DEVELOPMENT OF A NOVEL SYSTEM FOR THE EXPRESSION OF INTRACELLULAR PROTEINS

By

ANKITA BASANT

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ABSTRACT OF THE THESIS

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Thesis Director:

Dr. Joseph Marcotrigiano

Mammalian cell expression has become an important recombinant protein production system for a wide variety of applications because of the cells capacity for proper post-translational modification and molecular structure assembly. In the past, mammalian expression has been optimized for secreted, glycosylated proteins. However, intracellular protein expression leaves yet another layer of complexity to be solved when expressing proteins in mammalian cells. There is a set of challenges that associates with intracellular protein expression due to the post-translational modifications involved with the bioactivity of the protein. The benefits to cytoplasmic protein production in mammalian cells, particularly for eukaryotic proteins, are very significant. Improvements in this area have been slow, however, due to limited development of the cell culture processes needed for low-cost and higher-throughput expression in mammalian cells. Here, we describe the development of a novel robust protein expression system using

recombination cloning, suspension cells and stable lentiviral transfection to decrease the time and lower cost that can be combined to make mammalian cells much more amenable for routine recombinant protein expression.

Acknowledgements

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Chapter 1

Introduction

Expression of recombinant protein is extremely important for the study of underlying gene function, analyzing biological activity, determining structure function relationships, engineering proteins, producing antibodies and designing target specific drugs for therapy. Unfortunately, it is also a task where the outcome is extremely unpredictable. A protein that has evolved over millions of generations for expression at a level appropriate to a specific cell type or in a particular developmental stage may be toxic to a new host cell, or become insoluble (among many possible obstacles) when overexpressed. Bacterial expression has been considered to be the easiest and cheapest method to express protein targets. The variety of expression tools in *Escherichia coli* (*E. coli*) is far greater than any other expression system, but it does not work for proteins of more complex nature, such as those requiring post-translational modifications for proper folding and function. Eukaryotic systems, such as mammalian and insect cells, play an important role in expressing the complex proteins. Although baculovirus system is cheaper than mammalian expression systems, mammalian cells are more ideal environments for expressing human protein targets due to capability to express proteins that require post-translational modifications for their bioactivity. Since the costs associated with mammalian cell manipulation are relatively high, there are circumstances where quality is an overriding concern. For almost all purposes, the protein is desired to be as native as possible, i.e., resemble the protein made in the organism and folding along with activity state found in nature.

The main objective of this thesis was to develop a mammalian expression system, which is more time and cost efficient for intracellular proteins production with complex post-translational modifications.

1.1 DEVELOPMENT OF THE SYSTEM

Many human proteins are complex molecules that contain post-translational modifications that are necessary for bioactivity. Mammalian cells, although more expensive and time consuming as compared to the baculovirus expression system, have been considered to be the best host when it comes to the expression of human proteins for structural or therapeutic purposes for decades demanding deeper exploration (1). We developed a novel system for the expression of complex intracellular proteins in mammalian cells. This system combines high-density mammalian suspension cells (293H) with highly efficient gene transfection of lentiviruses.

Rapid growth in protein structural and functional studies for various purposes has led to a surge in bioreactor designs that are used to produce these protein targets on a much larger scale and development of cell culture processes (2). Key barriers to large scale mammalian culture were considered to include oxygen supply limitations, waste product accumulation, the need for more sophisticated process control, shear sensitivity of animal cells, and the challenges of growing adherent and suspension cell lines (2).

Protein expression in mammalian cells is divided into transient and stable expression. Both modes start with the introduction of plasmid DNA into a cell line, called transfection. Using transient transfection protocols, thousands of plasmid DNA molecules are transfected into a cell by either lipid based chemicals or $CaPO_4$; the transfected plasmids do not replicate but instead are linearized and ligated to each other; the plasmids

express the protein of interest but are lost by dilution as host cells replicate; and the plasmid molecules may be degraded or modified (silenced) over time so that protein expression is lost. The mammalian expression systems utilize the transient transfection with cationic liposomes in commonly used cell lines, such as human embryonic kidney (HEK) 293 and Chinese Hamster Ovary (CHO) (3, 4). These systems have a limited period to express protein since the transfected DNA is not stably integrated and lost during several rounds of cell division, thereby requiring a fresh transfection every time more material is needed. The time required to achieve a high-quality mammalian clone expressing a protein of interest is typically measured in months. This results in a loss of time and resources over a long period of time. Thus, improving yield in transient expression is governed by improving the percentage of cells that are transfected; decreasing the cost of the transfection reagent; making enough DNA; increasing the expression of the protein of interest through vector and gene optimization; and increasing the volume of cells transfected. With stable or permanent transfection, the transfected DNA is either integrated into the chromosomal DNA or maintained as an episome. Stable integration of plasmid DNA into the genome is a rare event. Stably transfected cells can be selected by co-transfection of a second plasmid carrying an antibiotic-resistance gene or by providing a resistance gene on the same vector as the gene of interest. When these antibiotics are added to the growth medium, they kill off any cells that have not incorporated the vector and those cells that survive can be expanded to create stable cell lines, which have incorporated the vector and express the insert.

Our system relies on the high-density, suspension cultures of 293H cells and the efficient lentiviruses stable transfection. The 293H are suspension cell lines derived from

the adherent HEK293T, which are widely used for lentiviral packaging and recombinant protein production (3, 4). In addition, 293H cells have been adapted to grow without serum in suspension, limiting protein contaminants from the media. These cells grow to very high densities in shaker/spinner flasks and bioreactors, are easy to maintain, can go through 30 passages with little or no loss in protein expression and double every 24 hours on average. 293H suspension cells have high efficiency and tolerance for infection using lentivirus. In addition, suspension cells can be easily harvested and lysed for protein extraction.

The second component of the suspension cell system is the high transfection efficiency of lentivirus vectors. Lentiviral vectors can be used to make stable cell lines. Many lentiviral vectors have selectable markers, such as the puromycin resistance gene, conferring resistance to antibiotics (Fig. 2). Many lentiviral transfer vectors do not have selectable markers conferring resistance to an antibiotic, but do express some other maker such as GFP. GFP can be used in conjunction with Fluorescence Activated Cell Sorting (FACS) to separate cells expressing fluorescent and other types of markers and later expand these cells into a cell line.

Lentiviruses are a genus of the *Retroviridea* family that can efficiently and conveniently infect a wide variety of mammalian cells, including non-dividing cells. Upon infection, the viral RNA genome is reverse-transcribed into DNA, which is transported to the nucleus and integrated into the genome by a viral-encoded integrase enzyme. They provide a cost and labor-effective method of generating stably expressing stable cell lines within a short period of time and have emerged over the last decade as powerful, reliable, and safe tools for stable gene transfer in a wide variety of mammalian

cells (5). Lentiviral expression vectors are effective vehicles for stably expressing different effector molecules (siRNA, cDNA, DNA fragments, antisense, ribozymes etc.) or reporter constructs in almost any mammalian cell, including non-dividing cells and whole model organisms. A common feature of lentiviral vector systems is that they are replication-deficient and require helper virus functions for propagation known as pseudoparticles. These pseudoparticles fuse with foreign envelope particles. Pseudoparticles do not carry the genetic material to produce additional viral envelope proteins, so the phenotypic changes cannot be passed on to progeny viral particles. Thus, the mechanism of viral entry to deliver foreign genes is exploited, whereas the harmful effects of a productive viral infection in recipient cells are avoided. By packaging the lentiviral expression construct into pseudoviral particles, one can obtain highly efficient transduction (up to 100 %), even with cell lines that are difficult to transfect using standard methods, such as primary, stem, and differentiated cells.

In contrast to the plasmid based transient transfections, lentiviruses are a quick and efficient means to make a cell line that stably expresses the protein of interest. The use of multiple plasmids in lentiviral delivery system prevents recombination of genetic material to produce an intact viral genome, resulting in a pseudotype lentivirus capable of only a single round of infection into a variety of mammalian cell lines. The retroviral envelope glycoproteins have been replaced with the G protein of vesicular stomatitis virus (VSV-G) to allow for greater host cell infection and added stability. The entire process of stable cell line production using lentiviral vectors can be performed in a week, bypassing the time consuming procedure of drug selection. The lentiviral system is more rapid and efficient than previous approaches because it circumvents the needs to: 1) use

special media or drugs for selection in cell culture, 2) isolate clonal cell lines, and 3) screen large numbers of cell lines to find a high producer. As a result the generation time to produce a stable cell line significantly shortens from 2 - 3 months to about one week. In addition to efficiency, typically lentivirus expression also results in a substantial increase of protein yield owing to the high copy number of expression cassettes. The number of copies of the expression cassette introduced into a cell is correlated to the number of virus particles entering the cell, or multiplicity of infection (MOI). Thus, protocols can be designed such that all cells are infected at extremely high MOIs (100-1,000), eliminating the need to culture cells in selective media to identify clones that contain the expression cassette.

The three components of lentiviral system used for the suspension cell expression system are; pMD2.G encodes VSV glycoproteins for incorporation into the viral envelope and versatility of cell tropism, and psPAX2 contains the standard HIV Gag/Pol cassette (5). The third plasmid, pJG, is the transfer vector in which the protein of interest is cloned into and was engineered to provide improved protein expression. pJG uses a CMV promoter to drive expression, with additional enhancement provided by the Rev Response Element and a Woodchuck Hepatitis promoter enhancer element (5). This vector contains a GFP gene immediately following the cloned gene separated by IRES. This provides an early visible marker for infection efficiency, which correlates to the expression of the protein of interest (6). Another version of pJG in which the GFP gene is replaced with a puromycin resistance gene was created, that allows for the selection of puromycin or neomycin resistant cells, further amplifying protein production (Fig. 1, 2 and 3).

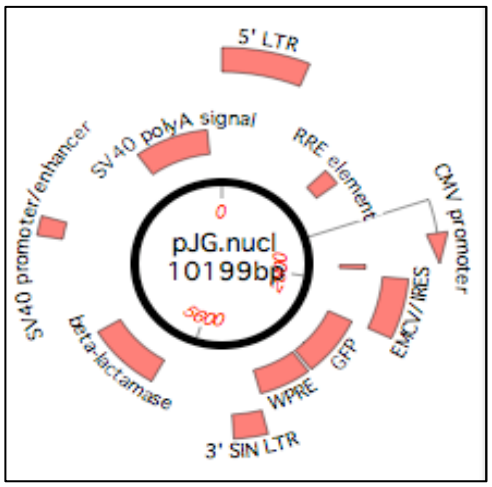

Figure 1: Schematic of pJG vector elements

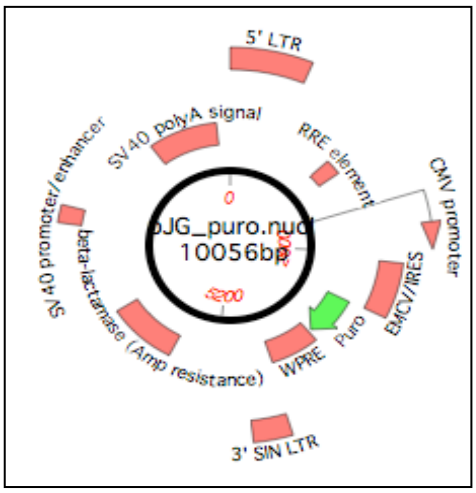

Figure 2: Schematic of pJG puromycin vector elements

The pseudoparticle carrying the gene of interest is produced by concurrent transfection of the three plasmids into HEK293T cells where they are packaged into a HIV shell that is replication incompetent (Fig. 3). Three days later the supernatant containing the pseudoparticles are harvested, concentrated by ultracentrifugation and infect the desired cell line at a certain MOI. Suspension cells can be efficiently infected, expanded from a 6 well plate into a spinner flask in a week's time. The GFP helps in early visualization of the infected cells and initial western blots can confirm the identity of the protein. These cells can be expanded to a 2 L scale in a fed batch bioreactor system in ~2 weeks from the infection to a density of 2.5-3x10^6 cells/mL. This time span is much shorter than the traditional methods used for protein production in suspension systems. This system can yield protein with no significant losses for up to a month, after which there is a need for a fresh infection and a new batch of cells. This system is not only, time and cost saving, but also environmentally friendly as it minimizes the use of disposable plastics since the glass spinner/shaker flasks can be effectively clean, sterilized and re-used.

This kind of fed batch bioreactor would offer an effective and sustainable solution for recombinant protein expression. The bioreactor would be able to maintain a steady supply of good quality protein for a few months with insignificant loss in yield. There has not been a lot of success in the arena of cytoplasmic protein expression as opposed to the secreted proteins owing to a number of reasons; mainly due to the lack of protein secretion into the media for ease of harvest over long periods of time and the interference of cell processes due to over expression of intracellular proteins in the cytoplasm. The literature on large-scale, intracellular expression of proteins in mammalian cells is scarce

and the techniques not much evolved. This system is a step forward in the direction of producing complex intracellular proteins in their native conformation.

1.2 mCHERRY

mFruits are second-generation monomeric red fluorescent proteins (mRFPs) that have improved brightness and photostability compared to the first-generation mRFP1. It is a red monomeric fluorescent protein, which matures extremely rapidly, making it possible to see results post infection due to activating transcription, in the form of red fluorescence. It is highly photostable and resistant to photobleaching (7, 8). The emission and excitation maxima are distributed over the remarkably large ranges of about 550-650 and 540-590 nm, respectively; however, the variations in the spectra can be traced to a few key amino acids.

To test and optimize the potential of this system, we expressed mCherry, a small simple red fluorescent protein using the suspension cells. mCherry expression served two main purposes; it was easy to understand the limits of the suspension cells and change parameters accordingly owing to its small size and less complex nature, and secondly the fluorescent nature of the protein made it very easy to visualize it from the post infection stage.

1.3 MAMMALIAN TARGET OF RAPAMYCIN COMPLEX (mTORC)

The mTOR protein is a 289-kDa serine-threonine kinase that belongs to the phospho-inositide 3-kinase (PI3K)-related kinase family and is conserved throughout evolution. mTOR regulates cell growth by controlling mRNA translation, ribosome biogenesis, autophagy, and metabolism.

mTOR was originally known as FKBP-12-rapamycin associated protein (FRAP 1) or Rapamycin and FKBP12 Targets (RAFT-1) in yeast. In 1965, Rapamycin was isolated from a microorganism in soil Streptomyces hygroscopicus and its antibiotic properties were confirmed (9). In the early 1990s, genetic screens in budding yeast identified *TOR1* and *TOR2* as mediators of the toxic effects of rapamycin on yeast (10, 11). Rapamycin is a potent immunosuppressive drug used in solid organ transplantation for the prevention of acute rejection.

mTOR nucleates at least two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (12). These complexes have different level of sensitivities to Rapamycin as well as upstream inputs and downstream outputs (Fig. 3 and 4).

mTORC1 is the main focus of this study. It has five components: mTOR, which is the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL); proline-rich AKT (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) (13). The exact function of most of the mTOR-interacting proteins in mTORC1 remains elusive. It has been proposed that Raptor might affect mTORC1 activity by regulating assembly of the complex and by recruiting substrates for mTOR (14, 15). The role of mLST8 in

mTORC1 function is also unclear, as deletion of this protein does not affect mTORC1 activity *in vivo* (16). PRAS40 and Deptor have been characterized as distinct negative regulators of mTORC1 (13, 17, 18).

mTORC2 comprises six different proteins, several of which are common to mTORC1 and mTORC2: rapamycin-insensitive companion of mTOR (Rictor); mammalian stress-activated protein kinase interacting protein (mSIN1); protein observed with Rictor-1 (Protor-1); mLST8; and Deptor.

The overall domain structure of TOR is well conserved (Fig, 5), some of the functions have been identified and some are still under scientific scrutiny. Just like any other Phosphatidylinositol 3-kinase-related kinase (PIKK) family proteins, mTOR's N-terminal is defined by tandem HEAT repeats (Huntington Elongation factor3, A subunit of protein phosphatase 2A and TOR1). The number and position of these repeats remains undetermined. Individual repeat units vary in length from 30 to 40 amino acid residues and number from as few as two repeats to more than dozens within a protein (19, 20). In other repeat-containing proteins these structures mediate protein-protein interactions (21). In general, internal repetition affords a protein enhanced evolutionary prospect due to an enlargement of its available binding surface area (19).

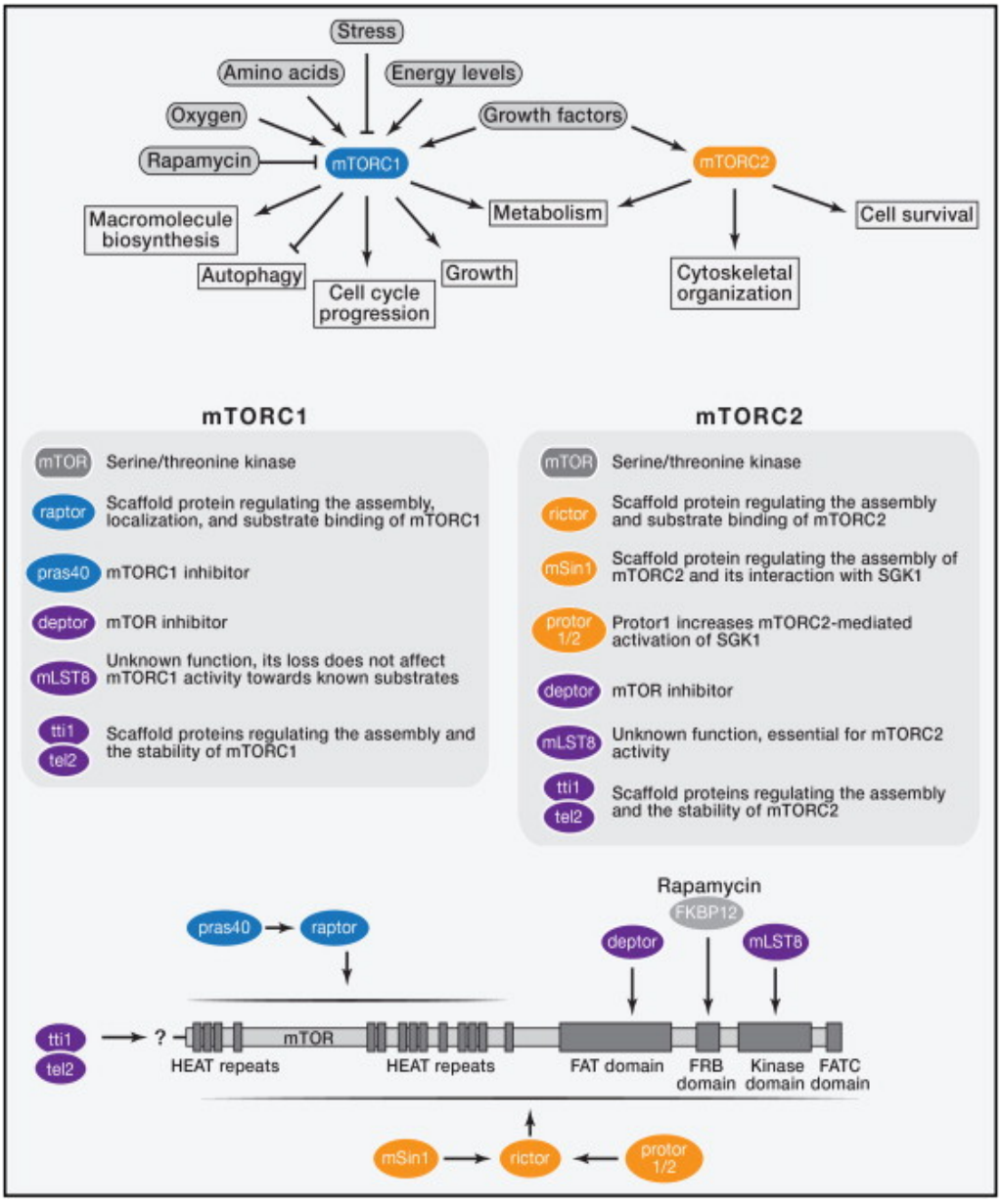

Figure 3: mTORC1 and mTORC2 Complexes. The mTOR kinase nucleates two distinct protein complexes. mTORC1 responds to amino acids, stress, oxygen, energy, and growth factors and is acutely sensitive to Rapamycin. (22)

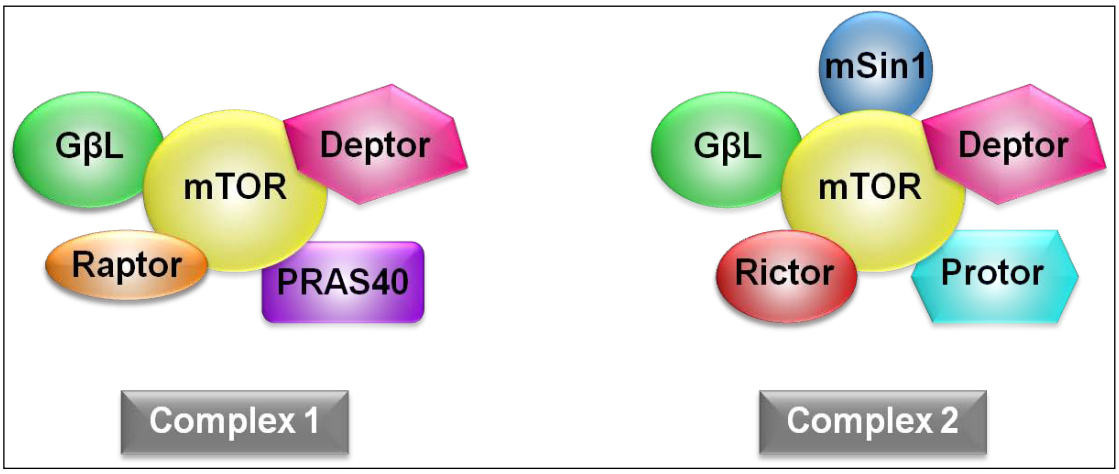

Figure 4: Two mTOR complexes. (23)

The C-terminal PI3K (phosphatidylinositol-3-kinase) catalytic domain of mTOR is immediately flanked by four different alpha helical domains (24) starting with the FRB (FKBP12 rapamycin binding) domain, the FAT (FRAP, ATM, and TRRAP) domain (25–27) and the FATC (FRAP, ATM and TRRAP C-terminal) domain (28) (Fig. 5). Understanding the repeat architectures of TOR is key to understanding how this protein ultimately functions in the cell and how TOR interacts with its targets. A major obstacle to detailed structural and functional analysis of TOR and other members of the PIKK protein family is their large size (~280-470 KDa), which renders experimental manipulation difficult. To date, only three structures of TOR have been solved: two high resolution structures of the FRB and FATC domains and a low resolution EM structure of the full-length protein (25, 27–29). Structural and functional studies of PIKKs have also been hindered by poor understanding of their repeat architectures, as traditional web-based repeat prediction methods detect few to none of the repeats in their HEAT and FAT domains due to their high variation.

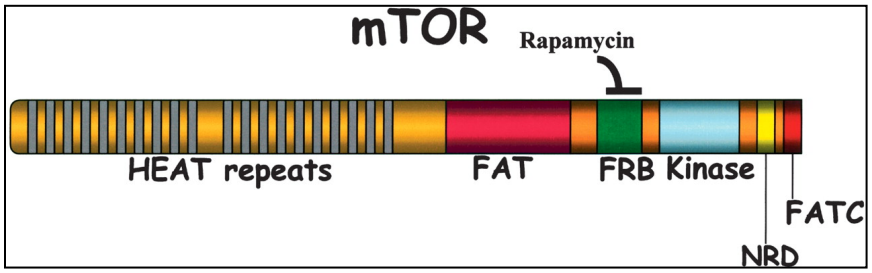

Figure 5: Domain organization of mTOR proteins

Raptor (Regulatory Associated Protein of TOR); (14, 30), is a conserved 150-kDa protein that also binds the downstream effectors of mTOR, S6K1, and 4E-BP1 (14, 31). All Raptor homologs contain a unique conserved region in the N-terminal half, followed by three HEAT repeats and seven WD-40 repeats in the C-terminal half. The N-terminal HEAT repeats of mTOR are required for the efficient interaction with Raptor, to which it binds avidly; however, the C-terminal half of mTOR can also bind weakly to Raptor (30). Although Raptor is normally a positive regulator of mTOR, one report indicates that, upon nutrient deprivation, Raptor-mTOR association is stabilized in a manner that inhibits mTOR kinase activity (14, 30, 32). All Raptor orthologs contain a unique conserved region in their N-terminal half (Raptor N-terminal Conserved, also called the RNC domain) followed by three HEAT (huntingtin, elongation factor 3, A subunit of protein phosphatase 2A and TOR1) repeats and seven WD-40 repeats near the C terminus (33). RNC (Raptor N terminal Conserved domain) binds the N terminal region of TOR and interacts with TORC1 substrates (Fig.6).

Another characterized mTOR-interacting protein from *S. cerevisiae,* LST8 its mammalian ortholog is mLST8/Gβ L (G protein β -subunit-like protein, pronounced "gable")—a highly conserved 36-kDa protein. It interacts specifically with the kinase domain of mTOR (independently of Raptor) and plays a positive role in mTOR activation by nutrients (34). mLST8/GβL stabilizes mTOR-Raptor association.

However, Sabatini and colleagues (30, 34) have suggested that the nature of the mTOR-Raptor complex changes upon amino acid deprivation; explaining the effect of amino acids on mTOR activity (Fig. 7). In the absence of amino acids, the mTOR-mLST8-Raptor complex precludes mTOR from binding avidly to its substrates and/or

prevents the access of mTOR (or mTOR-associated kinases) to the substrates. This model does not explain how amino acids elicit these putative conformational changes in mTOR-Raptor complex, and further studies are required to address this question and verify this model.

In order to gain clarity into the structure and binding sites of the different mTOR components and specify their role, there is a need to be able to express these wild type proteins in their native conformation. The suspension cell expression system with the efficient lentiviral transfection offers a potential solution to express these proteins in their wild type form for further functional and structural studies.

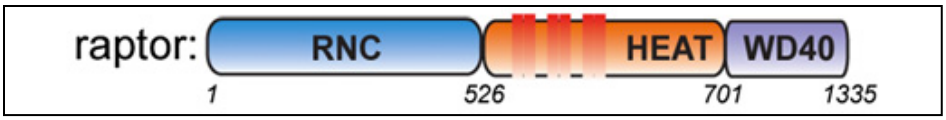

Figure 6: Domain organization of Raptor.

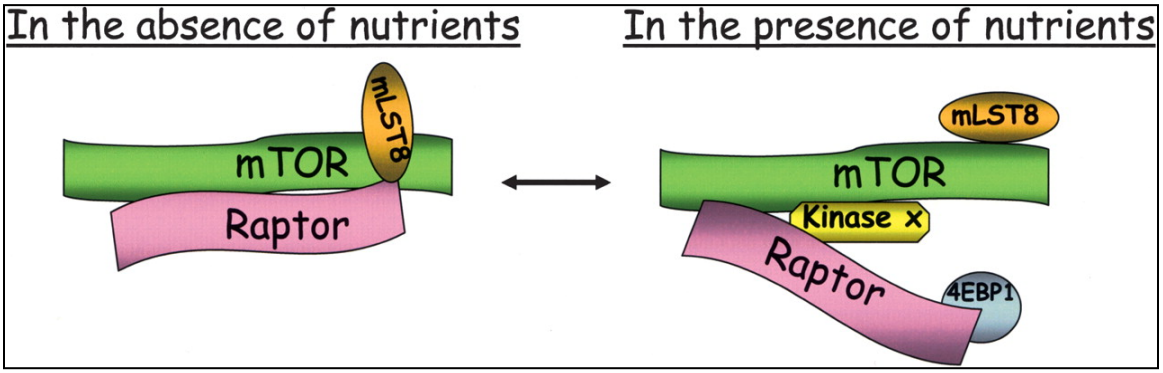

Figure 7: A model of mTOR-Raptor interaction in response to nutrients. In the absence of nutrients, a tight interaction between mTOR, Raptor, and mLST8 prevents the access of mTOR to its targets. In the presence of nutrients, a conformational change may disrupt Raptor/mLST8 interaction and enables the accessibility of mTOR (or an associated kinase) to its targets, 4E-BP1 or S6K1, which are bound to raptor. (32)

Chapter 2

Materials and Methods

2.1 MAMMALIAN EXPRESSION SYSTEM

The 293H and 293F cells were purchased from Invitrogen (Gibco). These are a variant of HEK293T cells that have been adapted to grow in suspension using a chemically defined serum free media CD293 (Invitrogen). The media supplements, L-glutamine, antibiotic (penicillin- streptomycin) and HEPES (1M stock) were purchased from Invitrogen for tissue culture use.

Unlike the adherent HEK293T cells these grow in suspension to a very high density. The technique and handling to grow these cells is different from the parent 293T. These cells are fragile requiring proper care in terms of feeding times, optimization of speed of the spinner/shaker flasks and close monitoring for days. The recommended seeding density for the 293H is >500,000 cells/mL. These can be grown to a density of $2.5\text{-}3x10^6$ cells/mL. The cells require 5-8 % of Carbon dioxide, 37 ^oC temperature and humidified atmosphere. The 9-position stirrer platform, the spinner flasks (100mL – 3 L) and shaker flasks (2.8 L) were purchased from Chemglass. The suspension 293H/F cell line was sub-cultured every 48 hours by centrifugation and re-suspended in fresh medium to maintain cell density of $<1x10^6$ cells/mL. All cultures were maintained at 37 ˚C incubator with 5 % carbon dioxide and agitated at 100 rpm. Cell densities were determined using the standard hemocytometer trypan blue exclusion method and reported as cells/mL.

The media used for adapting adherent HeLa cells to suspension Joklil MEM and Horse serum for adapting adherent HeLa cells to suspension, non-essential amino acids as a supplement to increase cell growth and viability and antibiotic-antimycotic used to prevent bacterial and fungal contaminations were all purchased from Invitrogen. The infections were done in adherent HeLa cells using the same protocol for adherent 293T cells and then they were adapted to suspension by changing the media to a different formulation.

2.1.1 Lentiviral Transfection and Infection in suspension 293H cells.

The aim was to develop a method using the suspension cells that has the ability to grow the suspension cells to a high density for production of intracellular proteins. This required modifying the existing lentiviral transfection and infection protocol that was intended for the HEK293T adherent cells, since it was too aggressive for the suspension cells. The existing protocol for adherent cells from Dr. John Shires (Emory University, School of Medicine) and Dr. Jillian Whidby, (Rutgers University) was optimized for suspension 293H cells. The best infection protocol would allow the cells to be efficiently infected using lentivirus without killing them.

In order to develop the various aspects of the system, different aspects of cell growth and expansion conditions were optimized. It involved comparing the effects of varying carbon dioxide, addition of antibiotic (penicillin-streptomycin), addition of surfactant like Pluronic F-68 and Hepes as a buffering agent for the media. Uninfected 293H/F cells were grown in 5-8 % Carbon dioxide, while being maintained at a density of $1x10^6$ cells/mL or less in a 40-100 mL volume. Antibiotic (penicillin-streptomycin) was added at a concentration of 1 % (v/v) compared to the control flask with no

antibiotic. PF68 was added to the cultures at a concentration of 2.5-5 mL/L (10 % Pluronic F 68) compared to the control flask without PF-68. Hepes (1M stock) was added at concentrations of 10 mM and 25 mM. The speed of the spinner had to be adjusted according the size of the flask used in order to reduce sheer on the cells. 100-120 rpm was used for the optimization and optimal growth. The cells were monitored and checked for changes in density and viability for upto 72 hours.

293H/F cells were infected at different cell densities. Different experiments were set up to optimize the number of cells to be used, amount of virus used for infection, drawing comparison between the 293H and 293F cells and varying the amount of polybrene used for infection. Polybrene (hexadimethrine bromide) is a cationic polymer used to increase the efficiency of infection of certain cells with a retrovirus in cell culture. Polybrene acts by neutralizing the charge repulsion between virions and sialic acid on the cell surface.

Lentiviruses obtained from 1xT225 flask were diluted 2-, 5- and 10-fold and added to 6 well plate, containing $2x10^6$ 293H cells in the presence of 8 µg/mL polybrene. The cells died on the fourth day post infection, while the uninfected cells continued to survive. Comparative experiments were also done between 293H and F cell lines. No significant difference was noticed in their growth and general behavioral characteristics so the rest of the work was done using 293H cells.

After a careful comparison of growth parameters, the cells survival rate was still limited to couple days. The investigation pointed towards the fact that the reagents used for the infections could be causing cell death. The chemical compound polybrene, used for increasing the lentiviral infection efficiency was the main suspect. Repeat infections

were done in the 6 well seeded with $2x10^6$ 293H and F cells in duplicate wells using the same amount of polybrene with infected and mock cells. The concentrations of polybrene used were 2, 4, 5, 8 μg/mL and no polybrene as control for the infections in 293H and 293F cells for 12-16 hours. The best infections of over 90 % efficiency were obtained with 4 μg/mL polybrene with post infection cell survival. It was important to change the media and the flask/6 well 24 hours post infection. Residual polybrene can cause havoc on these cells, stressing the cells and eventually killing them.

2.2 METHODS TO PRODUCE mCHERRY IN MAMMALIAN CELLS

The mCherry plasmid was a kind gift from Dr. Jim Millonig's lab, Center of Biotechnology and Medicine, Rutgers University. mCherry gene was cloned into the pJG lentiviral vector. pJG, PMD and PAX lentiviral vectors were a gift from Dr. John Shires from Emory University, School of Medicine, Atlanta. The adherent HEK293T cells for transfection were obtained from Invitrogen. The media CD293, DMEM with additives of 10 % serum and antibiotic-antimycotic were purchased from Invitrogen as well.

2.2.1 Cloning

Expression tags are very important considerations for the downstream processing of any protein. mCherry was cloned with a 6xHis tag that binds to Ni-NTA resin. For western blots, anti-his antibodies (Pierce, Thermo scientific) at a dilution of 1:1500 were used for detecting the protein expression. The pJG vector was digested using the PmeI restriction enzyme, followed by treatment with CIP to prevent re-ligation. The gene of interest (including the 6xHis tag) was amplified using PmeI complimentary forward and reverse primers, with InFusion-HD (Clontech) overhangs. InFusion-HD cloning was executed according to the manufacturer's instructions and the reaction was transformed into HB101 cells. The DNA was purified using the Hi-speed Plasmid Purification Kit from Qiagen for optimum yield and minimal ethanol contamination**.**

2.2.2 Lentiviral Production and infection in Suspension 293H cells

a) Seeding. One day prior to planned transfection, a single T-225 monolayer flask was seeded with $6.0x10^6$ HEK293T cells.

b) Transfection. The co-transfection protocol for these vectors was done via the $CaCl_2$ method with HEPES buffered saline. Combined in a 50 mL Falcon tube: 90 µg pJG-gene, 60 µg psPAX2, 30 µg pMD2.G, 450 µL $CaCl_2$, q.s. to 4.5 mL of ddH_20. 4.5 mL of 2X HEPES buffer was added at room temperature, and bubbled with a serological pipet for 10 seconds. The mixture was allowed to incubate at room temperature for 2 minutes and is then added directly to the culture media in the flask prepared as per 2.2.2a. The media was changed 6-8 hours later.

c) Preparing for infection. Three days after the transfection (on the morning of virus harvest), $2x10^6$ 293H cells each are seeded into two wells of a 6-well plate in a final volume of 1 mL per well.

d) Harvesting the virus. The supernatant from the transfection, containing the recombinant lentiviruses, is harvested and centrifuged for 30 mins at 4000xG at 4˚C to pellet major cellular debris. 37 mL of clarified supernatant is transferred to a Beckman Ultracentrifuge tube fitted for an SW28 rotor. Virus is then pelleted for 1.5 h at 25,000 rpm (80,000xG) at 4˚C using maximum acceleration and deceleration. Supernatant is decanted into a waste container filled with 1% vesphene. The pellet is dried, inverted, for 5 minutes, then re-suspended in 2 mL of CD293 media + 4 mM L-glutamine + 0.5 % antibiotic (penicillin-streptomycin) + 4 µg/mL polybrene.

e) Infection in suspension cells. 1 mL of the re-suspended virus is added to each of the seeded wells in the 6-well plate. The next day, the infected cells are pelleted at 100xG for 7 minutes in a 15 mL Falcon tubes. The media is aspirated and each cell pellet is re-

suspended in 2 mL of CD293 media + 4 mM L-glutamine+ 20 mM HEPES+ 0.5% pen/strep. All the infections are performed in duplicates.

f) Culture Maintenance. Once the infected cells in the 6-well plate become dense, the cells from two duplicate wells are pooled and transferred into a T-75 flask with 10 mL of media. After 3-4 days, the cells are split into 4x T-75 flasks with 10 mL media each. After another 3-4 days, the cells from all four flasks are counted, pooled, and transferred to a 100 mL spinner flask at a seeding density of 500,000 cells/mL. The spinner platform is fitted inside a standard cell culture incubator and set to 100 -120 rpm. The cells are grown to a density of 850,000 cells/mL in a final volume of 2.2 L, centrifuged to collect the cell pellet and lysed using the Emulsi Flex C3 Homogenizer.

2.2.3 Protein Purification of mCherry

The 293H expressing mCherry were harvested in Buffer A: 50 mM Sodium phosphate, 500 mM Sodium Chloride, pH 7.5, 5% glycerol. The cells were lysed using a Emulsi Flex C3 Homogenizor and trypan blue stain was used to make sure that the lysis went to completion. The lysate was centrifuged at 20,000 rpm for 40 minutes. The supernatant was collected and filtered using a 0.22 µm syringe filter. The supernatant was loaded on a His trap column (GE), washed using buffer A+ 40 mM Imidazole and eluted using buffer A+ 100 mM Imidazole. The fractions were collected and analyzed on a SDS PAGE gel. The 6xHis tag was cleaved overnight by dialyzing it into the buffer: 50 mM Hepes, 100 mM sodium chloride, pH 7.5 and 5% glycerol, adding precession protease in the dialysis bag (1:50 i.e. 1 mg of enzyme for every 50 mgs of protein). The protein concentration was determined using Bradford colorimetric assay from Bio rad analyzed

using Nanodrop spectrophorometer. The eluted samples and the cleaved His tag samples were send for Mass spectral analysis to confirm the molecular weight.

2.3 METHODS TO PRODUCE RAPTOR IN BACTERIAL AND MAMMALIAN CELLS

2.3.1 Cloning Raptor into Bacterial expression plasmids

The pRK5-Myc tagged Raptor-WT full-length vector was provided by Dr. Nahum Sonenberg, McGill University, Montreal, CA. In order to test the expression of Raptor in bacterial system, three different truncations of i.e. amino acid 1-697, 1-720 and 1-740 of Raptor were cloned into pGex 6P1, pet skb2 and pet-SUMO vectors. Multiple other constructs were cloned and expressed in bacterial system. (Table 1)

Cloning of Raptor fragments into pGEX-6P-1 (GST tag), pET-Skb2 (6xHis tag-C terminal) and pET-SUMO (N terminal- 6xHis-Sumo tag) vectors was done using restriction cloning using the sites BamHI and XhoI from New England Biolabs (NEB) with a precession protease site (LEVLFQGP) in pGEX-6P-1 and ULP1 protease in the pET-SUMO vector at the N terminal of the protein. The forward and reverse primers were designed with the BamHI and XhoI sites designed in them, and after PCR, the insert and the vectors were digested using the same enzymes. T4 ligation was done for 30 minutes at room temperature and transformed into DH5alpha cells. The ligation was done according to the protocol provided with T4 ligase from NEB. The positive clones were selected with the help of restriction digest done using enzymes NcoI and EcorV from NEB. The positive clones were sent for further confirmation with the help of DNA sequencing. The DNA for the confirmed positive clones was re transformed, prepped fresh and stored at -20^{o} C freezer.

2.3.2 Bacterial Transformation of Raptor constructs

The cells were thawed on ice after taking them out of -80^{o} C freezer. 4 µl of ligation product/DNA was added to 50 µl of cells aliquoted in a fresh eppendorf and gently mixed by tapping the eppendorf with index finger. The mixture is allowed to stay on ice for 10 minutes. Heat shock at 42 ^{o}C water bath for 45 seconds and then replaced back on ice for ten minutes. It was allowed to stay on the benchtop at room temperature for 5 minutes. 250 µl of fresh Luria Broth (LB) was added to the eppendorf and allowed to revive at 37^{o} C on a shaker for an hour. The cells were spun down and re-suspended back into 40 µl of the same media and plated on a compatible antibiotic agar plate required by the different vectors, ampicillin and kanamycin for pGEX-6P1 and pET vectors, respectively.

2.3.3 Expression of Raptor in bacterial cells

The pGEX-6P-1 clones of Raptor were transformed into UT5600 cells for test expression. For pET SUMO and pET-SKB2 clones, BL21-DE3 cells were used. The expression conditions were 37 ^{o}C and induced using 1 mM IPTG. The samples were collected from all the cultures, pre and post induction.

The 3 pGEX-6P-1 clones were expressed and a small-scale purification using 1 L of culture volume was done. The cells were induced and grown to an OD of 0.6 at 37 ^{o}C and then induced using 1 mM IPTG over night at 18 ^{o}C by passing it over a GST column (GE) and eluted using Tris buffer with fresh glutathione. The samples were analyzed on a SDS PAGE with coomassie staining for visualization. Overnight dialysis was done into

Hepes buffer, pH 7.5 with precession protease enzyme added to the dialysis bag at 1:100 to confirm the identity of protein.

2.3.4 Expression of Raptor in Mammalian cells (Transient and Lentiviral)

Transient transfections were done for myc-Raptor in HEK 293T, HeLa and ChoKI cells to check initial protein expression. The DNA was purified using the Hi-speed Plasmid Purification Kit from Qiagen for optimum yield and minimal ethanol contamination**.** DNA is diluted to the required concentration using sterile water. 400,000 cells per well were plated in 6 well plate in a total of 2 mL media volume one day before the transfection to obtain the desired density >80 % confluency at the time of transfection. FuGene HD transfection reagent from Roche was prepared in the 3:2 ratio (FuGene transfection reagent (µl): DNA (µg) in a final volume of 100 µl. This mixture is allowed to adjust to room temperature (~25˚C) and vigorously mix it. This transfection complex is added to the cells in a dropwise manner. The cells can be analyzed for expression after 48-72 hours. The protein expression was detected using western blot probed using anti-myc antibody.

To check the stable lentiviral expression of wild type (WT) Raptor full-length (FL) in mammalian HEK293T adherent cells, it was cloned into pJG vector backbone using the same cloning procedure of maxi prep as mentioned in 2.2.1. Lentiviral transfection was done in HEK293T cells. The protein tags used for the lentiviral transfection were N-terminal 6xHis, StrepII, GST tag in pJG and Tandem his.

2.3.5 Detection of Raptor expression by Western Blot

Western blots were probed using the anti-His antibody, by collecting samples from the 6 well plate, spun down and re-suspended in coomassie dye, sheared using a 23.5 gauge needle and then heated at 95 ˚C for 10 minutes before loading on the 8 % SDS PAGE gel.

SDS-PAGE gel was run with the samples and appropriate positive and negative controls. 10 µl of sample were loaded into the SDS PAGE wells. Transfer to a nitrocellulose membrane was done using trans blot semi dry transfer cell from Bio Rad using the transfer buffer (5.8 g tris base, 2.93 g glycine, 0.375 g SDS, 200 mL methanol volume made up to 1 liter with Millipore water). Membrane was blocked using Phosphate Buffered Saline tween 20 + 5 % milk for 1 hour at room temperature. Protein was probed using primary antibody anti myc (1:1000); anti Raptor (Millipore- 1:2000), anti His (Pierce- 1:1000) overnight at 4 ^{o}C. 3 washes were done 15 minutes with Phosphate Buffered Saline-tween. Secondary antibody conjugated with horseradish peroxidase (Pierce) diluted 1:5000 for visually detecting the protein at room temperature for 1 hour. 3x15 min washes were done with Phosphate Buffered Saline –Tween 20 to wash off the excessive secondary antibody. The western blot was developed exposing the film in the dark room in a cassette after exposing it to the film for 30 seconds - 2 minutes.

2.3.6 Lentiviral infection of Raptor in adherent HEK293T cells

a) Seeding. One day prior to planned transfection, a single T-225 monolayer flask is seeded with 6.0x10^{6} HEK293T cells.

b) **Transfection.** The co-transfection protocol for these vectors is done via the $CaCl_2$ method with HEPES buffered saline. Combined in a 50 mL Falcon tube: 90 µg pJG-gene, 60 µg psPAX2, 30 µg pMD2.G, 450 µL $CaCl_2$, q.s. to 4.5 mL of ddH_20. 4.5 mL of 2X HEPES buffer was added at room temperature, and bubbled with a serological pipet for 10 seconds. The mixture was allowed to incubate at room temperature for 2 minutes and was then added directly to the culture media in the flask prepared as per 2.3.5a. The media was changed 6-8 hours later.

c) **Preparing for infection.** Two days after the transfection, 10,000 cells are seeded into a single well of a 96-well plate in a final volume of 50 µL ($2x10^5$ cells/mL).

d) **Harvesting virus.** The supernatant from the transfection, containing the recombinant lentiviruses, is harvested and centrifuged for 30 minutes at 4000xG at 4 ˚C to pellet major cellular debris. 37 mL of clarified supernatant is transferred to a Beckman Ultracentrifuge tube fitted for an SW28 rotor. Virus is then pelleted for 1.5 hour at 25,000 rpm (80,000xG) at 4˚C using maximum acceleration and deceleration. Supernatant was decanted into a waste container filled with 1% vesphene. The pellet was dried, inverted, for 5 minutes, then re-suspended in 120 µL of: DMEM + 20 % FBS + 1 % Antibiotic-Antimycotic + 8 µg/mL polybrene.

e) **Infection.** The media is aspirated from the prepared well in the 96-well plate (2.d) and 50uL of virus suspension is added and left to incubate overnight. (The remaining 70uL can be frozen and stored at -20 ˚C). The following morning, 50 µL of fresh DMEM + 10 % FBS + 1 % Antibiotic-Antimycotic is added to the infected well. On the third day, the media is removed and replaced with 100 uL

of fresh DMEM + 10 % FBS + 1 % A/A. At this time, a view of GFP expression will indicate the approximate efficiency of infection. Cell expansion can begin as soon as confluence is reached.

f) **Bioreactor Maintenance**. A single BelloCell-500 bioreactor is typically seeded with ~5.0-8.0x10^7 HEK293T cells, the equivalent of 3-4 confluent T-175 monolayer flasks. The seeding protocol was followed in accordance with the manufacturer's instructions, with an oscillation rate of 2.0 mm/s up and down, a top hold time of 20 sec, and a down hold time of 0 sec. After 2-4 hours using the seeding protocol, the oscillation speed was reduced to 1.0 mm/s, with a top hold time of 10 sec and a bottom hold time of 1 min.

2.3.7 Checking expression of Raptor from adherent HEK293T cells

a) Using P150 plates. In order to achieve higher cell density in adherent cells, amounting to higher protein yield, lentiviral transfection and infection were done in HEK293T cells, further propagating the 293T adherent cells in 20xP150 plates. The cells were scraped using a cell scraper and collected in a 50 mL falcon tube in Phosphate Buffered Saline (Invitrogen). After two washes with Phosphate Buffered Saline, these cells were centrifuged at 500xG for ten minutes in a swinging bucket benchtop centrifuge (Beckman Coulter). The supernatant was discarded and the cell pellet was re suspended in 20 mM Sodium Phosphate pH 8, 500 mM Sodium Chloride, 5% glycerol. These cells were lysed using a sonicator and then bound to loose nickel resin, by incubating the cells with 200 µl nickel resin for 1 hour on a rocker at 4˚ C. The resin was collected by centrifugation. Three washing steps were done using 10 mM Imidazole. Samples were collected at each step after centrifugation at 4 ˚C for 10 minutes at 10,000 rpm to be

analyzed on SDS PAGE. After 3 washes, the resin was collected and heated at 95˚ C for 10 minutes, 10 µl of resin was loaded on an acrylamide gel. Western blot was probed using anti-Raptor primary and anti-rabbit HRP conjugated secondary antibody to confirm the identity of the protein.

b) Using BelloCell bottle with Dissolvable matrix. The BelloCell-500D bioreactor with dissolvable matrix (Cesco Bioengineering, Taiwan) was chosen for its ideal investment/output ratio and for its capacity to generate an increased number of cells. Its innovative design addresses several adherent cell culture challenges. Relative to the systems described above, the BelloCell a) consumes significantly less disposable products and waste, resulting in a substantial cost saving of almost 50 % b) utilizes a three dimensional attachment matrix, allowing for better use of space and a higher degree of cell density and c) provides a more ideal growth environment in terms of temperature distribution and gas exchange. The BelloCell operates on a programmable stage called the BelloStage 3000, which fits inside a standard cell culture incubator. The bottle is divided into two chambers: the top chamber houses a polyester matrix onto which adherent cells attach and the bottom chamber is a flexible bellows, which holds 500mL of media. During the upward oscillation, media flows over the matrix to feed the cells. During the downward oscillation, media is contained within the bellows, allowing gas exchange. The top hold time and bottom hold time can be adjusted for seeding, cell type, and growth rate control. The infected HEK293T cells expressing Raptor were expanded to 2xT175 flasks grown until the flasks were confluent. The BelloCell bottle was equilibrated with 450 mL of DMEM with 1 % antibiotic-antimycotic (Gibco, Invitrogen) and 10% Serum (Invitrogen) + 5 mL Reagent A (Cesco Bioengineering) for 1 hour in the

incubator on a BelloCell platform. The three T175 flasks were trypsinized and the cells were suspended in 50 mL of media. These cells were added to the BelloCell through the perforated film, drop by drop allowing them to settle on the matrix. The flask was placed back in the incubator on the BelloCell platform and allowed to multiply. The BelloCell was allowed to grow to confluency for 2 weeks. For harvesting, the media was removed and was replaced with 500 mL of Reagent B (Cesco Bioengineering).

The reagent B helps dissolve the matrix. The entire re-suspended solution along with cells, is centrifuged at 2000 rpm for 10 minutes at 4 ^{o}C. Three washes are done with Phosphate Buffered Saline (Invitrogen). 5 mL worth cells were obtained from one BelloCell bottle. The cells are lysed using a sonicator and lysis checked using trypan blue stain. The lysate is then centrifuged at 20,000 rpm for 40 mins to collect the cell pellet containing the protein of interest purified as described in section 2.2.3 using the Nickel-NTA column (GE) using the buffer 50 mM Hepes, 15 mM Imidazole, pH 7.5, 1 M Potassium Chloride, 5% glycerol eluted using 200 mM Imidazole. The elution fractions were collected and analyzed by SDS-PAGE and western blot. Samples from the eluted protein were analyzed using mass spectral analysis for molecular weight and phosphorylation sites.

2.3.8 Puromycin selection optimization of Raptor infected cells

In order to enrich the population of Raptor expressing cells, puromycin selection gene was cloned into pJG replacing the GFP gene. Puromycin kill curve was done to optimize the concentration of antibiotic to be used for selection of the best healthy and expressing cell population. Post infection the 293T cells were expanded into 6 well plate

from the 96 well plate, allowed to stabilize for 2 days and recover before applying antibiotic selection. The concentration of puromycin used were, 10, 5, 2.5 and 1.25, 0.675, 0.3125, 0.156 μg/mL and 239T cells without any puromycin added were used a control.

2.3.9 Expression of Raptor in suspension cell system

The 293H suspension cells were infected with pJG Raptor and grown in spinner flasks as mentioned in section 2.2.2. Post infection these cells were expanded using spinner flasks under close monitoring for days. The media used was CD293 (Gibco, Invitrogen) with additives.

2.4 METHODS OF EXPRESSION OF MAMMALIAN TARGET OF RAPAMYCIN COMPLEX 1 (mTORC1) IN BACTERIAL AND MAMMALIAN CELLS

The genes expressing the proteins from mTOR complex 1 namely, pBJ5-mTOR with N terminal FLAG tag, mLST8, Pras40 and Drosophila Target of Rapamycin (dTOR), *C. elegans* TOR were provided by Dr. Nahum Sonenberg (McGill University, Canada).

Transient transfection was done for pBJ5-mTOR in HEK293T, HeLa and ChokI cells to check expression with western blot probed with anti Flag antibody. The full-length mTOR was cloned into pJG vector using in fusion cloning (Clontech) as described in section 2.2.1. Also, the kinase dead mutants of mTOR D2338A and D2357E were cloned into pJG vector for lentiviral transfection.

Five different truncated constructs of mTOR were cloned into pJG vector with an N terminal 6xHis tag i.e. Δkinase, 1-1819, 1819-2549, 1260-2549 and 1370-2549 amino acids. Lentiviral transfections to produce pseudo particles were done in 293T cells.

The small-scale expression was checked using 2.3.6 (a. and b.) methods for the mTOR construct mTOR 1819-2549. Western blots were probed using anti-mTOR antibody (Cell Signaling) at a dilution of 1:1000. To obtain a highly enriched population of mTOR full-length and mTOR 1819-2549 expressing cells, the genes of interest were cloned into pJG vector with puromycin selection marker incorporated as mentioned in section 2.3.8.

Bacterial expression was attempted with Sumo-pET to mTOR cloned N-terminally to mTOR 1819-2549 construct. For lentiviral transfection, mTOR gene FL and 1819-2549 were cloned with N terminal Tandem his tag, His-StrepII tandem tag, 6xHis, 8xHis, 10xHis, StrepII, GST in pJG vector as described in sections 2.3.2 and 2.3.3.

A series of buffers were tested to check the stability of protein 1819-2549 mTOR. The buffers tried were, A) 20 mM Sodium acetate, pH 5, 250 mM NaCl, 5 % glycerol, B) 50 mM Tris, pH 9, 250 mM NaCl, 5% glycerol and C) 20 mM MES, pH6, 250 mM Nacl, 5 % glycerol.

Purified mTOR protein eluted from Nickel column was dialyzed overnight in the three buffers and next morning the dialysis bags were checked for precipitate. SDS page and anti mTOR western blot were done to determine the buffer compatibility to 50 mM Tris, pH 9, 250 mM NaCl, 5% glycerol.

Lentiviral transfection was done in 293H and F cells (Invitrogen) and in adherent HeLa cells that were adapted to grow in suspension for scaling up. The cells were expanded and collected for purification. The cells were centrifuged at 500xG for 10 minutes using swinging bucket benchtop rotor (Beckman centrifuge). The supernatant was discarded and the cell pellet was re suspended in four times the cell volume by gentle pipetting. After two washes with Phosphate Buffered Saline, the cells were re-suspended in the loading buffer 20 mM Sodium Phosphate, 500 mM Sodium chloride, pH 8, 5 % glycerol. After the lysate was loaded on the column, on column washes were done using 25 and 40 mM Imidazole and eluted using 250 mM Imidazole. Samples were collected and analyzed using SDS page and western blot probed using anti-mTOR antibody (Cell Signaling) used at 1:1000 following the protocol as described in section 2.3.1.

Chapter 3

Results and Discussion

3.1 Mammalian Expression system using 293H suspension cells

Previously, recombinant protein expression in mammalian cells was performed by transient transfection of suspension cells using $CaPO_4$ (35). In an attempt to improve expression yields and feasibility, Aricescu et al published a description of HEK293 adherent cells transiently transfected using polyethylenimine (36). Although, they reported recovery of 1-40mg/L of media amongst 24 targets, this method required excessive disposable plasticware and is both labor- and cost- intensive. In 2009, Lee et al. described a similar approach, where HEK293T cells are transfected with milligram quantities of DNA and grown in Corning CellStacks (37). In an additional publication, large-scale transfection of various suspension cell lines was described, outlining optimal transfection reagents, expression vectors, and media (3,46). While these methods have remained the most common for large-scale protein production in mammalian cells, there are significant drawbacks. For example, protein expression levels slowly decrease since the DNA plasmids are not integrated into the host genome and are therefore lost during several rounds of cell division. If more protein is needed, the entire process must be repeated. Consequently, the expression is limited to a few weeks per round of transfection. Still, large amounts of DNA and costly transfection reagents and plastics make this approach cost prohibited for many applications.

Despite of all the advances in the recombinant protein expression, there are still very limited resources to express challenging intracellular proteins in mammalian cells.

The three main reasons for choosing suspension mammalian cells 293H were; capability to grow up-to high cell densities of $3x10^6$ cells/mL, tolerance to withstand lentiviral infections and ease of access to cell for protein extraction. Adherent HEK293T cells have been adapted to grow in suspension, negating the need for serum that contain attachment factors to make them adhere to the surfaces of flasks. These cells can grow up to a density of $3x10^6$ cells/mL and survive for weeks. They are more fragile as compared to the adherent HEK293T cells and the protocols for their growth, maintenance; lentiviral infection needs to be adapted.

Initial experiments showed that in order to grow 293H suspension cells, there was a need to optimize the incubator conditions as well as the spinner speed and design. The glass spinner flasks were chosen because they are reusable; easy to maintain; cost effective as well as environmentally friendly; and fit standard tissue culture incubators, making them an ideal choice for laboratory scale setting. Amongst the different designs of spinner flasks available, the flasks with the following properties worked out the best: baffles to provide better aeration especially in spinner flasks of 1L and above volume capacity, paddles instead of stir bars to provide better aeration and with a dimpled bottom to prevent the cells from accumulation in the center of the flask. WAVE bag bioreactors and shaker flasks were also attempted however, these systems were not as efficient as the spinner flasks for cell growth.

Due to the lack of existing literature on the care, handling and infection of these suspension cells, it was a challenge to define culture condition, which allowed for prolonged growth. The 293H cells grow in suspension and have a high tendency to clump

together, thus preventing the cells at the core of these clumps from obtaining nutrition from the media. This is one of the major issues for scale up after a fresh infection. To keep the infection efficient and effective, there is a need to infect a smaller, healthy population of cells in a 96 or 6 well plate and to expand them in a way that permits the infected cell population to divide without excessive cell death. The best cell density for efficient infection was determined to be $2x10^6$ cells in a 6 well plate. The infections were performed in duplicates in a final volume of 2 mL media that resulted in most efficient infections. These cells survived the infection and were alive for a week.

The influence of different growth conditions upon cell culture of serum free adapted 293 cells including the surfactant Pluronic F68, carbon dioxide, HEPES buffer and antibiotic (penicillin-streptomycin) were tested for optimal growth of suspension 293H cells.

3.1.1 Effect of Pluronic F 68 (PF68) on 293H cells

The non-ionic surfactant Pluronic F68 has been used in cell culture to avoid damage caused by agitation and sparging of mammalian and insect cells (38, 39). The surfactant, increases cell viability when scaled-up at bioreactor scale in agitated cultures. The media used for these cells CD293 already contains surfactant. Previous studies in mammalian cells have shown that the interaction between PF68 and the cell membrane depended on the PF68 concentration varying from 0.05 % to 0.2 % v/v (41–42). Additional PF68 was added to the cultures to identify any visible effects of increased surfactant amounts on cell growth and viability. The concentrations of PF68 evaluated in this study included 0.05, 0.1 and 0.2 % (v/v).The cells were grown in a 250 mL spinner

flasks agitated at a speed of 100 rpm with a working volume of 100 mL at 37 ˚C with 5 % carbon dioxide. Samples were analyzed daily for 6 consecutive days to check viability and density as compared to the control. No significant difference was found in the cell viability or density with addition of more PF68. (Fig. 8 and 8)

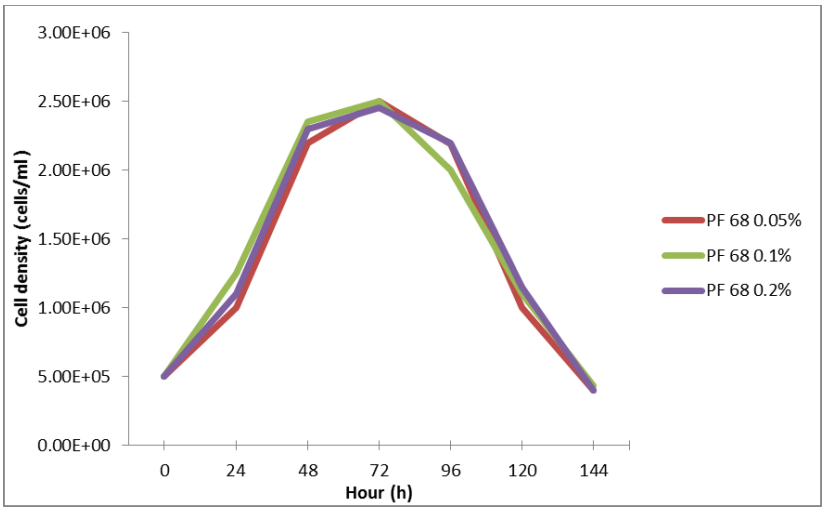

Figure 8: Effect of Pluronic F-68 upon cell density of suspension 293H cells growing in CD 293. Concentrations of Pluronic F-68 used included 0.05, 0.1 and 0.2 % (v/v) was compared with the flask control with no PF68.

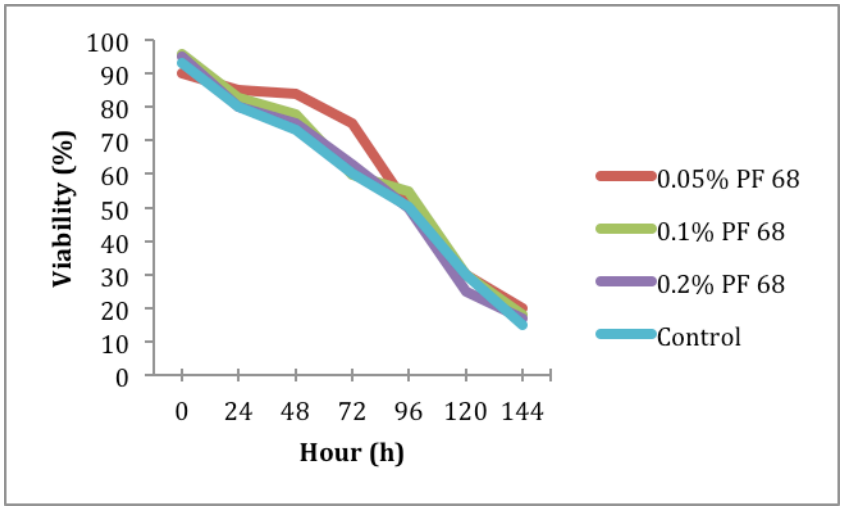

Figure 9: Effect of Pluronic F-68 upon cell viability of suspension 293H cells growing in CD293. Concentrations of Pluronic F-68 used included 0.05, 0.1 and 0.2 % (v/v) was compared with the control flask with no PF68.

3.1.2 Effect of Carbon Dioxide atmosphere on 293H cells

Oxygen supply is a major factor in the large- scale production of high-density mammalian cell cultures. From a technical point of view, the most convenient method of supplying sufficient oxygen to a large-scale suspension cell culture is by sparging air into the media. However, in many cases significant cell damage associated with cell-bubble interactions has been observed (43, 44). CO_2 is an anaerobic product and can accumulate to toxic levels, altering metabolism (45). This can be removed with proper aeration of the culture media. Usually cultures are incubated under 5 % CO_2 with appropriate Sodium Bicarbonate in the media to maintain the required pH. Carbon dioxide levels were altered to see if there were any changes in the buffering capacity of the media. This can be done by placing flasks in tissue culture incubators or sparging the large cultures with gas. The density and viability of the cells was checked with varying 5-8 % CO_2.

In a 250 mL spinner flasks were used with 100 mL volume and the CO_2 was controlled at 5 and 8 %. A control flask was placed in a 37 ˚C cell culture room without additional CO_2 and sealed to prevent the air exchange. The cells were seeded at 500,000 cells/mL. During the first 72 hours, there was no difference in the viability of the cells, and the cells divided as normal. Though the cell density was not as high for the cells grown in the presence of 5 and 8 % carbon dioxide (Fig. 10).

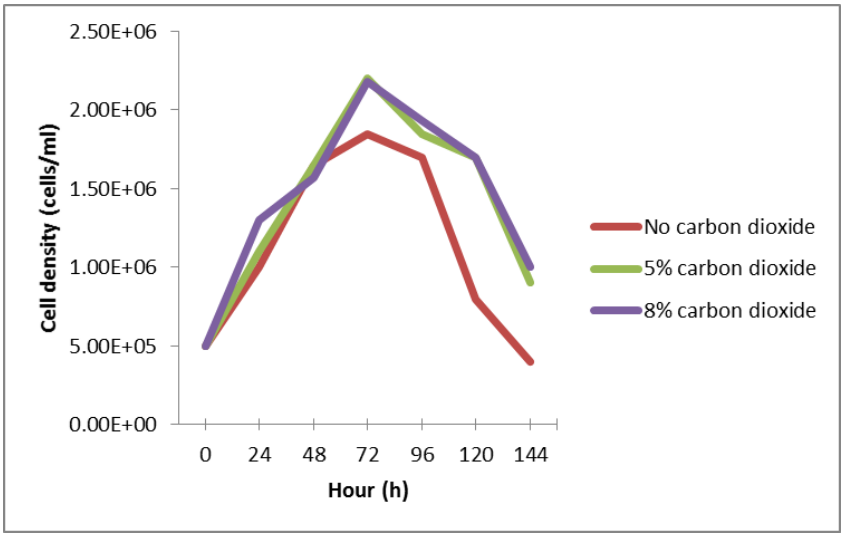

Figure 10: Effect of 5 and 8 % CO_2 atmosphere upon cell density of suspension 293H cells growing in CD293.

After 72 hours, the viability started to go down in all the flasks, due to lack of nutrition in the medium and possible accumulation of metabolites. This proved that these cells were capable of surviving and growing in the absence or presence of 5 % CO_2. This also proves that sparging of large-scale cultures with gas might potentially cause damage. Controlling the pH in the absence of carbon dioxide is a challenge; hence 8 % carbon dioxide is used while growing these cells.

3.1.3 Effect of HEPES on 293H cells

pH control is extremely important for these cells. In order to achieve effective pH control, addition of buffering agents like HEPES (N - 2 - hydroxyethyl - piperazine - N' - 2 - ethanesulfonic acid) was tested. According to the manufacturer, the media comes supplemented with 1 M NaOH or $NaHCO_3$ to maintain the pH of the culture. (46). In order to test if the buffering capacity of the media was an issue, addition of buffering agents like HEPES was tested.

HEPES has been described as one of the best buffers for biological research. HEPES has been used previously in cell culture usually in the range of 10 to 25 mM with no toxic effects upon cells (47). At most biological values of pH, the HEPES molecule is zwitterionic, and is effective as a buffer system at pH values from 6.8 to 8.2. In this study, two different concentrations of HEPES including 10 and 25 mM were used to determine the effect of the addition of such a compound upon cell density and viability of suspension adapted 293 cells grown in CD293 media. The concentration of HEPES used was 10 and 25 mM and a control flask with no HEPES and no CO_2. The density and viability was checked every 24 hours for 10 days. The culture volume of 100 mL was

used in 250 mL spinner flask, agitated at 100 rpm. The caps were loosened for air exchange. There was no significant difference between the flasks with 10 and 25 mM HEPES over the first 5 days. The cell density and viability decreased after the first 5 days, possibly because of lack of nutrition. Once fed, these cells survived for weeks. After the four days, cells in the control flask stopped dividing and most cells died, indicating that additional pH maintenance is needed for optimal cell growth. 20 mM HEPES was used for all subsequent infected and uninfected cultures, since additional HEPES helped to prolong the life span of the cells (Fig.11 and 12).

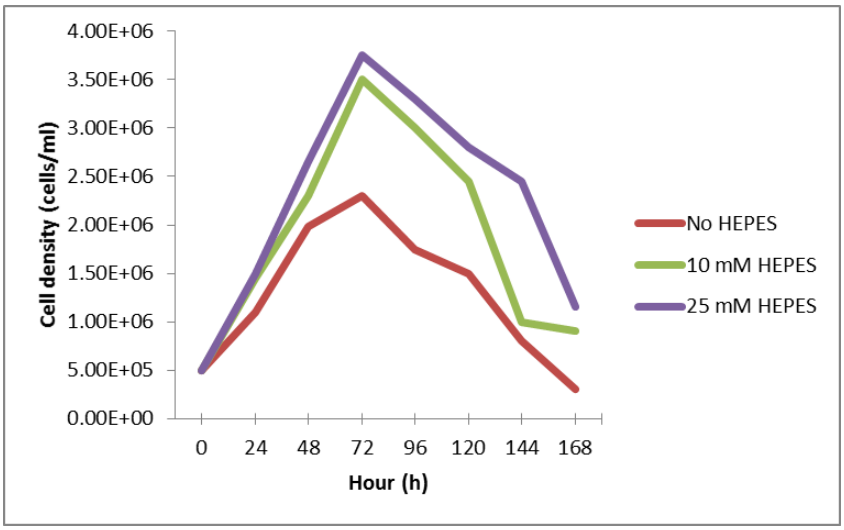

Figure 11: Effect of HEPES upon cell density of suspension 293H cells growing in CD293. HEPES concentrations used were 10 and 25 mM and then compared the flask control with no HEPES.

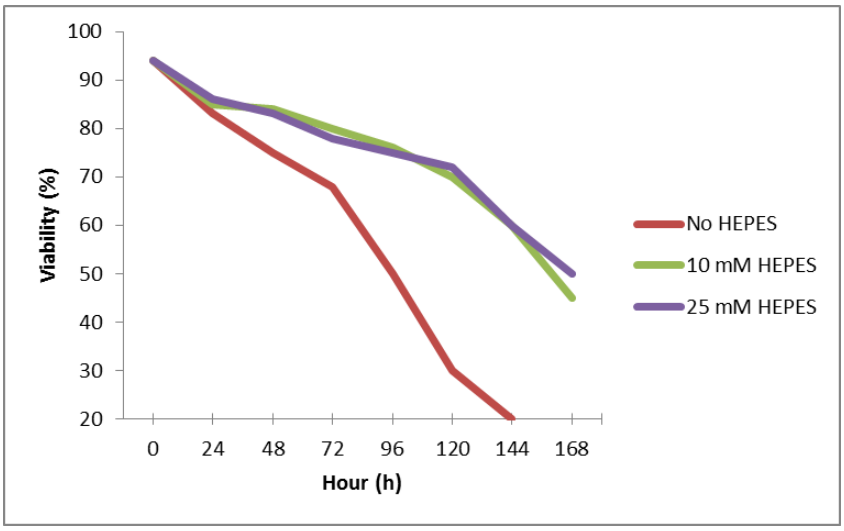

Figure 12: Effect of HEPES upon cell viability of suspension 293H cells growing in CD293. Control flask without HEPES.

3.2 Production of mCherry in mammalian cells.

With the cells growth conditions optimized, we next optimized lentivirus infection and protein expression. As a first attempt, mCherry was chosen for optimizing intracellular protein expression using suspension cells and lentivirus system, since it's a small monomeric fluorescent protein that can be visualize at different stages of infection, expression and purification. mCherry was cloned into the pJG backbone, lentivirus particles were made, and 293H cells were infection. Lentivirus infected cells had a pink color due to overexpression of mCherry when confluent even in the absence of UV stimulation, unlike the mock 293H cells that are yellow in color (Fig. 13 a and b). In addition mCherry expression could be easily visualized on a coomassie stained SDS PAGE using whole cell extracts. The expression of the N-terminal 6xHis mCherry was confirmed by an anti-His western blot (Fig.15 a and b).

We decided to test how long expression of mCherry could be maintained in these cells. The mCherry expressing cells were maintained at a density of approx. $1x10^6$ cells/mL in a 100 ml media volume. The cells were fed fresh CD293 media with 4 mM L-Glutamine, 20 mM HEPES and 5 mL/L antibiotic (Penicillin-streptomycin) and split regularly to prevent cell death due to overcrowding; the flasks were changed to a fresh one every 2 weeks and maintained in an incubator with 8 % carbon dioxide. The culture was centrifuged under sterile conditions to get rid of any dead cells. Dead cells secrete cytokines that can affect the growth of healthy cells, hence it is important to get rid of the dead cells from time to time. The culture of mCherry expressing cells can be grown for 2-3 months with insignificant loss in protein expression. For long term storage of the protein, the cell pellets were re-suspended in 50 mM Sodium Phosphate, pH 7.5, 500 mM

Sodium Chloride, 5 % glycerol and flash frozen in liquid nitrogen to be stored at -80 ˚C freezer. These cells can be thawed at a later time for purification.

For protein purification, mCherry infected cells were scaled up to the 2 L volume and harvested by centrifuging at 500xG for 10 minutes to collect the cell pellet while the supernatant was discarded. mCherry protein was purified by N- terminal 6xHis tag over Nickel column. 60 mgs of protein was obtained from 2 L culture (850,000 cells/mL) (Fig.14 and 16). The 6xHis tag was cleaved from the eluted samples using precission protease enzyme and the pre and post cleavage samples were sent for Mass spectrometry analysis (Fig. 17). Having successfully optimized mCherry expression in the suspension cells-lentiviral system, we attempted to express other challenging, intracellular proteins in this system.

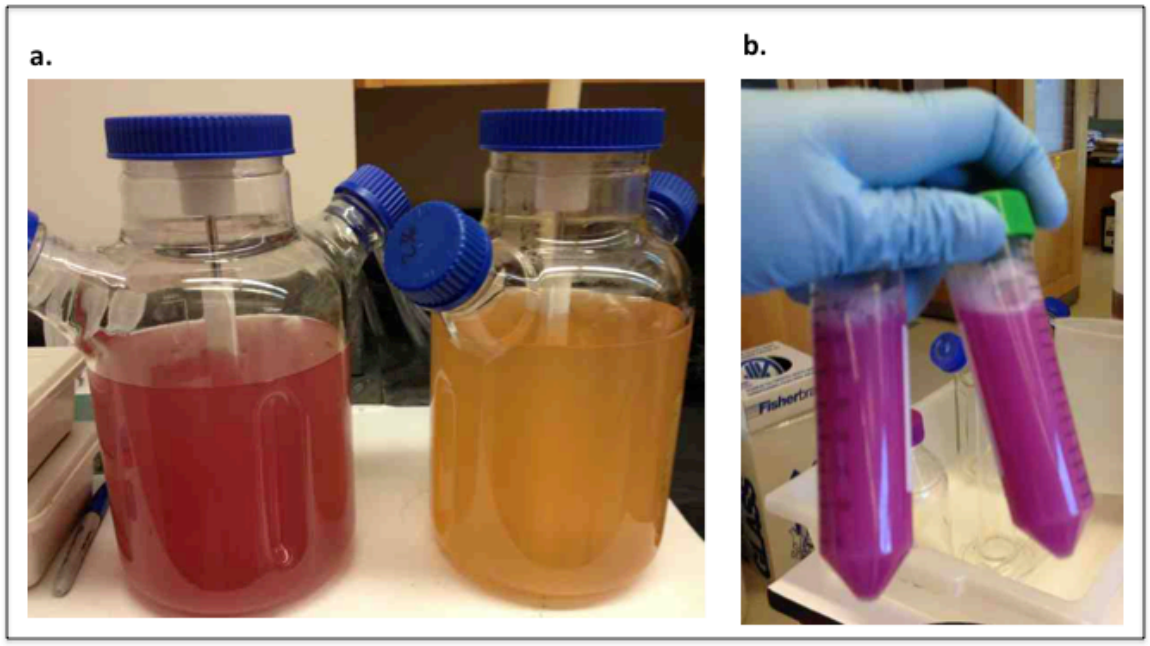

Figure 13: a) mCherry (left) culture vs. the uninfected 293H cells (right). b) mCherry expressing 239H cells harvested and re-suspended in Phosphate Buffer Saline.

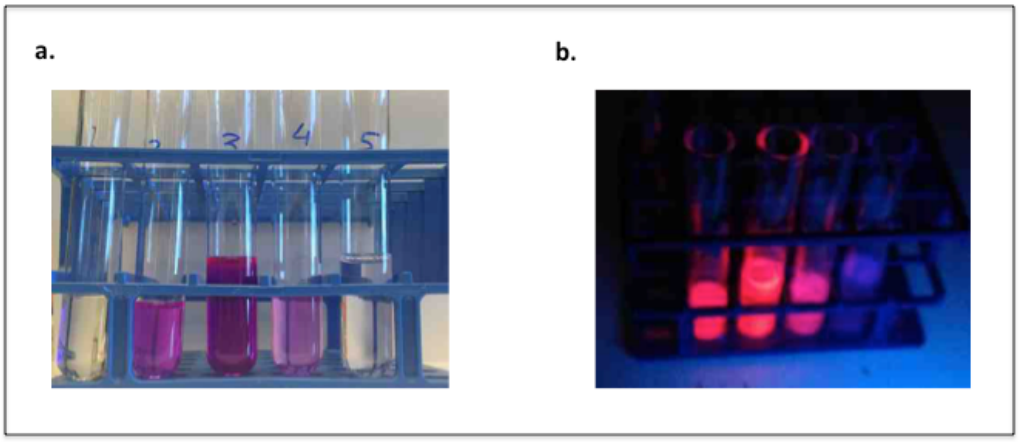

Figure 14: mCherry elution fractions from Nickel column under a) fluorescent light b) UV light

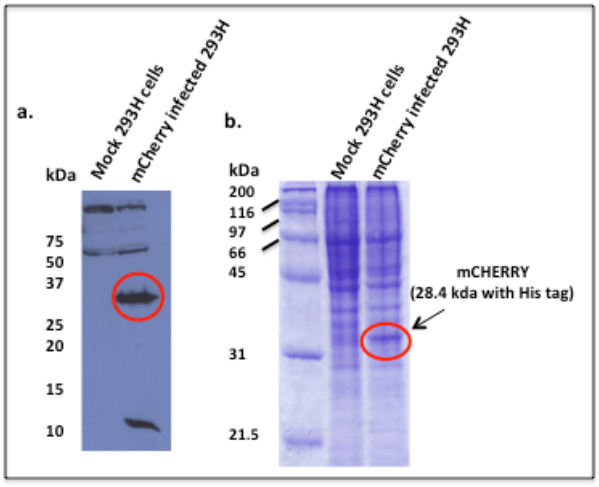

Figure 15: a) Anti-His western blot was done for detecting mCherry expression in 293H suspension cells. b) SDS PAGE with mCherry protein expression from 293H suspension cells.

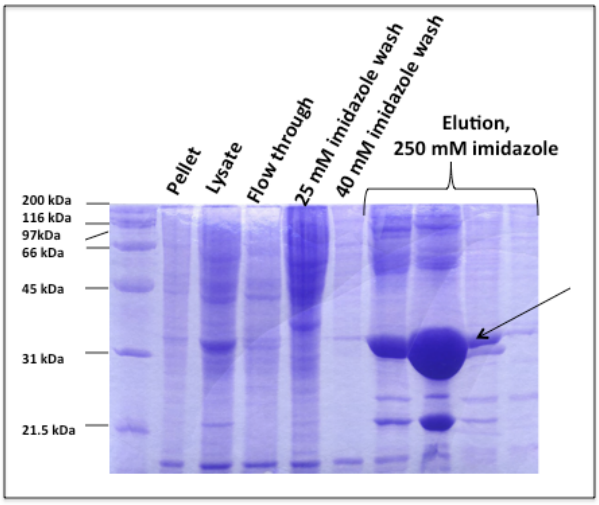

Figure 16: SDS PAGE with the samples from mCherry grown in 293H cells purified over Nickel column.

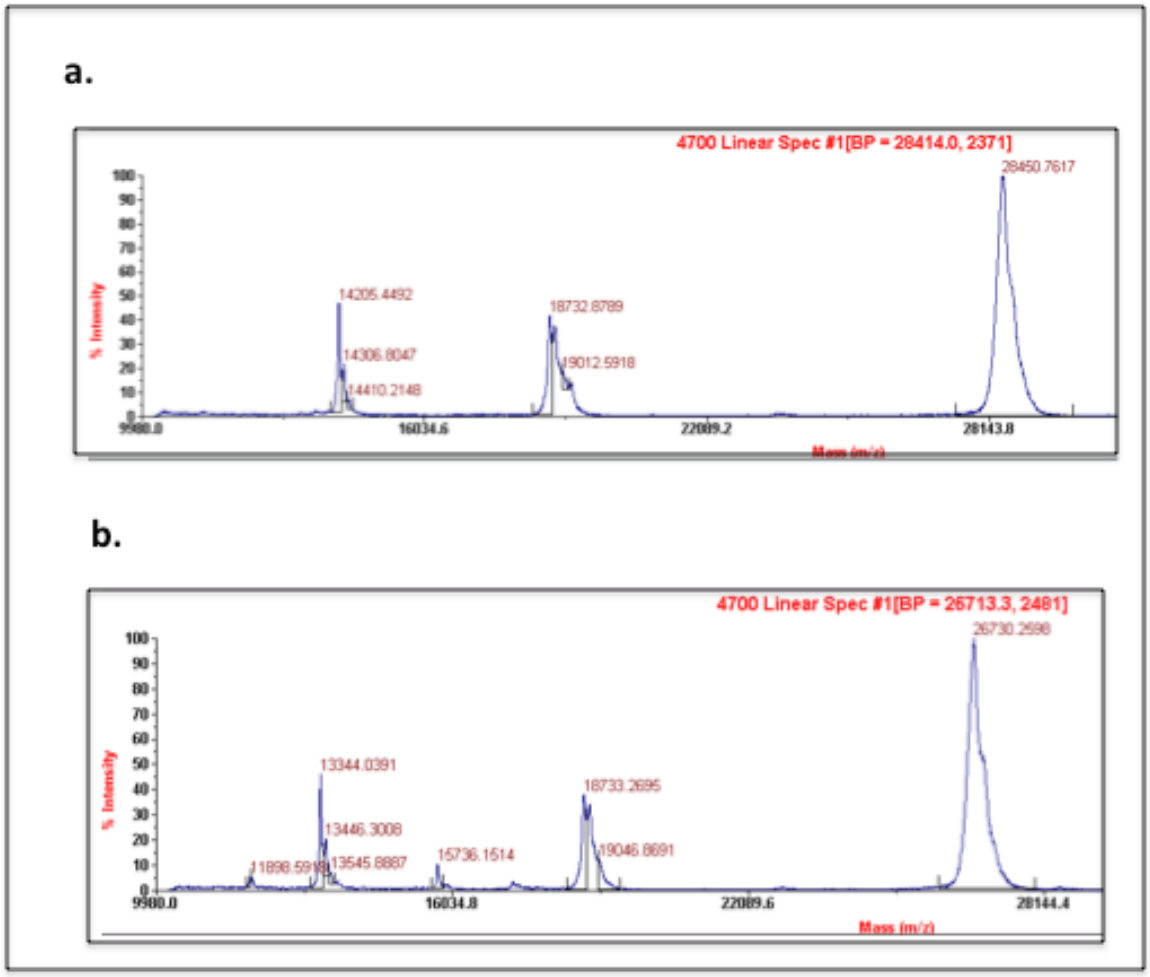

Figure 17: Mass spectra for mCherry samples a) un-cleaved His tag b) cleaved 6xHis tag sample. The mass corresponded the theoretical mass of mCherry 26720.02 Da.

3.3 Raptor

Raptor is a 150 kDa intracellular protein that acts as a regulator for mTOR and, is responsible for recruiting the substrates for phosphorylation by mTOR Kinase. Expression of Raptor has been unsuccessful in other expression systems owing to the phosphorylation modifications and large size of the protein. Shorter constructs were designed to determine any improvements in expression levels. The constructs were designed based on the online domain boundary and secondary structure prediction software (Dismeta). These truncated constructs were cloned into bacterial vectors with 6xHis, GST and 6xHis-Sumo tags (Table 1). The truncated constructs of Raptor with N-terminal GST tag expressed weakly but were grown to larger bacterial culture for purification. The lysate was passed over GST column and set up for overnight tag cleavage using precision protease enzyme. Shift in the protein bands were observed post cleavage and the protein identity was confirmed with the help of western blot (Fig. 18). The bacterial expression of Raptor 1-697, gave very poor yields of the material. Bacterial expression was not very successful in producing Raptor so we attempted the mammalian cell expression for the full-length protein.

Table 1: Raptor constructs expressed in bacterial and mammalian cells.

Raptor Construct (amino acids)	Vector Backbone	Tag	Expression	Solubility/ Yield
1-697	pGEX-6P1	N terminal GST	Yes	Low
1-720	pGEX-6P1	N terminal GST	Yes	Very Low
1-740	pGEX-6P1	N terminal GST	Yes	Very Low
1-697	pET-Sumo	N terminal Sumo	No	N/A
1-720	pET-Sumo	N terminal Sumo	No	N/A
1-740	pET-Sumo	N terminal Sumo	No	N/A
1-697	pET-Skb2	C terminal-his	No	N/A
1-720	pET-Skb2	C terminal-his	No	N/A
1-740	pET-Skb2	C terminal-his	No	N/A
Full-length	pJG	N terminal Tandem His	No	N/A
Full-length	pJG	N terminal His tag +Puromycin selection gene	Yes	Yes/Comparable to 6xHis tag
Full-length	pJG	N terminal GST tag	No	
Full-length	pJG	N terminal StrepII	Yes	Insignificant
WD40 domain	pGEX6P-1	N terminal GST	No	

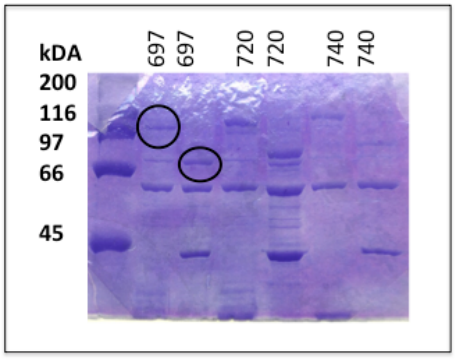

Figure 18: Different Raptor truncated constructs, passed over GST column and treated with precision protease overnight to cleave the tag off.

Mammalian cells should provide a better growth environment for the protein to fold correctly and be phosphorylated in the host cells containing the natural kinases. Full-length Raptor was cloned into pJG vector with a N-terminal 6xHis tag and lentiviruses were made as described in Sections 2.2.1 and 2.2.2, respectively. The initial transient transfections into adherent HEK293T demonstrated that these cells were capable of expressing the protein without any side effects. In order produce enough material for purification, the lentivirus infected cells were expanded to 24xP150 plates. The harvested cells were lysed and the cells were collected, re-suspended and purified over loose nickel resin followed by western blot analysis using anti-His antibodies. The purified full-length Raptor protein was visible as a coomassie stainable band on SDS PAGE (Fig. 19).

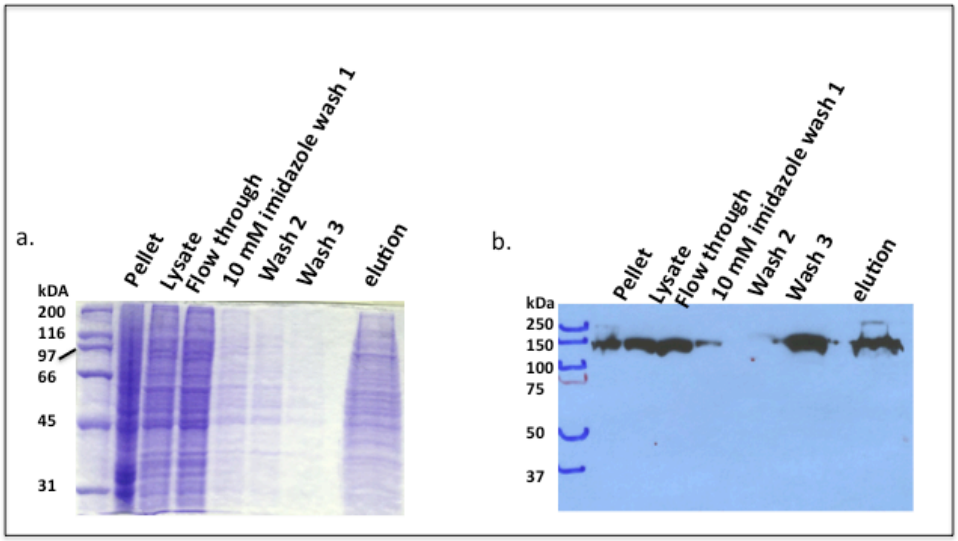

Figure 19: Raptor-FL purification over loose Nickel resin a) SDS PAGE b) Western blot probed using anti-his antibodies.

In order to optimize the protein purification, HEK293T cells expressing Raptor were placed in an adherent cell bioreactor with a dissolvable matrix (BelloCell) to obtain a larger quantity of cells. Infected adherent HEK293T cells were seeded using 2 confluent T175 flasks and grown for 2 weeks until confluent. The full-length Raptor was successfully purified over Nickel resin and analyzed using SDS PAGE and western blot. Mass Spectrometry was used to confirm the identity of the protein as Raptor and also few sites for potential phosphorylation (S722, S863 and S877) (Fig. 20 and 21). This proved that the full-length Raptor protein was well folded and had the appropriate post-translational modifications needed for mTOR activation. Attempts to express full-length Raptor in suspension cell system are in process.

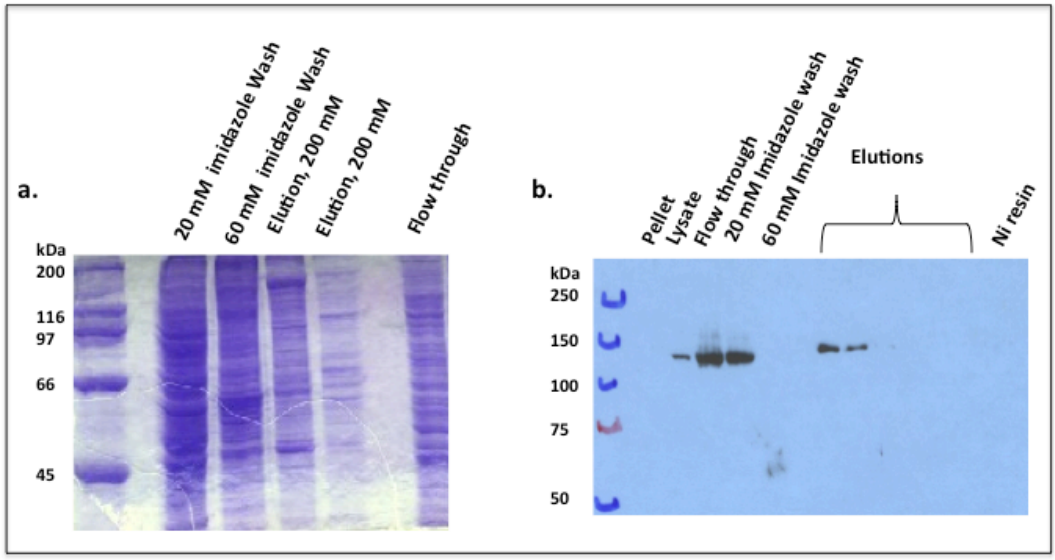

Figure 20: FL Raptor expressing 293T cells grown in a BelloCell bottle purified over nickel resin a) SDS PAGE b) Western blot probed using anti-His antibodies.

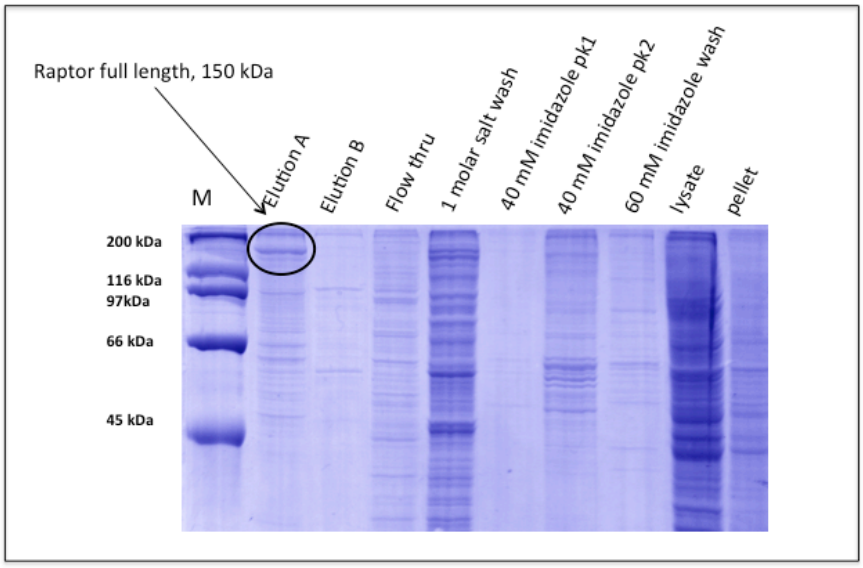

Figure 21: FL Raptor expressed in 293T cells, BelloCell flask and purified over 5 mL pre-packed Nickel column, samples from the purification analyzed on a SDS PAGE.

3.4 Mammalian Target of Rapamycin

mTOR is a 289 kDa intracellular serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. The large size of this protein poses a major challenge to produce it in any of the current expression systems. To test the expression of mTOR protein in our mammalian expression, system, full-length mTOR was cloned into pJG vector with an N-terminal 6xHis tag (Section 2.2.1). To test expression of the protein, the infection was done in adherent HEK293T cells. GFP quantification was used to monitor infection efficiency and expression of the desired protein. The expression of full-length mTOR was not very efficient in mammalian cells. We hypothesized that poor expression could be due to; 1) a failure of the lentiviral to package, owing to the large size of the FL mTOR gene or 2) the kinase activity of mTOR was toxic to the cells. A systematic study of lentivirus packaging showed an insert limit up to about 12 kb (48). In these experiments, viral titers were insignificantly affected with inserts 7 kb or less. Full-length mTOR FL is approximately 7 kb so lentivirus packaging could be affected. To rule out that low expression was due to kinase activity, two kinase dead mutants were designed for mTOR; D2338A and D2357E (49, 50). The protein expression did not improve with the kinase dead mutants. Hence, proving that mTOR kinase was not responsible for low infection and cell death.

To test the hypothesis that insert size was detrimental to lentivirus packaging, truncated constructs of mTOR were designed based on the secondary structure and domain boundary prediction software, (Dismeta, PFam) as well as a limited proteolysis results from the Shrieber's lab, using mTOR purified from bovine brain (51). One

particular trypsin stable fragment starting from amino acid 1819 to the C-terminus was particularly interesting. After aligning the TOR protein sequences from humans, Bovine, Drosophila, *C. Elegans* and Yeast, significant inter species domain conservation in the overall sequence of TOR was evident (Fig. 22). Using these experimental and computational data, several truncated constructs for mTOR (amino acids 1-1819, Δkinase and 1819-2549, 1260-2549 and 1370-2549) (Fig 23).

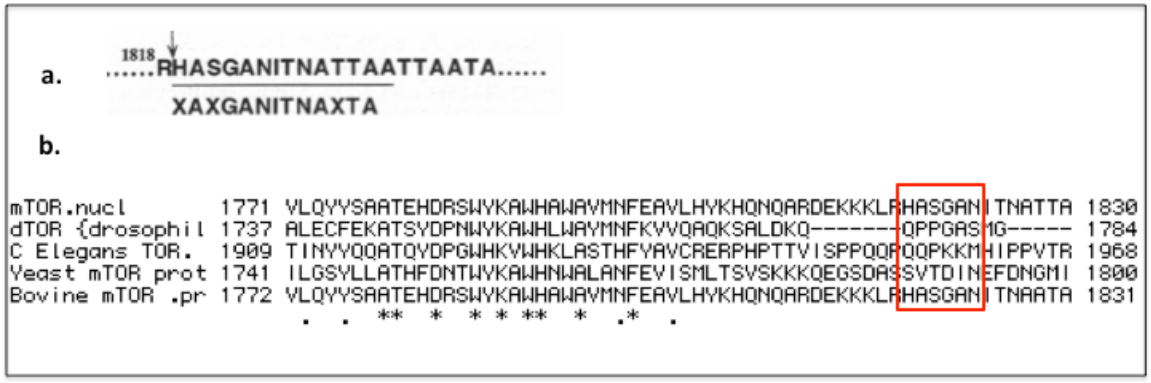

Figure 22: a) N terminus of the 75 kDa fragment obtained after trypsin digest. (51) b) Alignment of TOR from different species around the 1819 amino acid to design constructs.

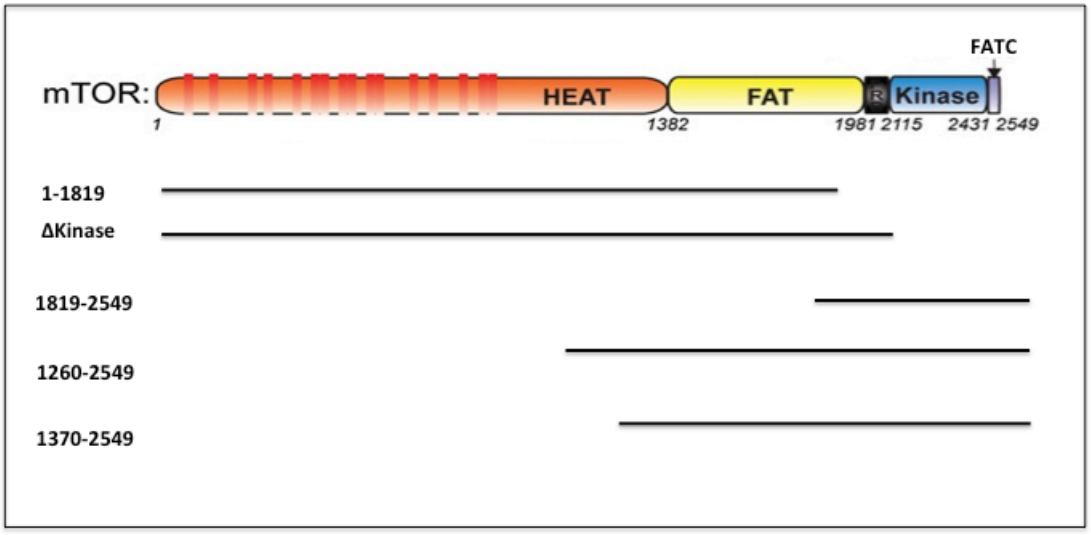

Figure 23: Schematic of mTOR constructs. The constructs have 6XHis tag and precession protease site the size of constructs are: mTOR- 1-1819= 207.7 kDa, mTOR- Δkinase= 245 kDa, mTOR- 1819-2549 = 85 kDa, mTOR 1260-2549= 147.5 kDa, mTOR 1370-2549= 135 kDa.

All fragments were cloned into pJG with N-terminal 6xHis tag and expression was tested in adherent HEK293T cells, due to ease of infection and expansion. The mTOR-1819-2549 fragment gave the best expression and was expanded to 24xP150 plates for small-scale purification over nickel resin (Fig. 24 and 25). The initial purification looked promising and expression of this construct was attempted in suspension 293H cells. In attempts to enrich the mTOR expressing cell population, the GFP was replaced with the Pac gene conferring puromycin resistance and cells were selected using 0.7 μg/mL of puromycin post infection. However, there was no significant improvement in the protein expression and this was not pursued further.

To check the expression of mTOR in other cell lines, lentiviral infections were done in adherent HeLa cells and then adapted into a suspension cell line. The cells divided extremely well in suspension, but as compared to 293H cells, these cells were hard to handle and process for purification. They were extremely viscous during lysis that prevented proper and complete lysing of cells. In an attempt to reduce the viscosity, Benzonase and DNAase were added, however the enzymes did not reduce viscosity. A small-scale purification was done using Nickel column to purify mTOR 1819-2549 construct. It proved that though other cell lines were capable of mTOR expression but 293H remained the best cell line due to the easy and hassle-free access to the cells for protein extraction (Fig. 26).

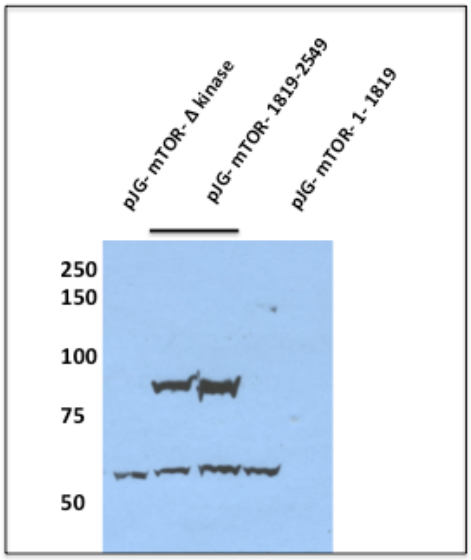

Figure 24: Expression of three truncated mTOR constructs in adherent HEK293T cells analyzed on a western blot using anti –his antibody.

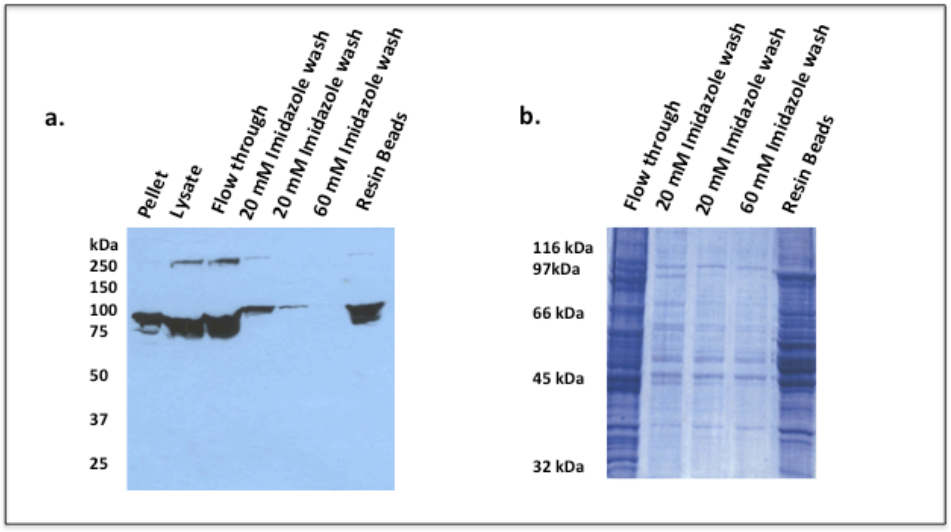

Figure 25: a) Anti-mTOR western blot for the purification of 6xHis-mTOR 1819-2549 over Nickel column, b) SDS PAGE with the same samples.

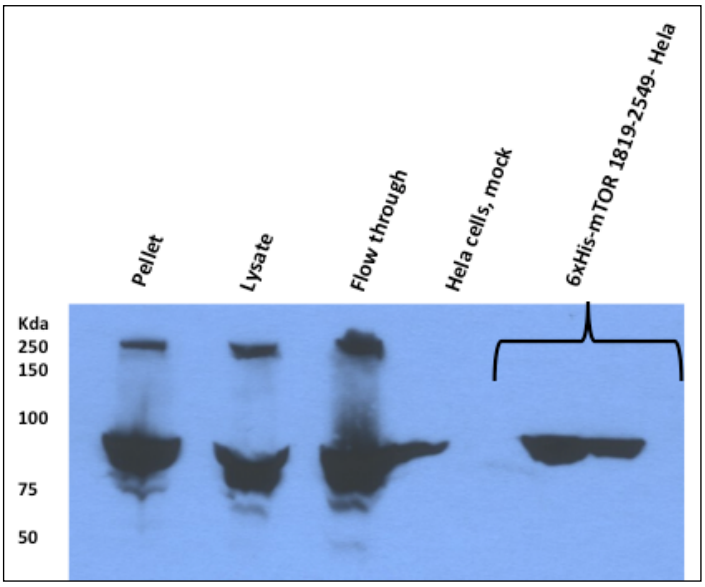

Figure 26: Anti-mTOR western blot, probing samples from purification of 6x His-mTOR 1819-2549 over nickel column produced in HeLa suspension cells.

Protein tag plays a very important role in proteins expression and further purification. An ideal tag would be small in size, would help in protein expression, solubility and aid in obtaining a cleaner protein after purification. The 6xHis tag that was initially used mTOR, was small, and did not hinder the expression, but it lead to a lot the binding of non specific proteins to the column during purification, resulting in protein with lot of contaminations. In order to improvise the purification tags for cleaner protein, the construct mTOR 1819-2549 was cloned with different tags; tandem His tag, Strep tag, His-Strep double tag, 8xHis and 10xHis tag were tried. More number of Histidine residues promised better binding to the column, so that the protein could be stringently washed using higher concentrations of imidazole while immobilized on the column. 8xHis mTOR 1819-2549 was successfully purified over Nickel column with more stringent washes. It yielded three bands in the elution fraction. The top band was identified to be mTOR when analyzed by western blot. Protein band identification results identified the top band as Splicing factor in addition to mTOR, second band as Hsp70 and third band as NONO gene. The proportion of mTOR found was very low in the first band (Fig. 27).

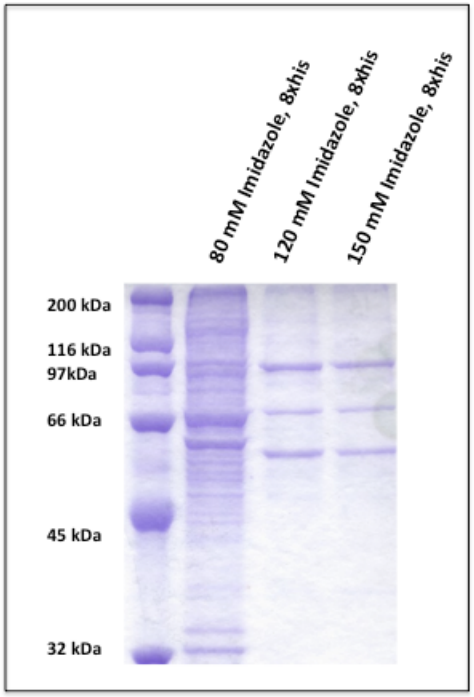

Figure 27: 8xHis-mTOR 1819-2549 purification over Nickel column.

The results for the expression of different proteins (full-length mTOR, mTOR 1819-2549, mCherry) in the suspension system were in agreement with the fact that increasing size of DNA inserted into lentivirus compromised their packaging. This results in a much lower efficiency of cell infection leading to low or null protein expression. In order to enrich the population of infected cells, Fluorescence Activated Cell Sorting (FACS) was employed to the full-length mTOR infected suspension 293H cells. Cell samples for mTOR-FL, mTOR 1819-2549 and mCherry were sent for FACS analysis with uninfected 293H cells as control. The different constructs helped draw a comparison between the efficiency of infection with increasing size of the gene being overexpressed (Fig. 28).

The FACS analysis was consistent with the fact that as the size of the gene insert increased, the infection efficiency went down, as determined by GFP or mCherry expression. FACS was used as a tool to enrich for a stable, GFP positive healthy cell line. Using FACS the population of GFP positive mTOR FL infected 293H cells increased from 19 % to 85 %, proving that the expression system is capable of expression large proteins (~300 kDa mTOR). FACS can increase the percentage of infected, GFP positive cells and consequently protein yields. Approximately 800,000 infected cells of 6xHis-mTOR-FL were FACS and expanded over a period of a week to 2 L culture volume. His-tagged, full-length mTOR from the enriched cells were purified over a Nickel column. The SDS PAGE and western were done to identify the band for full-length mTOR (Fig. 29). This experiment proved the efficacy of combining the suspension expression system with FACS to isolate cells expressing higher GFP/protein of interest levels, resulting in higher protein yields.

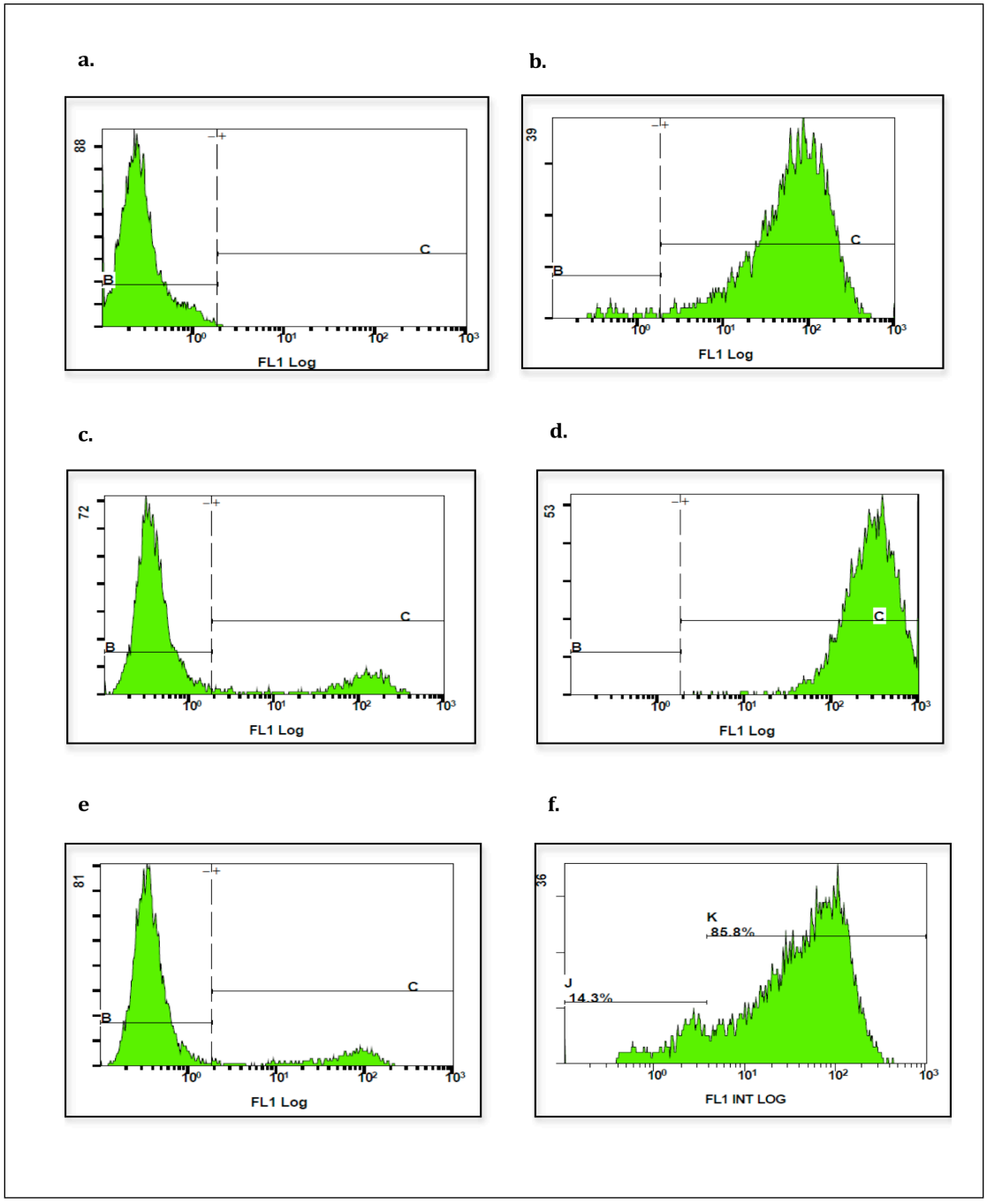

Figure 28: FACS analysis data: a.) Uninfected 293H cells as control, 0.93% GFP positive b.) 6xHis-mTOR1819-2549 (88 kDa), 94% GFP positive c.) 6xHis-Raptor FL (150 kDa), 23.93% GFP positive d.) 6xHis mCherry (26 kDa), 99.39% e.) 6xHis-mTOR-FL (289 kDa) initial GFP positive cells at 19.3% f.) mTOR FL cells after first round of FACS, 85.8% GFP positive

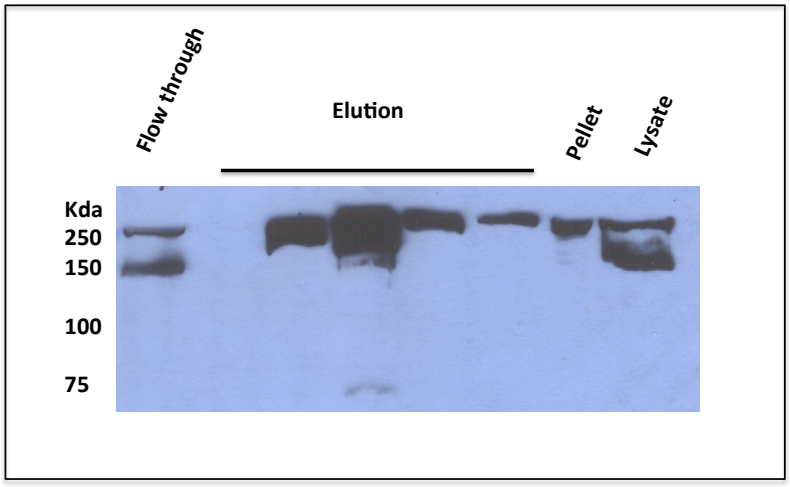

Figure 29: Anti-mTOR western blot probing samples from the mTOR full-length purification –FACS cells.

Chapter 4

Conclusion

In conclusion, the main objective of this thesis was to develop an expression system to facilitate the production of intracellular proteins in mammalian cells. Proteins such as mTOR are a major challenge for expression systems in general, due to their complexity and sizes. Studies on large intracellular proteins have taken a back seat due a lack of system to produce enough material for functional and structural studies. The suspension cell system combines the best of mammalian suspension cells, due to the high densities similar to bacterial cultures with the high efficiency of lentiviral transfection..

This system can be used to express other important cytoplasmic proteins/ drug targets. Combining this method with FACS increases its efficiency, making it useful for the expression of large proteins like mTOR that was not possible previously. This method is environment friendly and cost effective while shortening the time of production of proteins in mammalian cells from months to weeks. This could pave the way to study other difficult intracellular proteins that might have the potential to become excellent drug targets and to gain better understanding of their function and mechanism of action.

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