar residues in folding intermediates (Ellgaard & Helenius, 2003).

Immunocytolocalisation studies were therefore carried out to investigate the localisation of YFP-DHCR7 as DHCR7 has been reported to be localised in the ER. Stable YFP-DHCR7 clones were analysed for localisation studies. The presence of YFP can be used to highlight the subcellular localisation of YFP-DHCR7 as the intact YFP-DHCR7 molecule was shown to be present by western blot analysis. Microscopy was performed on five CHO-S clones of YFP-DHCR7 which were identified as high expressers relative to other clones as previously described. Detection was carried out using confocal imaging. Images obtained from immunoblotting are presented in three panels in figure 4.4 for each clone at different excitation wavelengths for respective colours; Calnexin, YFP, and an overlay image of the two colours to determine if there is co-localisation.

All images are shown at the same magnification as indicated by the 10 µm bar in each figure (Figure 5.9). Both the anti-calnexin antibody and YFP show cellular morphology of clones while the most prominent signals were present in a network/mesh pattern suggestive of endoplasmic reticulum.

Some of the clones analysed showed peculiarity of expression, for instance YFP-DHCR7 clone 6 cell nucleus appear clustered together whereas DHCR7 clone 7 show defined sparsely dispersed nucleus. There was considerable over-lap of the YFP and calnexin staining suggesting some of the YFP-DHCR7 was co-localised to the ER whilst other was cytoplasmic. This agrees with previous work (Zwerger et al., 2010) although the YFP-DHCR7 does not have a ER localisation signal at the N-terminal and suggests that the molecule interacts with ER membrane in some manner. Images presented here (Figure 5.9) show the morphology of YFP-DHCR7 clones only when counterstained with calnexin and DAPI for compartmentation studies for the localisation of the ER and do not show the presentation of the nuclear envelope of CHO-S expressing GFP and pcDNA3.1Hygro as controls which would be necessary for analytical discussion. This would be considered in the future. Another approach to an investigative study is to analyse CHO-S cells morphology with live cell imaging which would allow for some measurement of the ER and the presentation of the ER structure of clones in relation to CHO-S ER.

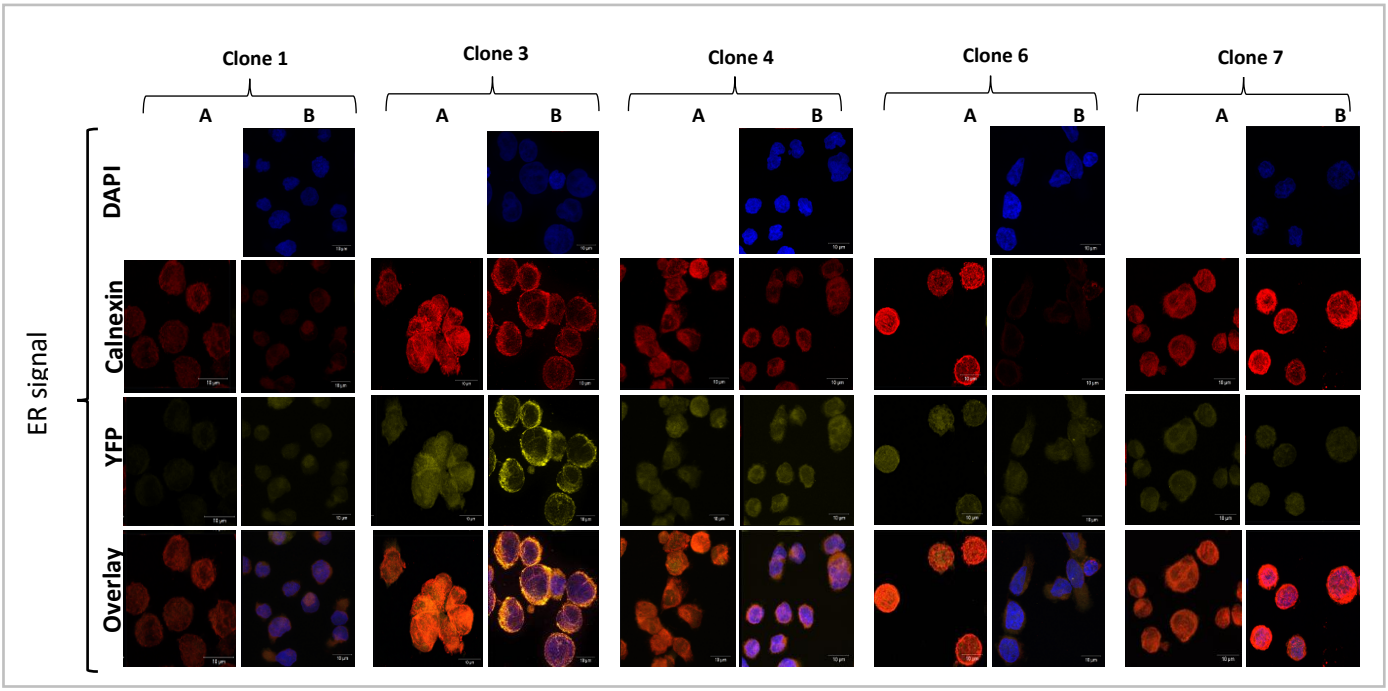


Figure 5.9 Immunocytolocalisation of YFP-DHCR7 clones to monitor ER by co-localisation of calnexin and YFP. Fixed CHO-S cells section were probed using 1° anti-calnexin/2° anti-rabbit TRIT.C and chromatin, counterstained with DAPI to monitor the potential cellular compartmentation of stably over-expressing YFP-DHCR7. Clones appear to show signals in nuclear membrane where the ER is located. Expansion has been predicted to occur in the ER when YFP-DHCR7 was over-expressed in U2OS cells (Zwerger et al., 2010). Images are expressed at 10 µm. Expression levels of YFP-DHCR7 vary in agreement with western blot analysis between clones. Images presented were generated from two different studies (A and B). Study "A" does not show DAPI whereas study "B" presents DAPI images.

5.6.0 Comparison of the Growth and Culture Viability of YFP-DHCR7 Clones of CHO-S with Controls during Batch Culture

While acknowledging the variation of YFP-DHCR7 expression amounts observed for clones, it was important to examine the relationship between their culture viability and proliferation rate with YFP-DHCR7 expression levels in the engineered CHO-S compared to the controls. Indeed, there is the potential to increase the productivity of cells by controlling cell growth (Du et al., 2015). The selected clones were analysed throughout a 10-day batch culture with reference to controls (Figure 5.10). Both culture viability and viable/total cell numbers were monitored over the batch-culture time course. Cells were seeded at $0.2x10^6$ viable cells/ml while 0.2 ml was taken for cell counts at each time these were assessed over the 10-day period.

The cell counts across the culture period of days 3 to 7, which fall within the exponential growth and decline phase, are shown in Figure 5.10. Although the time course for the study was a 10-day period, analyses discussed and shown here are the period between day 3 and day 7. It was observed that unlike CHO-S cells overexpressing YFP-TM7SF2, YFP-DHCR7 overexpressing cells exhibit great variation in specific proliferation rate and showed peak viable cell density usually within days 3 and 7, suggesting that cells may be extensively undergoing an exponential growth phase due to an initial stimulating effect on specific cell growth rate rather than to the higher availability of those nutrients at any other time of the culture. The peculiar growth pattern had been persistent and therefore, it became interesting to have a closer look at this period of culture in CHO-S over-expressing YFP-DHCR7 growth was necessary.

All clones had a peak viable cell concentration on day 6 of culture and had similar profiles with the pcDNA3.1Hygro control obtaining the lowest maximum viable cell number (Figure 5.10A). As cells expressing YFP-DHCR7 do not appear to be impacted in terms of toxicity to cells, it appears likely that the levels in the clones isolated is tolerated by the CHO-S cell and hence any impact of this amount of expression on the secretory capacity of the cell and ER membrane for increased recombinant protein production could be assessed. In order to determine whether the stable cells expressing YFP-DHCR7 functionally influences the stability and viability of CHO-S, the percentage culture viability was also analysed in replicate biological samples and average values plotted (Figure 5.10B).

All clones expressing YFP-DHCR7 showed sustained viability from day 3 to day 6 compared to the empty vector control and all except clone 1 (the lowest expressing YFP-DHCR7 clone) maintained higher culture viability than the GFP control cell pool (Figure 5.10B). Collectively the data in figure 5.10 shows that expression of the YFP-DHCR7 molecule in the CHO-S cells at the amounts isolated does not detrimentally impact upon the growth and culture viability and may have a positive impact on this compared to the empty vector control.

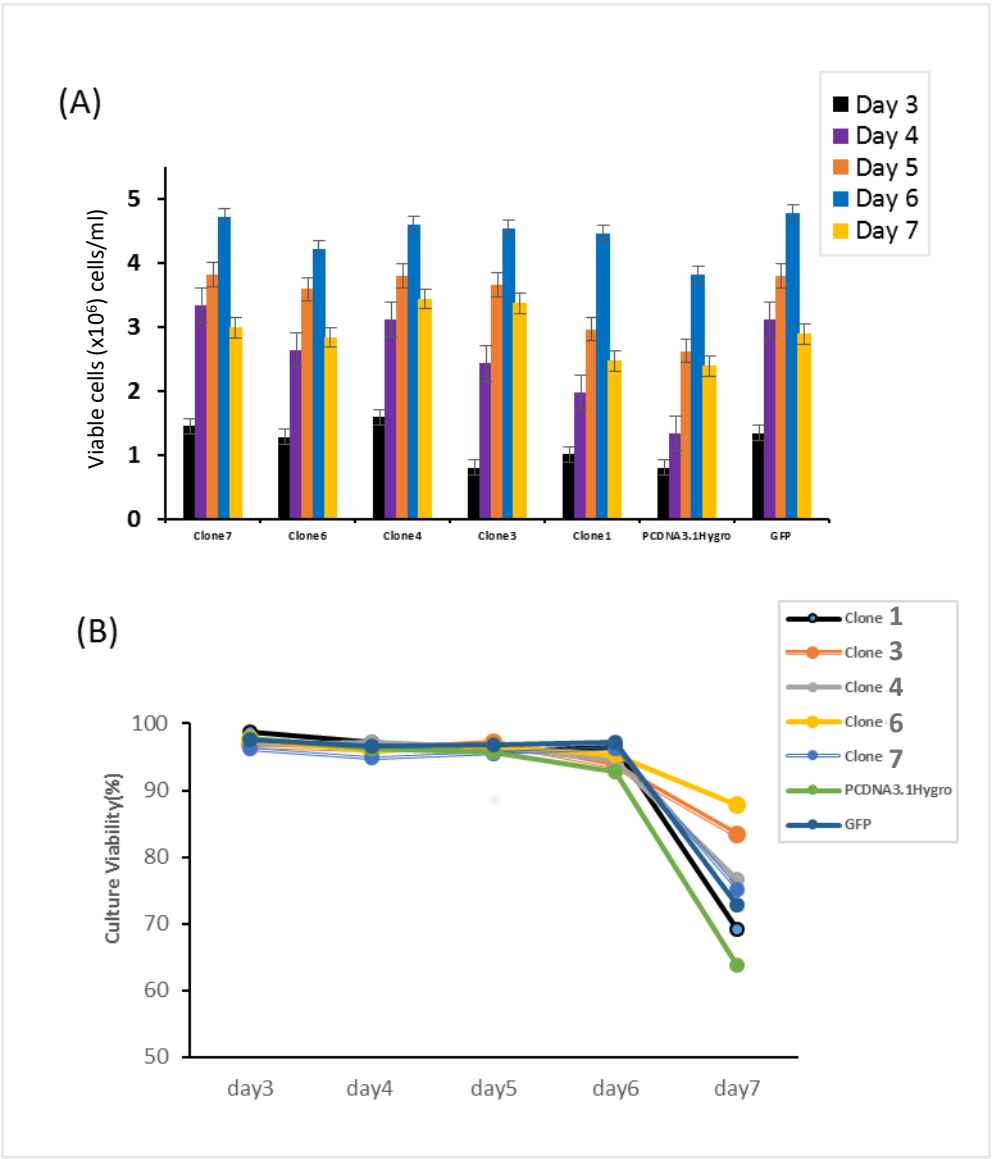


Figure 5.10 Batch culture growth profiles of CHO-S stable expressing YFP-DHCR7 clones and GFP and empty vector control pools. **(A)** Cells were seeded at 0.2x10^6 viable cells/ml in replicates for 5 clones of CHO-S expressing YFP-DHCR7 and control cells. A 10-day batch culture was studied and data from day 3 to day 7 is presented. Error bars represent standard error of replicate biological samples. **(B)** Percentage culture viability over days 3-7 of the 10-day batch culture experiment from (A). Data represents the averages of samples, where n=3.

5.6.1 Comparison of the Impact of Transient and Stable Expression of YFP-DHCR7 in CHO-S Cells on Growth Profiles during Batch Culture

Many studies use transient expression of target proteins in mammalian cells to assess the impact of their expression on cell phenotypes. In this study stable expression of YFP-DHCR7 and any impact on regulating growth and secretion processes in CHO-S was investigated. However, this was also compared to transient expression of YFP-DHCR7 in terms of the influence on phenotypic diversity and proliferation rate. For all assays, the maximum viable cell concentration obtained was on day 3 of culture. Interestingly, on day 2 the transient transfected samples of YFP-DHCR7A had a higher viable cell number and seemed to have thrived more than it did by day 3 (Figure 5.11). Cell concentration was more-or-less the same for most of the pools investigated for both the transient and stable pools. One of the causes of instability of recombinant protein production from CHO cells is the loss of the gene of interest from the recombinant host cells' genome, particularly for the CHO expression system (Kim et al., 2011) and hence it is possible that gene expression is lost over time in stable cells. However, this occurs over multiple passages and prolonged culture and hence would not explain any difference on day 2 between the transient and stably expressing cells investigated here. The data again confirms that the expression of YFP-DHCR7, whether transiently or stably, does not negatively impact upon CHO-S cell growth during batch culture.

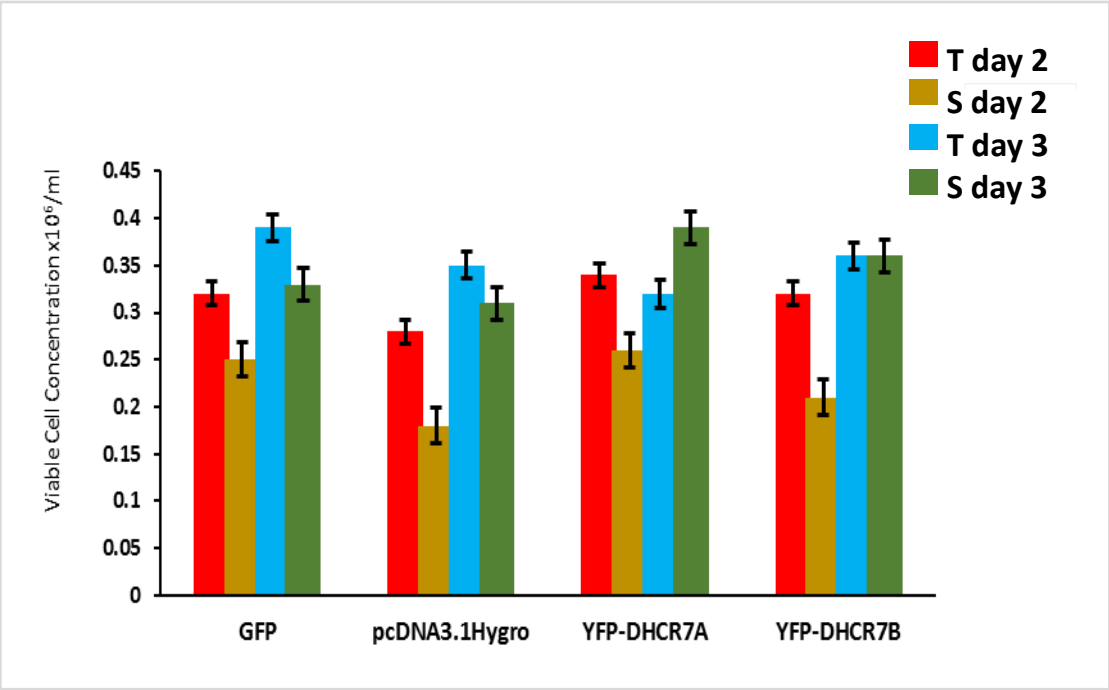


Figure 5.11 Growth profile of transient (designated by **T day 2** and **T day 3**) and stable (designated by **S day 2** and **S day 3**) YFP-DHCR7 expressing CHO-S cells during batch culture. Cells were seeded at $0.2x10^6$ viable cells/ml in replicates and viable cells counted on day 2 and day 3 post transfection for stably expressing cells or after transfection with the appropriate construct for the transiently expressing cells. Error bars represent standard error of the mean for replicate biological samples (n = 3).

5.6.2 Assessing the impact of EPO transient transfection on YFP-DHCR7 engineered CHO-S culture viability

The YFP-DHCR7 cells could respond differently to transfection and the load of EPO on them than the controls with respect to growth and viability, hence the viability was monitored across the batch culture. When the viability was assessed on CHO-S growth over a time course (section 5.4.4.2), the aim was to evaluate the influence on CHO-S growth as we hypothesised that by controlled expression of lipid biosynthesis ER membrane, cell growth and viability can be influenced for improved productivity. It was observed the DHCR7 pool A cells maintained a similar culture viability to the GFP control and both were higher than the pcDNA3.1Hygro control (Figure 5.12). Thus, there was no negative impact, nor a positive impact, of the expression of YFP-DHCR7

on the culture viability across the batch culture.

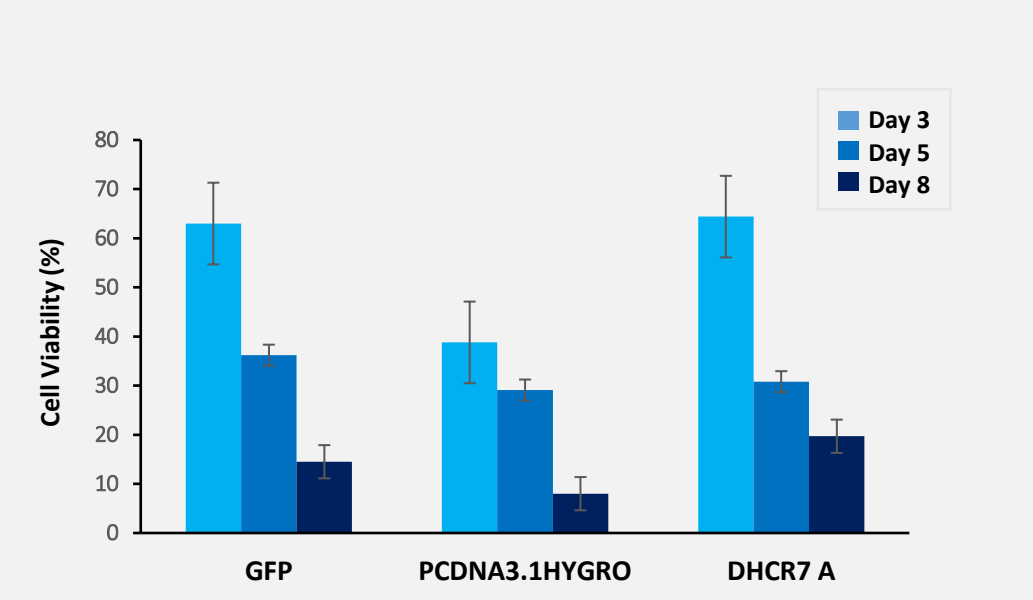


Figure 5.12 Percentage culture viability during transient EPO expression in YFP-DHCR7 pool A and the GFP and empty vector control. Analysis of cell viability at different days (day 3, day 5 and day 8) of an 8-day batch culture experiment, show DHCR7A pool to be steadily proliferating as compared with pcDNA3.1Hygro as a negative channel. Error bars represent standard error of replicate biological samples, where n=3.

5.7 Conclusions and Summary Statements from this Chapter

The main findings are discussed in the main discussion chapter (Chapter 6) but a brief discussion of the implications of the findings of the work in this chapter is presented. The results presented in this chapter describe the approaches taken to investigate improving CHO-S cells' secretory productivity by engineering YFP-DHCR7 expression. As approximately 30% of newly synthesised proteins have been reported to not fold properly and are thereby degraded (Goeckeler and Brodsky, 2010), there remains potential to improve secretory productivity by manipulation of the ER capacity to meet the increase in demand for protein synthesis, as a concomitant increase in the number of proteins will be degraded based on quality control (Teodoro 2012).

Here, an attempt to influence more recombinant protein production in the CHO-S secretory systems by overexpressing DHCR7, a key target protein in sterol biosynthesis, was undertaken. Our interest was to investigate an adaptive response approach that affords the cells secretory system the capacity to maximise its role effectively without activating the ER stress signal pathway which almost always ends in apoptosis. When stable CHO-S cells over-expressing YFP-tagged DHCR7 were generated, strategies carried out to characterise the engineered CHO-S cells resulted in the generation of two pools, named A and B, which showed distinct growth patterns. Although high protein yields are desirable, resultant proteins are less valuable if they are aggregated, misfolded, degraded or improperly glycosylated (Liu et al., 2015). Pool A appeared to express YFP-DHCR7 more than pool B when probed by western blotting.

Initial clonings undertaken in the research produced constructs that were engineered into expansive CHO-S transcriptome, for the generation of stable cell lines for gene expression analysis. Consequently, our data showed that over- expression of DHCR7 did not alter CHO-S cell's molecular dynamics as cellular differentiation and growth were not impeded neither was CHO-S cells' viability negativity impacted. From our stability analysis, we have shown that over a time course DHCR7 engineered cells were not toxic to cells and were not apoptotic as decay rates were consistently low when compared to controls. The heterogeneity of cells which was responsible for CHO-S cells' range of cellular behaviours led us to generate two major pools of DHCR7 producing cells.

With the generation of clones by limited dilution cloning methods, the focus was on investigating how over-expression of these may impact on cell productivity. According to Ley et al., (2015), production bottleneck is often reported to be independent of the heterologous target protein, indicating a general limitation of the secretory protein processing capacity, while in some cases secretory bottlenecks are linked to the synthesis of a specific post-translational protein modification. Previous reports have suggested that, when cells are not growing nor dividing they can concentrate cellular energy on protein synthesis (Anisimova et al., 2018). While high abundance of transmembrane proteins in the ER can induce extensive membrane growth, low expression does not alter ER morphology (Herrmann & Zwerger, 2010). Consequently, the aim of this study was to investigate the influence of reprogramming lipid synthesis on CHO-S cells' recombinant protein productivity.

Using western blot techniques, YFP-DHCR7 protein was confirmed to be stably expressed in CHO-S In order to obtain quantitative data for EPO and etanercept from western blots, we carried out densitometry. Densitometry is the quantitative measurement of optical density in light-sensitive materials, such as photographic films and as a first choice, it is considered the gold standard (Gallo-Oller et al., 2018). When EPO was transiently transfected and assayed in clones (3, 4, 6 and 7) of YFP-DHCR7 engineered cells in relation to pcDNA3.1Hygro control cell line, it was observed that the clonal cells proliferated by up to 0.5 fold over pcDNA3.1Hygro expressing cells when over an 8-day experimental period (Figure 5.7). This suggests that there perhaps was an improvement in the secretory vesicle system of the engineered cells allowing for some modulations of the cellular circuits which in turn results in a better maintained lipid homeostasis. Armstrong et al., (2017) had reported, on how cell engineering techniques such as genetic modification, metabolic labelling, and exogenous delivery have been used to change the surface expression and cargo of extracellular vesicles as the secretion of proteins from eukaryotic cells requires the coordinated function of multiple organelles and cellular machineries (Hua & Graham, 2013).

Despite the quantifiable difference observed when protein bands were quantified using densitometry, it was observed that statistically, the correlation was not significant at a probability less than 0.05 ($p<0.05$), in the levels of EPO observed in YFP-DHCR7 engineered CHO-S in relation to control. Several other assays which are applicable for the quantification of EPO and etanercept but not used in this study include the measurement of Ultraviolet (UV) absorbance at 280 nm, Bicinchoninic acid (BCA) include enzyme-linked immunosorbent assay (ELISA) assay, mass

spectrometry.

For confirmation studies of the localisation of YFP-DHCR7 to the ER, the calnexin an ER resident protein was probed for co-localisation of calnexin and DHCR7 to the ER. This may present a promising approach in identifying the role of DHCR7 which catalysis the final step in sterol biosynthesis. Our data showed that both DHCR7 and calnexin co-localise and therefore informs the subcellular localisation of DHCR7.Many of the analysis have allowed the identification of the implications of over-expression DHCR7 in CHO-S cells while analysing the biochemical implications of what might be occurring to cell at molecular level with reference to cell's phenotypic and biochemical function.

DHCR7 is a transmembrane protein involved in cholesterol biosynthesis. Regulation of DHCR7 at the transcriptional level by the sterol regulatory element-binding protein-2 (SREBP-2) transcription factor is widely reported (Prabhu et al., 2014). By manipulating cellular processes of cholesterol biosynthesis, there might be a feedback on SREBP-2 which functions in a cascade mechanism to preferentially activate DHCR7 and some other sterol reductases. In examining the possible implications of causing an expansion of CHO-S ER membrane capacity by overexpression of YFP-DHCR7 and the feedback effect on cell proliferation and product yield, expression of EPO and etanercept by transient transfection of lipid engineered cells with control channels showed variations in expression levels of these bio molecules with expression levels higher when compared to pcDNA3.1Hygro.

Knowing that there is an accumulating evidence which suggests that cancer cells show alterations in different aspects of lipid metabolism. The potential dependence of cancer cells on the deregulated lipid metabolism also suggests that enzymes and regulating factors involved in this process are promising targets for cancer treatment (Cha & Lee, 2017). The molecular implications of these are the changes proffered on cellular processes such as cell growth, proliferation, differentiation, and survival by changes to lipid bioprocesses. However some questions are yet unanswered. If deregulated lopid biosynthesis is known to trigger alterations that may cause cancer cells to proliferate, would the overexpression of DHCR7 be impacting on lipids levels and cholesterol amounts? Could the reprogramming of cholesterol biosynthesis alter the chemical conformation of lipids at molecular level, thereby affecting their functional roles? We have not looked into this yet. It would be a major work going forward.

Chapter 6

General Discussion

Here, an overall discussion of the results from studies described in the previous result chapters are detailed.

6.1 TM7S72 and DHCR7 are potential lipid biosynthesis targets for ER expansion in CHO-S cells

Over the last years, the biopharmaceutical industry has significantly turned its biologics production towards mammalian cell expression systems as recent advances in omics technologies have allowed for new possibilities which will improve these expression platforms, including developing new strategies, in particular for Chinese Hamster Ovary (CHO) cells (Lalonde & Durocher, 2017). Therefore, recent advances in systems biology and data-driven approaches aim to unravel how molecular pathways, cellular processes and extrinsic factors influence recombinant protein production (Kuo et al., 2018).

Although O'Callaghan & James (2008) had reported that improvement of productivity in mammalian cell production systems have been achieved despite a dearth of specific knowledge of how engineered cells successfully function in vitro, the metabolic burden imposed by heterologous protein production in mammalian cells is still not well characterized and thus may offer opportunities for further improvement of protein productivity (Ley et al., 2015). In the production and synthesis of proteins, the endoplasmic reticulum (ER) remains the central organelle where proteins traffic through the secretory pathway. As protein secretion is not only essential for cellular function but also provides the driving force for cell growth via delivery of newly synthesized lipid and protein that permit cell expansion (Barlowe & Miller, 2013), it is therefore critical to determine how the engineering of the CHO lipid biosynthesis machinery may improve productivity.

The work presented in this project has exploited the functional roles of Transmembrane 7 Superfamily member 2 (TM7SF2) and 7-dehydrocholesterol reductase (DHCR7) sterol reductases of the cholesterol biosynthesis pathway by over-expression of their capacities for recombinant protein production. We also briefly investigated the role of Lamin B receptor (LBR). Interest for this work was

drawn from the publication by Zwerger et al., 2010 who had reported on a novel, as yet undescribed cellular phenotype observed in the endoplasmic reticulum (ER) lumen and the perinuclear space (PNS) with large dilations throughout the entire cell and specifically at the nuclear periphery when TM7SF2 and DHCR7 were over-expressed. Samples used in this work were obtained from their group as our findings sought to corroborate their work.

Literature studies have reported on the crucial role of TM7SF2 and DHCR7 in cholesterol biosynthesis. Bellezza et al., (2013) reported that TM7SF2 functions in cellular response to stressful conditions. While Bellezza et al., (2015) demonstrated that the loss of Tm7sf2 alters the expression of proteins involved in epidermal differentiation by reducing the levels of cholesterol sulfate and accelerating skin papilloma formation, Herrmann & Zwerger (2010), had reported that the very same step in cholesterol synthesis that is catalysed by LBR can also be catalysed by a closely related sterol reductase, TM7SF2. For instance, Bennati et al., (2008) reported, that TM7SF2 knockout mice (which should not have active 3ß-hydroxysterol Δ14-reductase) continued to show a 3ß-hydroxysterol Δ14-reductase activity required for cholesterol biosynthesis in the presence of Lamin B receptor (LBR), which although resides in the nuclear membranes, may be contributing to cholesterol biosynthesis in Tm7sf2(-/-) mice. Another study investigated the long-term effects of altered TM6SF2, a variant of TM7SF2 levels in cholesterol metabolism (Fan et al., 2016).

While, several functional proteins involved in the various posttranslational steps of the secretory pathway and of exocytosis have been studied where some of these pathways were successfully engineered to remove bottlenecks and cellular limitations caused by recombinant protein overflow (Mariati et al., 2010), we have identified TM7SF2 and DHCR7 sterol reductases as potential engineering targets of the CHO secretory machinery and have exploited these sterol reductases while also investigating the molecular implications.

6.2 Stable Overexpression of TM7SF2 and DHCR7 in CHO-S shows beneficial phenotypes over control CHO-S cells

The propensity as to whether or not over-expression of resident proteins can influence productivity have been a major focus in the recent years. In some cases, protein secretion have been significantly increased such as by manipulation of BiP chaperones (Payne et al., 2008), Xbp1, a transcription factor that regulates secretory cell differentiation, ER maintenance and expansion, to decrease ER stress and increase protein processing and secretion (Mohan & Lee, 2010) . Stable cell lines over-expressing TM7SF2 and DHCR7 were therefore generated for the characterization of stably over-expressing hygromycin-resistant CHO-S cells. To investigate whether the genetically engineered cells could impact beneficial phenotypic properties on CHO-S for improved productivity, two different controls to monitor expression levels of lipid engineered cells. With no insert cloned, the empty vector was stably inserted as a negative control. Qualitative analysis of growth profiles showed that as culture viability was on the increase when compared with stably generated controls expressing GFP and pcDNA3.1Hygro vectors (Figure 3.18), the rate of growth of CHO-S cells over-expressing DHCR7 and TM7SF2 was significantly higher than control CHO-S expressing GFP and pcDNA3.1Hygro cells (Figure 3.20).

When CHO-S cells were analysed with stable TM7SF2 over- expression, there was a higher secretory EPO amount as analysed by western blot (Figure 4.7) (Figure 5.5). This initial indication was a pointer to the fact that the presence of over-expressing TM7SF2 protein was impacting upon the secretory processes of CHO-S but there was no detailed indication as to whether the ER size was influenced. Meanwhile, two pools of cells were generated for both TM7SF2 and DHCR7. Although these parental pools (A and B) showed higher growth rate and robust viability compared to the CHO-S host, there remained a peculiarity in their growth pattern. For instance, TM7SF2 Pool B maintained an increased and robust growth rate over TM7SF2 Pool A and there was consistency in their proliferation. For DHCR7 parental pools, DHCR7 Pool A showed a consistently better growth rate than DHCR7 Pool B. Other techniques such as western blotting, fluorescence microscopy were also used for the characterisation of cells which confirmed variance in expression signals of distinct pools.

The growth rate analysis was carried out to monitor expression levels in cells and data obtained from fluorescence microscopy suggested that not all cells were actively over-expressing these lipid targets. The analysis of heterogeneous cell populations in bulk is only able to provide

averaged data about the population, by which important information about a small but potentially relevant subpopulation is possibly lost in the background (Gross et al., 2015)For example, stable cell lines that constitutively expressed SERT grew very slowly and only about 25% of the cells actually expressed the transporter, which precluded large scale culture (Andréll & Tate, 2013). This resulted in an approach to isolate some of the high expressers as against the low expressers by using a limiting dilution cloning approach. The focus of the study remained on the high expressers of lipid-engineered CHO-S cells following selective measures by diluting cloning. For relative impact of engineered CHO-S over-expressing DHCR7 and TM7SF2 on the secretory capacity of ER to be investigated, it was imperative to identify the possibly high producing cells for further studies.

6.3 Stable Integration and Overexpression of GFP and pcDNA3.1Hygro as control for investigation into TM7SF2 and DHCR7 engineered cells.

Two DNA integration systems were cloned and stably generated in CHO cells to enable the monitoring and quantification analysis of expression levels in TM7SF2 AND DHCR7 engineered cell lines. The choice to use GFP as a control was to ensure that the process of generating stably expressing cell lines and the presence of a protein load was in the control CHO-S pools and could be related to the observations when the YFP-tagged proteins were overexpressed (Figure 3.8). GFP is a quantitative reporter of gene expression (Soboleski et al., 2005) and has been shown to have no toxic effects on cells Chalfie et al., 2008) as it is devoid of cellular function. Also, as a widely used reporter, GFP is not known to show any high-affinity with other proteins hence would not influence the phenotypic characteristics of cells. pcDNA3.1Hygro expression vector with empty backbone stably transfected in cells generated blank pools upon analysis by western blotting and was used in this thesis as a verification point of control for TM7SF2 and DHCR7 expression levels in cells (Figure 3.14).

6.4 TM7SF2 and DHCR7 show characteristic clonal variation in stable CHO-S cells

Having observed beneficial phenotypes attributed to both TM7SF2 and DHCR7 overexpression in CHO-S cells, it was confirmed that there was a high degree of heterogeneity within cultures, leading to the identification of different parental pools for both TM7SF2 and DHCR7, referred to as A and B (Figure 3.16). Phenotypic heterogeneity is observed not only between CHO host cell lines but also within the cell population of CHO host lines. In fact, CHO cells are known for being able to adapt to changes in process conditions, which has been exploited in the industry to generate clonally derived cell lines with enhanced manufacturing capabilities (Frye et al., 2016). Some of the factors when considering stable cell lines include those that showed tendencies for high growth rate, slow death rate and also high specific cell productivity (Qp).

Further, more investigations into the presence of heterogeneity in cells were observed during growth profiling of cells where cells showed variation in growth rate. When microscopic analysis was done, cells were observed to show different levels of YFP expression suggesting the variations in the expression level of cells of the population (Figure 3.17). The desire to investigate clones with a high specific productivity rate was necessary as the heterogeneity of CHO cells confers individual clones with different copy numbers, integration sites resulting in varying specific productivities. By comparing clones for their highest growth and productivity rate, the best clones were isolated.

Clone-specific variations at the functional genetic level have been extensively described. In one study, high and low producer CHO-mAb sub-clones were observed to differ mainly in their DNA fragment sizes where high numbers of differentially expressed genes were identified **(**Ghorbaniaghdam et al., 2014). In another study, clonal variations in response to culture condition variation was reported (Datta et al., 2013). Peculiarity of clonal cells is relative to their specific productivity, strong variations in cell density, nutrient uptake and metabolic generation patterns. Clones generated from TM7SF2 Pool B following limiting dilution cloning methods were analysed by western blotting techniques for investigating expression levels of the various CHO-S clonal cells.

In addressing the heterogeneity in clonal behaviour, a crucial factor is due to the genetic instability that occurs during integration of copy number variant and vector-integration transformants during genomic loci. With the identification of the clonal CHO-S cell lines (Figure 4.2) (Figure 5.2), the

ultimate propensity was for these clonal cells to reach higher growth densities and have longer viability in culture while still maintaining cellular homeostasis. Phenotypes of such, are actively being sought for bioprocessing engineering in order to increase batch productivity and yield, (Dorai et al. 2010).

6.5 Localisation of the YFP-tagged TM7SF2 and DHCR7 proteins

All constructs used in this study were designed as detailed in the Zwerger et al., (2010) published paper. The YFP fusion proteins they express, following the N- to C-terminal order of the expressed entities had been cloned into the constructs. Target genes were subsequently cloned into different expression vectors which were expressed in CHO-S cells for this study. When a YFP fusion protein of TM7SF2/DHCR7 is expressed in mammalian cells, it is efficiently targeted to the ER membrane. Leader sequences are very important for guiding protein along the secretory pathway. However there was no ER leader sequence cloned into these constructs as published. Their approach was to characterize the structural consequences on both the nuclear and the cytoplasmic side of the NAV of U2OS cells overexpressing YFP-TM7SF2 and YFP-DHCR7, where they monitored the distance between the INM and ONM segregates.

We took a different approach as the focus was the functional implications an expansive CHO-S ER membrane secretory capacity might have on recombinant protein production. We monitored the ER by staining calnexin for localization studies (Figure 4.4) (Figure 5.9) where expression levels were monitored. In order to effectively monitor changes leading to ER expansion by the over-expression of TM7SF2 and DHCR7 for productivity however, the need to monitor ER by other approaches, such as using ER tracker dye may be necessary as we did not design new ER leader sequences compatible with the new cassette system which was important (as discussed in section 4.3).

Several studies however, have shown that sequence variation among signal sequences can affect the efficiency of protein targeting, translocation, and signal sequence cleavage (Randhawa et al., 2004). With reference to the paper published by Zwerger et al., (2010), they had reported that the N-terminal domains of both TM7SF2 and DHCR7, which they compared to LBR, did not interfere with the C-terminal domain and upon over-expression, induced prominent nuclear compaction and caused vacuolisation in U2OS cells. When CHO-S over-expressing YFP-TM7SF2 and YFP-DHCR7 were

investigated using confocal microscopy approach where we monitored YFP signals (Figure 3.17), it is observed that cells showed nuclear compaction but whether expression was in the peri nuclear space was not confirmed.

6.6 Transient expression of YFP-TM7SF2 and YFP-DHCR7 in CHO-S cells

Transient recombinant protein production is a promising alternative to stable systems, particularly for emergency situations in which rapid production of novel therapeutics is needed (Sukenik et al., 2018) An initial investigation to confirm the presence of TM7SF2 and DHCR7 where YFP reporter gene tagged to the N terminal of TM7SF2 and DHCR7 was monitored using fluorescence microscopy techniques (Figure 3.13). A brief study was also undertaken on mCherry-tagged LBR although this was later dropped to allow focus on TM7SF2 and DHCR7. Findings from the transient transfection studies showed some cells expressed the YFP signal for TM7SF2 and DHCR7 while mCherry was also expressed in LBR as red. The viability of CHO-S cells, which had been confirmed to be above 95%, prior to the experimental study, however declined following transfection procedure, with only about 50% of cells successfully transfected.

As it is a promising alternative to stable transgenic systems, particularly for emergency situations in which rapid production of novel therapeutics is needed (Sukenik et al., 2018)transient expression of five YFP-LBR mutants in human U2OS cells showed that they localize to the nuclear rim and also formed aggregates in the cytoplasm, similar to those seen in cells expressing YFP-LBR wild-type and they do not alter nuclear morphology Zwerger et al., (2010)**.** When transient over-expression studies of these protein were carried out along with wild-type untransfected CHO cells (without TM7SF2 and DHCR7 DNA plasmids), wild-type untransfected CHO-S cells did not show any expression signals, whereas TM7SF2 and DHCR7 showed increased expression levels over the time course suggestive of the continued translation activities between day 2 and day 3 as expression on day 3 also doubled expression on day 2 post transfection as confirmed by western blotting (Figure 3.9). DHCR7 was also studied on day 2 and day 3 post transfection and similar expression level was observed (Figure 3.10) with an increase in secretion amount over time.

6.7 Effect of TM7SF2 and DHCR7 overexpression on recombinant protein productivity

The essence of this research was to investigate the relationship between engineered overexpressing TM7SF2 and DHCR7 and specific recombinant protein productivity. Literature has it well documented, that the production of proteins in appropriate quantity and quality is an essential requirement of the present time as there now appears to be a progressive increase in the application of mammalian cells for proteins production as seen in a review by Hayat Khan, (2013). Mammalian cells are currently the main hosts for commercial production of therapeutic proteins, including monoclonal antibodies (mAbs) (Lee et al., 2009) after it was first used to produce active antibodies in 1987.

Quite a number of strategies have been used to engineer mammalian cells for improved quantity and quality of therapeutics such as chaperone engineering (Jossē et al., 2012), unfolded protein response (UPR)-based engineering (Chien et al., 2014) and secretion engineering (Peng et al., 2010). There has been significant progress in developing and engineering new cell lines, introducing novel genetic mechanisms in expression, gene silencing, and gene targeting have been achieved through optimal expression system for productivity, bioactivity, purpose, and physicochemical characteristics of the interest protein while taking into consideration the cost, convenience and safety of the system itself Hayat Khan,(2013).

The productivity of EPO and etanercept following the over-expression of genes have been investigated before using other targets (e.g. not DHCR7 or TM7SF2), albeit with mixed results (Pybus et al., 2014) reported no effects of XBP1s over-expression on EPO productivity in stable cell lines but significantly enhanced transient production in EPO-saturated CHO cells. Pybus et al., (2014) showed that the effects of xbp1s overexpression were more pronounced on CHO cells expressing ‘difficult-to-express’ recombinant proteins with limiting folding and assembly reactions, than cells expressing an ‘easy-to-express’ recombinant proteins.

Bioprocess engineering often focuses on growth/proliferation strategies to improve productivity (Kumar & Starly, 2015). Alternatively, yields can be improved by targeting specific pathways, examples of targeting pathways/genes via miRNAs is becoming more prevalent (Fischer et al., 2013) in order to increase CHO cell specific productivity. Overexpression of myc in CHO cells have been shown to increase the specific growth rate without negatively affecting the specific protein

productivity (Kuystermans & Al-Rubeai, 2009).

Engineered CHO cells overexpressing XBP1s have enabled a significant enhancement in protein productivity due to an improvement of the secretory capacity of cells, although, there were not any improvement of recombinant protein production. Here, we target lipid biosynthesis pathway where a cascade of regulatory genes interplay which regulate the TM7SF2 and DHCR7 signal levels are functional. Another reason for the increased recombinant EPO and etanercept transient expression may depend on high transcription levels of the genes of interest. It must be noted however, that depending on the cell type being used for overexpression, expression levels may vary. Additionally, productivity can easily be improved by increasing the gene copy number of the gene of interest independent of the promoter system used (Vassileva et al., 2001).

Sometimes, there may be a decline in productivity upon increasing the copy number and this might be a limitation in protein folding and secretion (Hohenblum et al., 2004). We monitored the culture behaviour shown by the TM7SF2 and DHCR7 overexpressing clones transiently expressing EPO and etanercept during this project where we compared data to stable GFP and pcDNA3.1hygro controls, although only over a period of 3 days (Figure 4.7). Our findings showed improved growth pattern for lipid engineered cells transiently transfected with EPO and etanercept ultimately translating into improved yield of both types of recombinant protein products in stable producer lines.

As the greatest increase in recombinant protein productivity was seen in cells overexpressing TM7SF2 pool B and DHCR7 pool A as well as the high producer clones, it is imperative to establish an overall consequences of TM7SF2 and DHCR7 overexpression on the phenotype of the cell with reference to cell productivity. Our analysis of extracellular protein expression of EPO and etanercept showed a direct positive impact of recombinant protein levels (Figure 4.7) (Figure 5.6) with increased TM7SF2 and DHCR7 overexpression levels of engineered cells.

However, conclusions drawn from the findings would require further work as there were no loading controls to ascertain equal loading of sample materials of GFP and pcDNA3.1Hygro as well as YFP-TM7S2 pools and YFP-DHCT7A pool investigated in this study. Subject to further work, it is safe to state, that there was indeed, a massive out turn of EPO secretory protein when YFP-TM7SF2 B pool

of lipid engineered CHO-S was investigated with control cells using western blots technique, following the equal amount of samples loaded, resulting in the direct positive impact, here reported.

6.8 Quantification of recombinant proteins for relevance to commercial value

Sensitive analytical methods for the quantification of protein concentration in solution are important in biological laboratories (You et al., 1997). However, the available methods have limitations in many respects, such as sensitivity, protein-to-protein variability and dynamic range (Pihlasalo, 2011). When EPO and etanercept secretory amounts were expressed, the hypothesis that the engineering of the CHO-S secretory machinery would result in the enhancement recombinant protein production, would not have been ascertained without a means to accurately quantify how much of these products were secreted in the lipid engineered cells , in relation to control. Absolute values of protein expression levels in cells are crucial information for understanding cellular biological systems (Narumi et al., 2016). Generally, for the purpose of analysis, western blotting was used to assay for proteins.

When transient transfection was carried out over a 2 day period, YFP-TM7SF2 (Figure 3.9) and YFP-DHCR7 (Figure 3.10) were probed with GFP control and the predicted molecular weights investigated on western blots. For stably expressing cells, western blots were used to investigate whether the genes of interest had been successfully integrated into the chromosome of CHO-S cells (Figure 3.16). However this methodology is not without its limitations such as error during loading and poor migration of proteins. Densitometry data generated for western blots are commonly used to compare protein abundance between samples ((Butler et al., 2019).

We have quantified EPO and etanercept expression level using Densitometry, Data generated on densitometry analysis were obtained from western blots. For instance, densitometry data of Figure 3.12 had analysed the western blots obtained in Figure 3.10 for the quantification of YFP-TM7SF2 lysates as well as GFP transiently produced on different days, post transfection (day 1, day 2 and day 3). While western blots confirmed the presence and sizes of these proteins, it was not certain how much of these endogenously secreting proteins was recovered each day. However by densitometry analysis, the number of folds per expression could be determined. For YFP-TM7SF2 assay, western blots of Figures 4.5, 4.7 and 4.10 were analysed using densitometry in Figures 4.6, 4.9 and 4.11 respectively while for YFP-DHCR7 assay, western blots of proteins expressed in Figure 5.3 and 5.6

were quantified in Figures 5.4 and 5.7 respectively. Bennati et al., (2006) by densitometry analysis of lipids separated by TLC, quantified cholesterol in plasma and liver microsomal membranes. Prabhu et al., (2016) quantified the effects of various sterols on DHCR7 protein stability using densitometry.

Zwerger et al., (2010) had quantified protein concentrations of the lysates using the method of Bradford (Bradford, 1976) using the Bio-Rad Protein Assay (Bio-Rad #500-0006) and bovine serum albumin (BSA) as a standard, a method also used in this work. Lysates expressed on western blots were first analysed for respective concentration and calculations for equal loading up to 20 µg across each well was done. Several studies have used Bradford as a means to quantifying the concentration of proteins. Kim et al., (2004) quantified proteins (Carbamoyl Phosphate Synthetase I and Ornithine Transcarbamylase) using a Coomassie brilliant blue binding assay according to Bradford while EPO was measured by an ELISA for the enhancement of Erythropoietin Production from CHO Cells using Urea Cycle Enzymes, Carbamoyl Phosphate Synthetase I and Ornithine Transcarbamylase.

Several other methods have been used to quantify proteins. These include the ultraviolet absorbance methods which are based on the absorption of ultraviolet (UV) light by proteins in solution. With a peak of approximately 280 nm, resulting from the amino acids phenylalanine, tryptophan, histidine, tyrosine, cysteine, and cysteine-containing aromatic rings, and at approximately 200 nm, primarily from peptide bonds and secondarily from tryptophan and tyrosine (Albani, 2004).

Analysis using Enzyme-linked immunosorbent assay (ELISA) is also very useful for scaling Proteins. This method is based on the concept of antigen–antibody reactions, representing the chemical interaction between antibodies produced by the B cells of leukocytes and antigens. Hence, by exploiting this reaction, ELISA permits the highly sensitive and selective quantitative/qualitative analysis of antigens, including proteins, peptides, nucleic acids, hormones, herbicides, and plant secondary metabolites (Sakamoto et al., 2018). The Biuret method is known to be the oldest method for the quantification of proteins and is still commercially available. It involves only one reagent addition and a single incubation of 20 min. The divalent copper ion in the reagent forms a complex with the amide groups of the protein and is reduced to a monovalent ion under alkaline conditions. The purple complex formed absorbs at 550 nm and the absorbance is directly proportional to the quantity of proteins (Pihlasalo, 2011). .

6.9 Content Analysis in relation to Zwerger et al., (2010) reference paper

The present study reviews our findings as against the findings of the publication that informed this work. Although many of the assays and methodologies differ as well as certain findings, a common ground lie in the influence generated when TM7SF2, DHCR7 and LBR were over-expressed. Zwerger et al (2010) extensively reported on LBR and introduced the relationship of LBR with the two sterol reductase (TM7SF2 and DHCR7); its C-terminal domain being 58% identical to TM7SF2 and 37% identical to DHCR7. It is important to note, that Zwerger et al., (2010) investigated their work using several human cell lines, such as skin (A431), ovary (HeLa), breast (MCF7), liver (PLC), bone (U2OS), and brain (T98G), except CHO-S - the platform for this work.

When Zwerger et al., (2010) investigated the nuclear compaction and vacuolization observed in U2OS cells, both intact YFP-TM7SF2/YFP-DHCR7 and C-terminally truncated TM7SF2/DHCR7 were transfected and over-expressed in U2OS cells. They observed that, not only did the C-terminally truncated TM7SF2/DHCR7 induce comparable effects, similar to the LBR truncations already observed, but even the wild type YFP-TM7SF2/YFP-DHCR7 caused a compaction of the U2OS cells nuclei as well as dilation of the Perinuclear space and even the endoplasmic lumen. We have over-expressed the wild type YFP-TM7SF2/YFP-DHCR7 (samples kindly provided by Monika Zwerger) in CHO-S and have observed that the YFP reporter signal of these sterol reductases was traced to the portion around the nuclei of CHO-S believed to be the ER lumen. Further assay to confirm whether expression observed was from the ER, was done by co-expressing YFP-TM7SF2 and YFP-DHCR7 with calnexin a molecular chaperone in the ER lumen to investigate co-localization.

Data from this work corroborates the findings, by the Zwerger et al., (2010) on the localisation of these sterol reductases and where the influence was going on (Figure 5.9). However, whether the influence on the ER led to some vacuolization was not confirmed in this work. However, with the generation of clones having observed variation in expression levels of the YFP-tagged sterol reductases, we generated nine clones (in total) between the two sterol reductases, considered to be high expresser in CHO-S cells (Figure 4.2) (Figure 5.2), a different approach to the Zwerger et al (2010) work.

While Zwerger et al., (2010) reported a comparable phenotype upon overexpression in the related sterol reductases, we have reported a comparable phenotype among clones over-expressing YFP-TM7SF2 and YFP-DHCR7 which might not be unconnected to their functional role at the molecular level. For instance, when we observed that TM7SF2 clone 7 showed a similar phenotype to TM7SF2 clone 11. We have also observed how TM7SF2 clone 3 presents a layered cellular formation similar to TM7SF2 clone 10 (Figure 4.3). The implications of these phenotypic conformations would need to be further investigated, going toward.

Zwerger et al., (2010) extensively reported on Lamin B receptor (LBR) protein. We have only briefly investigated this protein. However LBR has been reported as a dual function protein with functional activities occurring both in the Nucleus and also in the cytoplasm (ER Lumen). When Zwerger et al., (2010) investigated the impact of LBR mutant variants on the integrity of the lumenal compartment, they had hypothesized, as published, an effect on this compartment consisting of the ER and the Perinuclear space (PNS) mainly due to the relation of TM7SF2 and DHCR7, which had induced a comparable phenotype according to their submission.

Their findings however reveal a correlation with the loss of nuclear size and an increase in nucleus-associated vacuole NAV volume. When we studied the localisation of LBR, we monitored the mCherry-tagged protein (kindly supplied by Monika Zwerger) using transient fluorescent microscopy techniques. 24 h after transfection, the CHO-S cells were fixed with paraformaldehyde and analysed. With control cells having DAPI, the nuclei were marked and cells expressed mCherry signal, in a region around the nuclei suggesting the presence of LBR in the Perinuclear space (PNS) where it is believed to localise to the ER lumen. The carboxy-terminal domain has enzymatic activity, that is, it is a sterol reductase acting at a distinct step of the cholesterol biosynthesis pathway (Herrmann & Zwerger, 2010). Subramanian et al.,(2012) had reported that Lamin B receptor (LBR) is a bifunctional nuclear membrane protein with N-terminal lamin B and chromatin-binding domains plus a C-terminal sterol D14 reductase domain, hence its exhibition of 3β-hydroxysterol Δ14-reductase activity (Bennati et al., 2006). While the focus on this work was on TM7SF2 and DHCR7, the molecular implications of the over-expression of these sterol reductases may as well be influencing the C-terminal enzymatic activity of LBR, which though not investigated in this work, but was established in the Zwerger et al., (2010) paper.

An important aspect to this work was to gain an understanding of the impact, as to whether positive or negative, induced on the mammalian cell lines as a result of the phenotypic characteristics observed when mCherry-LBR, YFP-TM7SF2 and YFP-DHCR7 was over-expressed. Again, Zwerger et al., (2010) investigated whether the nuclear compaction, NAV and CV formation observed were not due to ER stress, a condition known to trigger the unfolded protein response (UPR) to maintain ER homeostasis. Analysis of xbp1 mRNA has a stress marker and an autophagy marker protein to probe the impact of LBR and the sterol reductases on U2OS cells led to the conclusion that the increased vacuolisation observed do not interfere with cellular processes as autophagy pathway was not present as observed. We have also looked at the influence of over-expression on CHO-S cells. The generation of the stable cell lines over-expressing these sterol reductases in pcDNA3.1Hygro allowed for the generating on an empty cassette vector (pcDNA3.1Hygro) as a negative control channel.

Unlike Zwerger et al., (2010), a GFP control was also generated as control. GFP and YFP only differ in a few amino acids and the plasmid was readily available for use. A growth profile assay carried out on culture for a 10 day period showed a sustained growth pattern for the engineered cells as compared to controls (Figure 3.20). When cells where further investigated, we confirmed that there was indeed an heterogeneous population and not all cells were over-expressing YFP-TM7SF2 and YFP-DHCR7 using flow cytometry and western blots (Figure 3.19) (Figure 4.1) profiling of the growth behaviour of clones agreed with submissions of Zwerger et al., (2010) as there were no tendencies for apoptosis and engineered cells were clearly not undergoing autophagy. Despite these findings, the statistical inference drawn at a probability less than 0.05 ($p<0.05$) showed there was no significant difference in growth pattern.

On the molecular implications of these findings, Zwerger et al., (2010) discussed that while they had observed a remarkable membrane extension, which did not activate ER stress, nor resulted in autophagy nor apoptosis, the molecular mechanism involved remained elusive. To understand what might be going on at the molecular level, we looked that a novel approach to attempt to influence cholesterol biosynthesis, a pathway that takes place in the ER lumen. While Zwerger et al., (2010) did exclude the possibility that minor changes in cholesterol concentration of ER and NE membranes or the presence of low amounts of intermediate sterol, the bulk of the activities is vested upon the capacity of the ER lumen to synthesize proteins. Cholesterol regulates HMG-CoA reductase activity in the cell and the gene corresponding to HMG-CoA reductase is controlled by a family of proteins

called separator of regulatory element-binding proteins (SREBP) and when intracellular cholesterol levels are high, SREBP are inactive; if cholesterol decreases, the N-terminal domain of SREBP is cleaved by hydrolysis. In future, it assays to quantify cholesterol and lipid amounts will be beneficial if the molecular implications of the influence and positive phenotypic impact of overexpressing LBR, TM7SF2 and DHCR7 is to be unravelled.

6.10 Overexpression Engineering strategies in some other cell expression system

In December 1983, a seminal paper appeared on the overexpression of human IFN-b in insect cells with a genetically engineered baculovirus (Oers et al., 2015). Overexpression first gained prominence as a screening tool in the molecular genetics era shortly after the development of yeast transformation techniques and the construction of genomic libraries in vectors derived from the endogenous 2 μ plasmid, which are maintained at 10 – 30 copies per cell (Prelich, 2012). Since its inception, several studies have continue to manipulate this molecular technique for therapeutic purposes. For instance, Zeng et al., (2014) observed the overexpression of Bmal1 gene inhibited colorectal cancer cell proliferation and increased colorectal cancer sensitivity to oxaliplatin in three colorectal cancer cell lines and HCT116 cells model in vivo. Lee et al., (2009) showed that the overexpression of Heat Shock Proteins (HSP27 and HSP70), either individually or in combination, can delay the onset of apoptosis in CHO cell cultures and thus extend culture lifespan and improve recombinant protein production in fed-batch cultures. By overexpressing engineering, (Wilkens & Gerdtzen, 2015) showed that PYC2 in CHO cells were able to improve their exponential growth rate and cells transfected with the fructose transporter gene were able to increase cell density and reach the same volumetric protein production as parental CHO cells in glucose, to state but a few. However, lessons learned from overexpression studies have several implications for human health, either positive or negative, impacting our understanding of the causes and treatment of diseases, as there are numerous human diseases directly caused by increased gene expression (Prelich, 2012).Till date, several studies continue to explore the strategy of overexpression engineering for therapeutic purposes.

6.11 Impact of overexpression of YFP-TM7SF2 and YFP-DHCR7 sterol reductases on the manufacturing processes and the downstream processing

When commercial scale biopharmaceutical manufacturing began in the middle of the 1980's, the average titre started out at 0.5 g/l (McAuliffe, 2018). As at 2015, however the average reported titre was averaging at greater than 3g/l, with top end values reaching 7 & 10g/l, while average yields being reported at 70% (Rader & Langer, 2015). A typical bioprocess is split into two sections, upstream processing where the cell line is grown and the product synthesised and downstream processing where the cell mass and other contaminants are removed and the product is captured in a pure form (Mclachlan, 2017).

The upstream process is due to advances in culture media and its optimisation, in expression systems, genetic engineering and in cell line development as advances in process development have come in the genetic engineering and modification of cell lines, rather than the equipment (McAuliffe, 2018). Hence, there has been a significant progress in the optimisation of upstream processing in the last two decades and this is largely because Upstream titres depend mostly on biological limits (for example, cell line or media optimisation) and can be raised without an increase in costs whereas downstream capacity always scales at least linearly with costs due to its physical principles for separation (Gronemeyer et al., 2014). It is expected, that the genetic engineering of CHO-S secretory machinery by overexpression of sterol reductases (TM7SF2 and DHCR7) ordinarily will reduce the cost on downstream purification which should be beneficial for production processes.

This may explain why the biopharmaceutical industry continues to change rapidly as the production portfolio steadily expands for new biologically active molecules resulting in the need for fast and reliable process development and optimisation tools (Baumann & Hubbuch, 2017). In addressing the need for a scale up for commercial manufacturing, there is a need to look into omics-based approaches, such as transcriptomics, proteomics and metabolomics, which have been used in the whole process of developing recombinant CHO cell-based production in both upstream and downstream processes (Kim et al., 2012) and these range from clone selection, cell engineering, culture media and culture environments in the upstream process, to protein purification and characterisation in the downstream process (Gupta & Lee 2007).

Without doubt, demands for high quality biologics will continue to increase in the coming decades whereby, the boundary conditions will change substantially with regards to the amounts to be produced such that the number of products will increase (Gronemeyer et al., 2014). One area of interest in biopharmaceutical manufacturing is the invention of new designs for downstream processes, namely, the protein separations. Such a development, if successful, would be a major shift in the way that biopharmaceuticals are manufactured (Hong et al., 2018) and as the downstream is an integral part of the entire production process and is influenced by many factors, downstream equipment which at present, has been designed with the intent of lower cell titres of the previously mentioned 0.5 to 3 g/l range, resulting in this equipment reaching is physical limits (McAuliffe, 2018), would need to be reviewed.

Meanwhile, as recombinant therapeutic proteins are usually produced by genetically-engineered prokaryotic or eukaryotic host cells using fermentation/cell culture technology (Li, 2017), the genetic engineering of YFP-TM7SF2 and YFP-DHCR7 sterol reductases in CHO-S cells for the production of recombinant therapeutic proteins, for instance, would require a more robust downstream processes in order to meet the increased challenges.

6.12 Molecular mechanism involved in cholesterol (lipid) biosynthesis which may be used to enhance secretory productivity of CHO-S cells

The big question which defines the aim of this research is whether cholesterol biosynthesis manipulation can be used to control and channel increased productivity in CHO-S secretory machinery.

Firstly, to address this curiosity, we review the role of cholesterol/lipid biosynthesis in membrane secretion and unravel how some key players of the biosynthesis pathway modulate signalling within the ER to achieve biochemical conformation of their functions. The secretion of proteins by mammalian cells is a complex pathway involving an initial polypeptide translocation from the cytosol into the lumen of the endoplasmic reticulum (ER), where folding and assembling happen before being targeted to their final destination. Fourn et al., (2014) had reported, that for cells to reach a consistent and high-yield production of recombinant proteins, there is the need to identify the limiting bottlenecks, as well as specific engineering strategies that modify and improve the post

translational protein processing and secretion machineries. Although recent studies have reported that the bioengineering of host cell lines may improve the modification or the secretion of heterologous proteins and of other therapeutics, the molecular mechanisms by which these ER proteins may improve protein secretion and their ability to act on various recombinant proteins have not been studied systematically (Fourn et al., 2014).

A review by Fagone & Jackowski, (2009) reported on the role the ER, which together with the Golgi apparatus, is a major site of *de-novo* bulk membrane lipid synthesis and how they constitute the endomembrane compartment, which is a major site of lipid biosynthesis in eukaryotic cells. Lipid (including cholesterol) biosynthesis is highly conserved in all the eukaryotes and is needed to build new membranes and maintain active signalling. TM7SF2/DHCR7 genes transcribe for C14-SR, involved in cholesterol biosynthesis. By overexpressing these sterol reductases, what we have done, was to alter key players of the cholesterol biosynthesis pathway, exploiting its over-expression capacity, such that it would lead to an expansion of the ER membrane capacity which is speculated, to result, in turn, to an increased cell growth and productivity. According to Snapp et al., (2003), high abundance of transmembrane proteins in the ER can induce extensive membrane growth, whereas low expression does not alter ER morphology.

From the literature an awareness into the functional role of TM7SF2 and DHCR7 had been established. Zwerger et al., (2010) had suggested from its findings that the presence of wild-type TM7SF2 and DHCR7 as well as mutant LBR might alter certain membrane properties as the overexpression of the wild-type forms of TM7SF2 and DHCR7 was observed to have mimicked the phenotype observed for mutant LBR. They had found, that even fully functional proteins can critically affect the ER and NE morphology. However, the molecular mechanism that leads to the remarkable membrane extension, as reported, remains elusive. LBR, which was the main focus in the previously reported study, is not an SREBP-2 target gene unlike TM7SF2 and DHCR7.

Some studies have suggested that this may be due to LBR being constitutive, whereas TM7SF2 encodes the inducible sterol Delta (14)-reductase activity. Further, the molecular mechanism of TM7SF2 and DHCR7 overexpression may be observed in the SREBP cascade signalling mechanism during cholesterol biosynthesis. SREBPs are weak activators and require additional transcription factors to achieve optimal regulation of sterol sensitive genes. While SREBP1a/-2 have been reported

to up regulate cholesterol synthesis genes, SREBP-1c is responsible for the regulation of genes involved in FA and TG synthesis pathways (Horton et al., 2002). SREBP-2 however is the main transcription factor responsible for regulating the cholesterol biosynthetic pathways (Sato 2010). TM7SF2 is responsible for cholesterol biosynthesis and is regulated by SREBP-2 (Schiavoni et al., 2010).

Therefore, cholesterol metabolism are controlled in a coordinated manner by SREBP. A review by Sharpe and Brown (2013), reported that although nearly all the genes encoding cholesterol synthesis enzymes are SREBP targets, a notable exception is the LBR, a protein of the inner nuclear membrane mainly involved in heterochromatin organisation, but also possessing sterol Delta (14)-reductase activity. It is interesting how regulating cholesterol synthesis in cells underpins cholesterol homeostasis of the whole organism, and forms the basis of successful therapies for treating human disease. As such, investigation of the SREBP control and interaction with DHCR7 and TM7SF2 should be a focus in trying to understand how the over-expression of these two proteins leads to the phenotypes observed.

6.13 Future work and Directions

Although the outcome from this work suggests that the overexpression of either YFP-TM7SF2 or YFP-DHCR7 protein can result in the generation of CHO cells that give transient enhanced yields of bio therapeutic proteins, much further work needs to be undertaken to confirm this and determine the mechanism(s) by which this effect is impacted on the cell. It is clear that there are many opportunities that could be explored in this area while improving on current engineering strategies by engineering different parts of the protein secretion pathway through knowledge of the flux through the secretory pathway for improvement of heterologous protein production. For instance comparative analysis of 'omics data gathered under specific physiological conditions has revealed differentially regulated molecular mechanisms responsible for desirable phenotypes in isogenic clone populations and guided the design of improved cell factories (Ley et al., 2015).

Literature reports show that the process of clonal cell line generation has inherent pitfalls as random integration of the experimental vector/transgene can result in various genetic phenotypes such as intensity of expression potentially due to insertional mutagenesis and/or copy number variation (Griffith 2015). While absolute care was used during the generation of the clonal cells, further investigation may be carried out to get a better and clearer understanding on the cellular machinery of clones, such as clone 10, 11 and 7 of TM7SF2 engineered cells and determine if selective pressure in identifying clones resulted in their isolation while also carrying out further studies on the influence of transient expression of recombinant proteins such as EPO and etanercept on the clonal cell lines. Further work determining the locus of integration, the transcript and protein amounts of TM7SF2 in the different cell lines and the half-life of these, how the amounts changes throughout culture and with extended passage number (the stability) and obtaining a more precise measurement on the volume and amount of ER could be investigated.

It would also be of interest to investigate the influence of the over-expression of these molecules on general metabolism and lipid metabolism of the CHO cells to see how each clone impacts growth by studies that involve the molecular mechanisms of genome dynamics with reference to cell proliferation. Growth being a dynamic process results from cells accumulating mass. While the growth rates of clones investigated are related and proportional to their respective RNA and protein synthesis rates, there is a need to explore the role of gene regulation in cellular growth of the pools

and clones. Heterogeneous protein production has been reported to often result in a decrease in the maximal specific growth rate while stating that it is obvious to expect some changes in heterologous protein production during prolonged growth (Krogh, 2009).

While this study has demonstrated recombinant protein productivity and biomass-influenced generation using stable batch cultures of engineered CHO-S cells, it would be interesting to investigate whether the specific effect of engineering the cells is maintained at large scale production in the fermenters and during fed-batch culture. We have showed that by overexpressing TM7SF2 and DHCR7 more EPO and etanercept could be generated (as determined by western blot) when compared to stable GFP and the ocDNA3.1Hygro control cells. However, questions such as whether these are reproducible in large scale batch and fed-batch fermenters remain, as it is known that some choices of cell type may also have influences on the subsequent downstream processing (Hansen et al., 2017). The makeup of the CHO-S model may have also contributed to the expression profiles observed for both parental pools and clones of TM7SF2 and DHCR7 engineered cells. Going forward therefore, it would be interesting to analyse other CHO cell lines as reported in literature, that they respond differentially to process platforms further downstream (media, feeding strategy, bioreactor conditions, purification, etc).

A future direction for this project would be the genetic manipulation of the host cell lines by co-overexpression of gene(s) which promote protein secretion, for example co-expressing TM7SF2, DHCR7, TMS1, a J domain protein, localized in ER lumen and is induced by heat stress Sterol C5 desaturase (SC5D) while down-regulating processes that hinder production, for example, Follicle Stimulating Hormone (FSH), Cholesterol 7α-hydroxylase (CYP7A1). During the project there was not enough time to generate an inducible multi-gene vector cassette system, an aspect of great interest. With the manipulation of multiple genes simultaneously using either constructive or regulatable promoters, we can control the expression of exogenous gene and investigate cells during gene silencing by transient knockdown of gene expression while also expressing the DHCR7 and TM7SF2 genes.

The role or impacts of the proteins over-expressed in this study on lipid biosynthesis and in driving ER expansion and changes to the lipid nature of the ER and other membranous structures was not investigated in this study. Many studies have been raised about the functional relevance of lipid

signalling pathways in response to ER expansion (Madeline et al., 2018). Therefore, it would be important to look into the influence that engineering of the two proteins investigated here have on lipid biosynthesis by determining any impact on the lipids present in the cell upon over-expression using methods to determine lipids and the study of lipidomics. A study investigating CHO cell lipids has been reported and used mass spectrometry to determine the lipid profile of industrially relevant CHO cells. Whether any ER expansion actually does occur upon overexpression of the target proteins could then be related to impacts on lipid biosynthesis.

Data from this research suggests that the overexpression of the two proteins influences recombinant protein production, the mechanism by which this occurs, as described above, was not determined. In addition to determining if the ER had expanded, investigation of the contents of the ER could be investigated. For example, if the ER was expanded, was this just the total ER membrane space or was there also an increase in the total amount of ER machinery (e.g all the chaperones and proteins within the ER were also increased in amounts) or was any impact restricted to just the membrane component of the ER. A further level of investigation could be to investigate if there was any impact on vesicle transport or other organelles in the secretory pathway, including the Golgi. As part of this, it would be interesting to determine if there was any impact on post-translational glycosylation of recombinant proteins and the recombinant product characteristics across different engineered cells.

There are limitations identified in this work. For validation studies, the need to analyse recombinant protein secretion levels along with a loading control is crucial. Although protein samples were quantified using Bradford assay for equal loading on SDS PAGE gel, a loading controls generally conveys numerous benefits particularly in relation to quality. Issues such as edge effect when proteins in outer lanes drift towards the centre can be removed through the use of a loading control. Loading controls can also be used to minimize the impact of erratic protein expression levels. In any situation in which proteins may be affected by either loading or protein transfer errors. Therefore ,it is of essence that this is considered for future work In future, loading controls such as Beta-actin Tubulins (Alpha tubulin and Beta tubulin), Lamin B1, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) among others may be considered for use. Also enzyme quantification assays used in this work can be improved. Apart from western blots, Bradford assay and densitometry, it might be quite useful to investigate protein concentration using ELISA (enzyme-linked immunosorbent assay), in which an antigen immobilised on a solid surface forms a complex with an antibody that is linked to

an enzyme. This research could equally benefit from other quantification methods for EPO and etanercept, and these include measurement of Ultraviolet (UV) absorbance at 280 nm, Bicinchoninic acid (BCA), Lowry, Biuret and many more.

6.14 General Conclusions

Overall, findings from this project demonstrate how TM7SF2 and DHCR7 overexpression in CHO-S cells can be beneficial in the specific productivity capacity of recombinant proteins and therapeutic agents. By overexpressing TM7SF2 and DHCR7, two sterol reductases of cholesterol biosynthesis with altered signal expression levels, we have maintained an unusual expression levels of these proteins as they influence CHO-S cellular growth when expressed with GFP and pcDNA3.1Hygro stably expressing cells and although densitometry data for western blots suggest there might have been some increase in EPO and etanercept expression levels in lipid engineered CHO-S cells, these are not considered statistically significant at a set probability value less 0.05 ($p < 0.05$). Given all functional validation and cytological analysis, it is not known whether, coordinated lipid biosynthesis has actually resulted in an expansive ER membrane system. Generation of different clones with beneficial phenotype suggests that the effect of overexpression of TM7SF2 and DHCR7 may be influential towards ER expansion.

This project has shown that:

1. Transient and stable overexpression of TM7SF2 and DHCR7 tagged with YFP were achieved in the commercial CHO-S host cell line.

   YFP reporter gene tagged to the N-terminal of TM7SF2 and DHCR7 allowed for the cellular monitoring of YFP signal of proteins both transiently and stably. However, the localization studies of TM7SF2 and DHCR7 was not confirmed to be confined to the ER. Although in the literature which informed this research, Zwerger et al., (2010) had hypothesized that the related sterol reductases TM7SF2 and DHCR7 induce a comparable phenotype and that two proteins are predicted to reside in the cytoplasm.

2. Fluorescence microscopy of both transiently and stably expressing TM7SF2 and DHCR7 YFP CHO-S cells suggests that both proteins are mainly localised to the ER.

   Functional validation studies showed that clones exhibit different phenotypic expression levels such as cell viability, cell densities which was observed when growth profiling was carried out on cells while protein yield was confirmed using western blotting techniques, when assayed for, along with GFP and pcDNA3.1Hygro stable cells as positive and negative channels respectively. High expressers of both TM7SF2 and DHCR7 were identified further characterised. Of all eleven

clones generated for TM7SF2, clones 3, 4, 7, 8, 10 and 11 were selected as top expressers of TM7SF2 whereas of seven generated DHCR7 clones, clones 4, 6 and 7 were selected as top expressers of DHCR7. Data could have benefitted from a loading control on western bots images to confirm equal amounts of proteins were loaded, although all western blots of lysates were first quantified by Bradford assay for the purpose of equal loading for western analysis.

3. Overexpression of these cholesterol biosynthesis proteins (TM7SF2 and DHCR7) did not negatively impact upon CHO-S cell growth or culture viability although no very high expressing pools or clones were isolated.

   Stable overexpression of TM7SF2 and DHCR7 in CHO-S cells favours beneficial phenotypes as increase in cellular growth and recombinant protein yield was observed. These findings have been observed at culture temperature at 37°C and may not be same in event of a temperature shift. Therefore, stable over-expression of TM7SF2 and DHCR7 have shown increased proliferation and expression levels of recombinant proteins when cultured by 37°C.

4. A TM7SF2 pool (pool B) with higher expression levels than other pools gave increased transient expression of EPO and etanercept amounts compared to a pool with lower expression (pool A). On productivity, pools and clones showed various amount of EPO and etanercept expression levels as there was a significant out performance, in many cases, over pcDNA3.1Hygro. Interestingly, high expresser clones maintained a similar pattern of recombinant EPO and etanercept expresser levels according to their secretory capacity as confirmed by the TM7SF2 expression levels. TM7SF2 Clone 11 remained the highest expresser of EPO and etanercept whereas DHCR7 Clone 7 expressed highest amount of EPO and etanercept. However, data could have benefitted from more quantification methods to ascertain how much of recombinant protein materials were generated in relation to controls.

5. Clones of YFP-DHCR7 showed more ‘even’ expression between cells and variable EPO and etanercept expression.

   It is not clear if cells with increased recombinant protein expression do so because of over-expression of DHCR7 or is due to clonal variation although there appears to be some relationship between YFP-DHCR7 amounts and secreted recombinant protein amounts.

6. Further work should be undertaken to determine if overexpression of these proteins impacts cholesterol biosynthesis and the secretory capacity of CHO-S cells as well as explore more quantification assays to establish these findings.

6.15.1 Concluding Remarks

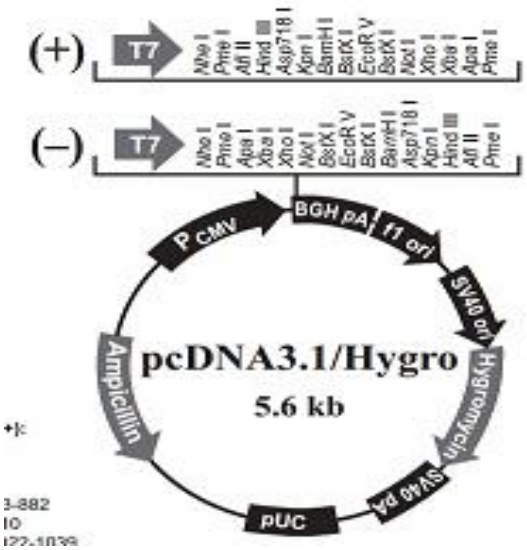
Advances in systems and synthetic biology applications have made it possible to undertake findings that cut across use for industrial and academic applications. The scope of this work has highlighted the possibility of engineering the protein secretory pathway of the ER to improve heterologous protein production by controlled manipulation of desired pathways. The most promising direction for this work is the genetic manipulation of host cell lines, re-engineering its secretory system. The data reported here provide a better understanding of the effects of overexpression of TM7SF2 and DHCR7 on CHO-S cells. Our findings suggest that the overexpression of proteins that impact upon cholesterol biosynthesis can be manipulated for the expansion CHO secretory capacity. By stably overexpressing TM7SF2 and DHCR7, we generated the parental pools of TM7SF2 engineered cells as well as DHCR7 engineered cells. Due to the highly reported heterogeneity of CHO cells, we carefully identified the high clonal producers of TM7SF2 and DHCR7. Clones 3, 4, 7,8,10 and 11 as the top expressers of TM7SF2 while clones 4, 6 and 7 were considered top producers. When we monitored the growth rate and viability of the engineered cells over a 10 day period, with control, the clones showed increased growth rates and higher viabilities and do not show any toxicity to the cells when compared to controls. Each clone displayed adjudged peculiarity in presentation of nucleus during confocal imaging suggesting that they differ in phenotypes variation that exist among the clones. Therefore we have confirmed that CHO-S cells exhibit functional heterogeneity and high heritability. It has been successfully demonstrated that by engineering the secretory processes of the CHO cell machinery, we produced more transient recombinant EPO and etanercept-a difficult-to-express TNFα fusion protein. Interestingly, amounts secreted from the extracellular channel correlated with the expression levels of engineered TM7SF2 and DHCR7 cells. When the clones were transfected transiently, they produced EPO and etanercept accordingly to their expression level. It has been noted, that there were no loading controls presented with western blots data and this serve as a limitation to findings. Also, data could have benefitted from more quantitative assay methods. Overall, overexpression of the cholesterol biosynthesis proteins

did not impact upon cell growth behaviour, however transient expression of two model recombinant proteins (EPO and etanercept – a TNFR-Fc fusion protein) that are difficult to express in CHO cells was enhanced in CHO cells engineered to overexpress the cholesterol biosynthesis proteins.

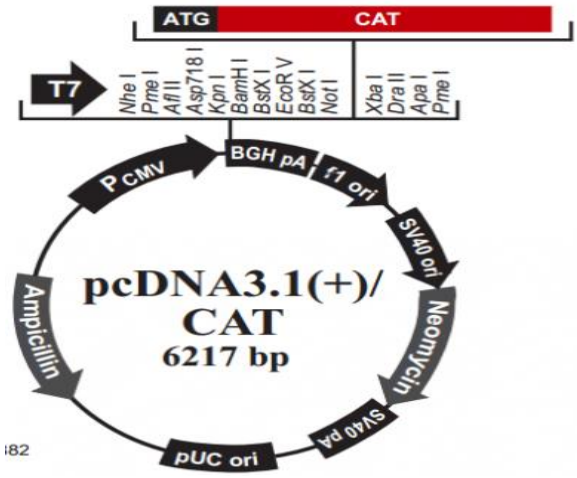
Appendices

7.1 Schematics of commercial vectors used in the cloning and expression of Transmembrane7superfamily member 2 (TM7SF2) and 7-DeHydroCholesterol Reductase (DHCR7)

(A)



(B)



Schematic 7.1 Vectors used in the transient and stable expression of TM7SF2 and DHCR7

(A) Vector map of pcDNA3.1Hygro used in the stable expression of TM7SF2 and DHCR7.

(B) Vector map used in the transient expression of TM7SF2 and DHCR7.

7.2 YFP-Tagged Sequence for TM7SF2 and DHCR7

7.2.1 Sequence for YFP-TM7SF2 Wildtype

ATGGCCCCCACTCAGGGCCCCCGGGCCCCGCTGGAATTCGGAGGGCCCCTGGGCGCCGCGGCTC
TGCTACTGCTGCTGCCCGCCACCATGTTCCACCTGCTCCTGGCGGCCCGTTCGGGCCCCGCGCGCC
TGCTGGGTCCACCCGCGTCCCTGCCGGGGCTGGAGGTGCTGTGGAGCCCACGGGCGCTGCTGCT
GTGGCTCGCCTGGCTCGGCCTGCAGGCGGCGCTCTACCTACTGCCGGCGCGCAAGGTGGCCGAG
GGGCAGGAATTGAAGGACAAGAGTCGCCTGCGCTATCCTATTAACGGCTTCCAGGCCCTGGTGCT
GACAGCCCTGTTGGTGGGGCTGGGGATGTCAGCGGGGCTGCCTCTGGGGGCGCTCCCGGAAATG
CTCCTGCCCTTGGCGTTTGTCGCCACCCTCACCGCTTTCATCTTCAGCCTCTTTCTCTACATGAAGGC
GCAGGTAGCCCCAGTTTCGGCCCTGGCACCTGGGGGGAACTCAGGCAATCCGATTTACGACTTTT
TTCTGGGACGAGAGCTCAACCCTCGTATCTGTTTCTTCGACTTCAAATATTTCTGTGAACTGCGACC
CGGCCTCATCGGCTGGGTCCTCATCAACCTGGCCCTGTTGATGAAGGAGGCAGAGCTTCGAGGCA
GTCCCTCACTGGCCATGTGGCTGGTCAATGGCTTCCAGTTGCTCTACGTGGGTGATGCCCTCTGGC
ACGAGGAGGCCGTCCTCACCACCATGGATATCACACATGACGGGTTTGGCTTCATGCTGGCGTTT
GGGGACATGGCCTGGGTGCCCTTCACCTACAGCCTGCAGGCCCAGTTCCTGCTGCACCACCCGCA
GCCCCTGGGGTTGCCCATGGCCTCTGTCATCTGCCTCATCAATGCTACTGGTTACTACATCTTCCGT
GGGGCGAATTCCCAGAAAAACACTTTCCGAAAGAATCCTTCTGACCCCAGAGTGGCTGGGCTTGA
GACCATCTCTACAGCCACAGGGCGGAAACTGCTGGTGTCTGGGTGGTGGGGTATGGTCCGCCAT
CCCAACTATCTTGGAGACCTCATCATGGCTCTGGCTTGGTCCTTGCCCTGCGGGGTGTCACACCTG
CTGCCCTACTTCTACCTCCTCTACTTCACCGCGCTGCTGGTGCACCGTGAGGCCCGGGATGAGCGG
CAGTGCCTGCAGAAGTACGGCCTGGCCTGGCAGGAGTACTGCCGGCGTGTGCCTTACCGCATCAT
GCCCTACATCTACTGA

Word count= 1257

YFP=720

Total= 1977bp

TRANSLATION **418Aminoacids**

MAPTQGPRAPLEFGGPLGAAALLLLLPATMFHLLLAARSGPARLLGPPASLPGLEVLWSPRALLLWLA
WLGLQAALYLLPARKVAEGQELKDKSRLRYPINGFQALVLTALLVGLGMSAGLPLGALPEMLLPLAFVA
TLTAFIFSLFLYMKAQVAPVSALAPGGNSGNPIYDFFLGRELNPRICFFDFKYFCELRPGLIGWVLINLAL
LMKEAELRGSPSLAMWLVNGFQLLYVGDALWHEEAVLTTMDITHDGFGFMLAFGDMAWVPFTYSL
QAQFLLHHPQPLGLPMASVICLINATGYYIFRGANSQKNTFRKNPSDPRVAGLETISTATGRKLLVSGW
WGMVRHPNYLGDLIMALAWSLPCGVSHLLPYFYLLYFTALLVHREARDERQCLQKYGLAWQEYCRR
VPYRIMPYIY

7.2.2 Sequence for YFP-DHCR7 Wildtype

ATGGCTGCAAAATCGCAACCCAACATTCCCAAAGCCAAGAGTCTAGATGGCGTCACCAATGACAG
AACCGCATCTCAAGGGCAGTGGGGCCGTGCCTGGGAGGTGGACTGGTTTTCACTGGCGAGCGTC
ATCTTCCTACTGCTGTTCGCCCCCTTCATCGTCTACTACTTCATCATGGCTTGTGACCAGTACAGCT
GCGCCCTGACTGGCCCTGTGGTGGACATCGTCACCGGACATGCTCGGCTCTCGGACATCTGGGCC
AAGACTCCACCTATAACGAGGAAAGCCGCCCAGCTCTATACCTTGTGGGTCACCTTCCAGGTGCTT
CTGTACACGTCTCTCCCTGACTTCTGCCATAAGTTTCTACCCGGCTACGTAGGAGGCATCCAGGAG
GGGGCCGTGACTCCTGCAGGGGTTGTGAACAAGTATCAGATCAATGGCCTGCAAGCCTGGCTCCT
CACGCACCTGCTCTGGTTTGCAAACGCTCATCTCCTGTCCTGGTTCTCGCCCACCATCATCTTCGAC
AACTGGATCCCACTGCTGTGGTGCGCCAACATCCTTGGCTATGCCGTCTCCACCTTCGCCATGGTC
AAGGGCTACTTCTTCCCCACCAGCGCCAGAGACTGCAAATTCACAGGCAATTTCTTTTACAACTAC
ATGATGGGCATCGAGTTTAACCCTCGGATCGGGAAGTGGTTTGACTTCAAGCTGTTCTTCAATGG
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ACAGCCATGTGACCAATGCCATGGTCCTGGTCAACGTCCTGCAGGCCATCTACGTGATTGACTTCT
TCTGGAACGAAACCTGGTACCTGAAGACCATTGACATCTGCCATGACCACTTCGGGTGGTACCTG
GGCTGGGGCGACTGTGTCTGGCTGCCTTATCTTTACACGCTGCAGGGTCTGTACTTGGTGTACCAC
CCCGTGCAGCTGTCCACCCCGCACGCCGTGGGCGTCCTGCTGCTGGGCCTGGTGGGCTACTACAT
CTTCCGGGTGGCCAACCACCAGAAGGACCTGTTCCGCCGCACGGATGGGCGCTGCCTCATCTGGG
GCAGGAAGCCCAAGGTCATCGAGTGCTCCTACACATCCGCCGATGGGCAGAGGCACCACAGCAA
GCTGCTGGTGTCGGGCTTCTGGGGCGTGGCCCGCCACTTCAACTACGTCGGCGACCTGATGGGCA
GCCTGGCCTACTGCCTGGCCTGTGGCGGCGGCCACCTGCTGCCCTACTTCTACATCATCTACATGG

CCATCCTGCTGACCCACCGCTGCCTCCGGGACGAGCACCGCTGCGCCAGCAAGTACGGCCGGGAC
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Word count= 1427

YFP=720

Total= 2147bp

TRANSLATION=475Aminoacids

"MAAKSQPNIPKAKSLDGVTNDRTASQGQWGRAWEVDWFSLASVIFLLLFAPFIVYYFIMACDQYSC
ALTGPVVDIVTGHARLSDIWAKTPPITRKAAQLYTLWVTFQVLLYTSLPDFCHKFLPGYVGGIQEGAVT
PAGVVNKYQINGLQAWLLTHLLWFANAHLLSWFSPTIIFDNWIPLLWCANILGYAVSTFAMVKGYFF
PTSARDCKFTGNFFYNYMMGIEFNPRIGKWFDFKLFFNGRPGIVAWTLINLSFAAKQRELHSHVTNA
MVLVNVLQAIYVIDFFWNETWYLKTIDICHDHFGWYLGWGDCVWLPYLYTLQGLYLVYHPVQLSTPH
AVGVLLLGLVGYYIFRVANHQKDLFRRTDGRCLIWGRKPKVIECSYTSADGQRHHSKLLVSGFWGVAR
HFNYVGDLMGSLAYCLACGGGHLLPYFYIIYMAILLTHRCLRDEHRCASKYGRDWERYTAAVPYRLLPG
IF"

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