Role of the Phosphorylation of mTOR in the Differentiation of AML Cells Triggered with CD44 Antigen

Thesis by

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In partial fulfillment of the requirements
For the degree of
Master of science

King Abdullah University of Science and Technology
Thuwal, Kingdom of Saudi Arabia

May 2013
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ABSTRACT

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Manar Darwish

Acute myeloid leukemia (AML) is a hematological disorder characterized by blockage of differentiation of myeloblasts. To date, the main therapy for AML is chemotherapy. Yet, studies are seeking a better treatment to enhance the survival rate of patients and minimize the relapsing of the disease. Since the major problem in these cells is that they are arrested in cellular differentiation, drugs that could induce their differentiation have proven to be efficient and of major interest for AML therapy.

CD44 triggering appeared as a promising target for AML therapy as it has been shown that specific monoclonal antibodies, such as A3D8 and H90, reversed the blockage of differentiation, inhibited the proliferation of all AML subtypes, and in some cases, induced cell apoptosis. Studies conducted in our laboratory have added strength to these antibodies as potential treatment for AML. Indeed, our laboratory found that treating
HL60 cells with A3D8 show a decrease in the phosphorylation of the mammalian target of Rapamycin (mTOR) kinase correlated with the inhibition of proliferation/induction of differentiation of AML cells. The relationship between the induction of differentiation and the inhibition of proliferation and the decrease of mTOR phosphorylation remains to be clarified.

To study the importance of the de-phosphorylation of mTOR and the observed effect of CD44 triggering on differentiation and/or proliferation, we sought to prepare phospho-mimic mutants of the mTOR kinase that will code for a constitutively phosphorylated form of mTOR and used two main methods to express this mutant in HL60 cells: lentiviral and simple transfection (cationic-liposomal transfection).
ACKNOWLEDGEMENTS

First and foremost, I thank Allah for endowing me with patience, and knowledge to complete this work. Thereafter, acknowledgement is due to the King Abdullah University of Science and Technology for the support given to this research through its excellent facilities and for granting me the opportunity to pursue my graduate studies with financial support.

I acknowledge, with deep gratitude and appreciation, the inspiration, encouragement, valuable time and guidance given to me by Professors Jasmeen Merzaban and Samah Gadhoum and for giving me the chance to work in this laboratory. It has been an amazing experience to join such a collaborative team, I thank every student in the laboratory for their continuous welcoming help and for being patient to give me some of their precious times to answer any question along the way. A sincere thank you to Dr. Kosuke Sakashita, for his extensive guidance, continuous support, and personal involvement in all phases of this research.

I am sincerely grateful to my parents, family and friends, who were by my side with their lovely words of encouragements and prayers.

Last but not least, I am very thankful to my husband for the tremendous and endless support and help he offered me through my thesis, and to my kids for their love and support.
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1. INTRODUCTION

1.1 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is a malignant disease, affecting hematopoietic stem cells or myeloid progenitors, characterized by the accumulation of immature myeloblasts which are blocked in cellular differentiation and resistant to cell death (Smith et al., 2004). These myeloid cells are arrested at various stages of granulocytic and monocytic differentiation, defining distinct AML subtypes according to the French British American (FAB) classification (AML0 to AML5) (Bennett et al., 1985).

Just like in the normal hematopoiesis, we believe that a rare population of cells called Leukemic Stem Cells (LSCs) with self-renewal capacity, are responsible for maintaining this malignant clone. The leukemic stem cells (LSCs) are very similar in phenotype to their normal counterpart (the hematopoietic stem cells or HSCs) and give rise to leukemic progenitors and blasts that overpopulate the blood stream thus leading to leukemia (Altman et al., 2011).
1.2 Potential therapy to AML

Chemotherapy remains the main treatment for all types of AML. Despite recent progress in understanding the biology of AML and the progress in chemotherapy regimens with stem cell transplantation for young patients, new treatments are still required as the disease relapse in a large number of cases and progression and death frequently occurs (Récher et al., 2005).

Chemotherapy, rarely eradicates the leukemic clone as it eliminates the leukemic blasts but not the quiescent leukemic stem cells (Fig 1.1). Extensive research is aiming toward a less toxic and more effective and targeted therapy that works side by side with chemotherapy to improve the life quality of AML patients and decrease the chances of relapse of the disease.
Leukemia mostly relapses after the treatment with chemotherapy because this treatment eliminates dividing cells, leaving the quiescent LSCs unharmed.

### 1.2.1 Differentiation therapy and the promise of CD44

Since the blockage of differentiation in cellular maturation is a key characteristic feature of leukemic cells, studies demonstrating the capabilities of certain chemicals to induce differentiation of the leukemic blasts have been of a great interest. In contrast to chemotherapy alone, the differentiation therapy, which reverses the blockage of differentiation of the leukemic blasts, has succeeded, when combined to chemotherapy, to
greatly improve the survival of some AML patients. The best example is the use of retinoic acid, which, used along with chemotherapy significantly improved the cure of AML-type 3 patients. A new promising therapy targeting CD44 antigen using specific antibodies, has recently been shown to induce a differentiation of all Leukemia types, making it a great therapeutic target in the treatment of AML (Gadhoum et al., 2004).

CD44 is a broadly distributed transmembrane glycoprotein expressed on the surface of hematopoietic cells. CD44 has been shown to be over-expressed in leukemic blasts and is sometimes considered as a marker of cancer cells. CD44 is also a signaling receptor that plays an important role in myelopoiesis (anti-CD44 antibodies are capable of inducing the production of myeloid cells in long-term bone marrow cultures). Moreover, it has been shown that specific monoclonal antibodies (mAbs), such as A3D8 and H90, directed to the CD44 on human AML blasts, are capable of inhibiting the proliferation of all AML cell lines, inducing terminal differentiation of THP-1, HL60, and NB4 cell lines (models of AML5, AML2 and AML3 respectively) and inducing apoptotic cell death, moderately in THP-1 and HL60 and massively in NB4 (Gadhoum et al., 2002). Furthermore, CD44 is required for
the homing and engraftment of leukemic stem cells (Krause, 2006). Therefore, targeting CD44 eliminates quiescent AML LSCs, as these stem cells need the interaction to a niche to maintain their stem cell properties. For all of those reasons, CD44 targeted therapy is considered promising for AML patients.

1.3 Aberrant signaling pathways in AML

Deregulation of signal transduction pathways is common in most cancers including AML (Willems et al., 2012). This deregulation enhances the survival and proliferation of abnormal hematopoietic progenitor cells and induces leukemia. Genetic alterations affect different signaling molecules and proteins, making them attractive therapeutic targets. Nevertheless, most of these signaling proteins seem to activate a limited number of downstream effectors pathways, such as the RAF/MERK/ERK and PI3K/Akt/mTOR cascades (Scholl et al., 2008). For this reason, it is attractive to target these pathways for treatment.
Regarding the PI3K/Akt/mTOR pathway, it is known that constitutive phosphorylation of the proteins in the pathway, such as Akt and mTOR, as well as overexpression of proteins such as PI3K, are correlated with an overall shorter survival of cancer patients (Park et al., 2009). These discoveries have led scientists to target the inhibition of such key proteins, reducing their phosphorylation activity and subsequently inducing cell death in cancers.

The mammalian target of rapamycin (mTOR) controls intracellular mechanisms involved in cell growth, proliferation and survival. It is present in two distinctive complexes, mTORC1 and mTORC2. Each of these complexes contributes to different functions in the cell. The activation of mTORC1 lead to the activation of its downstream substrates including the ribosomal protein S6 kinase (P70S6K) and the eukaryotic initiation factor 4E binding protein 1 (eIF-4E-BP1), and ultimately initiating protein translation in the cell. On the other hand, mTORC2 has a key role in cell survival and proliferation by inducing the activation of Akt kinase (Willems et al., 2012).
As mTOR appears to be a key regulator in the PI3k/Akt pathway, it logically appeared as a potential target for the treatment of cancer. Many studies on mTOR inhibitors show promising results in cancer patients.

1.4 **The mammalian target of rapamycin (mTOR)**

The mammalian target of rapamycin (mTOR) is an evolutionary conserved checkpoint protein kinase that is involved in the regulation of the proliferation, growth, apoptosis and angiogenesis (Récher et al., 2005).

mTOR is a central element of the PI3K/Akt pathway. It was originally identified in yeast through a screen for the drug rapamycin, hence the name, Target Of Rapamycin. Subsequently, it was cloned in mammals. mTOR is present in two distinct and functionally diverse cellular complexes (TORC1; rapamycin-sensitive, that binds with raptor protein and TORC2; rapamycin-insensitive that binds to rictor protein) (Hoeffer and Klann, 2009). Each complex has common and distinct subunits and effectors (Fig.1.2A). According to which of these complexes is activated, different downstream effectors controlling different functions in the cell will be activated (Fig.1.2B) (Altman et al., 2011).
1.4.1 *mTOR in cancers and AML and its potential role as a therapeutic target*

The PI3K/Akt/mTOR pathway is aberrantly activated in cancers including AML. Its constitutive activation plays a critical role in survival, growth and proliferation of malignant cells. In addition, this activation is believed to be related to chemotherapy resistance (West, Castillo and Dennis, 2002). Therefore, understanding this important signaling cascade and developing pharmacologic inhibitors to deactivate this pathway is of high interest.

Since alterations in the mTOR signaling pathway are common in cancer, mTOR is considered a therapeutic target in malignancies. Rapamycin and its analogues have been approved for the treatment of only some types of cancers for two main reasons: (i) this drug targets only one of the two main mTOR complexes and, (ii) mTOR signaling network is
Figure 1.2 mTOR is present as two complexes, each binding to different proteins and leading to different functions in the cell. (A) outlines the structure of mTORC1 and mTORC2 and the unique proteins that interact to make up each complex. This figure is from (Bhaskar and Hay, 2007). (B) outlines the main pathways, transcription factors and cellular processes involved in signaling through mTORC1 and mTORC2. This figure is from (Gao et al., 2012).
complex and rapamycin treatment leads to different signaling responses in different cell types (Guertin and Sabatini, 2007). Thus, there was a need to find inhibitors that act to inhibit the catalytic activity of both mTORC1 and mTORC2.

1.4.2 mTOR structure and phosphorylation sites

mTOR is a large serine/threonine kinase (2549 amino acids, ~250KDa) that comprise several conserved structural domains. The amino terminus of mTOR is composed of multiple tandem HEAT repeats, which are implicated in protein-protein interaction. On the other side, the carboxy terminus contains several important elements including the highly conserved kinase domain (KIN). Immediately upstream of this catalytic domain is the FRB that binds with the inhibitory rapamycin-FRBK12 complex that inhibits the activity of mTOR. The carboxy terminus also contains two more domains, FAT and FATC domains, responsible for the regulation of the kinase activity. When these domains interact with each other, they fold in a configuration that exposes the catalytic domain (Hoeffer and Klann, 2009) (Fig. 1.2A and Fig. 1.3).
In the KIN domain there is a small region called the negative regulatory domain (NRD). This domain contains phosphorylation sites (as threonine 2446, serine 2448 and ser2481) that are correlated with overall higher levels of mTOR activity. Recent studies demonstrated that ser2448 phosphorylation is primarily correlated to mTORC1, whereas ser2481 phosphorylation can be considered as a marker for the activity of mTORC2 (Copp, Manning and Hunter, 2009).

**Fig 1.3** mTOR structure. This figure illustrates the main functional domains in the mTOR kinase. This figure is from (Hoeffer and Klann, 2009).
**PRIMINARY RESULTS**

Our laboratory recently found that the inhibition of proliferation/induction of differentiation observed in AML cells (here we used 2 models of AML: HL60, a model of AML2 subtype and KG1a: a model of AML-1), is accompanied by a marked decrease in the phosphorylation of mTOR, without affecting the total amount of mTOR protein. (Fig. 1.4A). Moreover, since mTOR can be part of mTORC1 as well as mTORC2 complexes, we investigated which of these two complexes was inhibited by the anti-CD44 treatment. To this purpose, we used antibodies against phospho-p70S6K (Thr 389) and phospho-Akt (Ser 473), which directly reflect the activity of mTORC1 and mTORC2 respectively. We observed that treatment with the anti-CD44 mAb A3D8 drastically decreased the phosphorylation of p70S6K on Thr 389 (Fig. 1.4B). This decrease is correlated with the decrease of phosphorylation in mTOR protein strongly suggesting an inhibition of mTORC1 complex after CD44 triggering. In addition, treatment of HL60 cells with the anti-CD44 mAb A3D8 for the same time points significantly decreased the phosphorylation of AKT on Ser
This decrease directly reflects the inhibition of mTORC2 in the AML cell line HL60 after treatment with anti-CD44 mAbs.

These findings reveal a new role for anti-CD44 mAbs as potent mTOR inhibitors, giving CD44 an additional positive argument to be used as a targeted therapy in AML.

**Objective**

Our study aims to investigate the correlation between the importance of the decrease in mTOR phosphorylation and the inhibition of proliferation/induction of differentiation of leukemic cells caused by anti-CD44 mAbs treatment. To this end, we will construct and use dominant phospho-mimic mutants of mTOR, resembling the phosphorylated structure. The following mTOR mutations will be produced:

1) Point mutation targeting Ser2448Ala (mTORC1) and Ser2481Ala (mTORC2) to produce a form of mTOR that is not able to be phosphorylated; and
2) Point mutation targeting Ser2448Glu/Asp (mTORC1) and Ser2481Glu/Asp (mTORC2) that mimics constitutive phosphorylation.

Following that, HL60 cells, transfected with one of these mutants at a time, will be treated with anti-CD44 antibodies to examine the relevance of the inhibition of mTOR phosphorylation for proliferation and differentiation of leukemic cells.
**Expression of mTor/phospho-mTor in HL60**

A

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- **P-mTor**
- **mTor**
- **Beta-actin**

B

<table>
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- **AKT-P(Ser 473)**
- **P-p70S6K**
Fig. 1.4 Decrease of mTOR phosphorylation without affecting the total mTOR. Treating HL60 cells with an anti-CD44 antibody inhibits the phosphorylation of mTOR without affecting the total amount of mTOR protein. (A) HL60 cells were either treated with A3D8 or not and were subsequently collected at different time points (5 minutes, 1 hour, 24 hours), washed, lysed and prepared for Western blot analysis to measure phosphorylated mTOR, total mTOR and beta-actin. Note that the expression of phosphorylated mTOR decreases significantly in treated cells compared to untreated cells as early as 5 minutes and continues to 24 hours whereas the total mTOR expression was unaffected by treatment during the timepoints tested. β-Actin (a housekeeping protein) staining revealed that similar levels of protein were loaded per sample. (B) To study the effect of A3D8 on each of the mTOR complexes (mTORC1 and mTORC2), the phosphorylation of downstream proteins of each mTOR complex was examined through western blot analysis of the whole cell lysates of A3D8-treated or untreated HL60 cells with antibodies against specific for phosphorylated p70S6K and phosphorylated AKT kinase to show effects on mTORC1 and mTORC2 respectively. A significant decrease in the phosphorylation of p70S6K beginning after 5 minutes of A3D8 treatment was observed up to 24 hours compared with untreated cells suggesting an inhibition of the mTORC1. Likewise a significant decrease of the phosphorylation of AKT on Ser 473 was also evident starting at 5 minutes up to 24 hours suggesting an additional inhibition of the mTORC2.
2. MATERIALS AND METHODS

2.1 Generating mTOR phospho-mimcs constructs

Mutants of human mTOR genes that mimic the phosphorylation at two critical sites, Ser2448 and Ser2481, are created using QuickChange site-directed mutagenesis method. Glutamic acid or Aspartic acid, as well as Alanine as a non-phosphorylation control, are used for substitution of corresponding Serine residues. pCMV-mTOR (OriGene, RC220457; GenBank accession number NM_004958.2) was used as a parental plasmid. Mugagenic PCR reaction was conducted with corresponding primers for each mutant (shown in Table 1) and the template plasmid using Pfu DNA polymerase (provided by KAUST Bioscience Corelab). After the reaction, 1 μl of DpnI (New England Biolabs, R0176S) was added and incubated at 37°C for one hour to eliminate residential template plasmid. PCR product was then precipitated and dissolved in distilled water, followed by transformation into GeneHogs (Invitrogen, C80005) bacterial electrocompetent cells. After overnight incubation at 37°C, several colonies were picked up and inoculated in 20 ml of LB broth (Difco, 244620) in
conical tubes and incubated overnight at 37°C with 300 rpm shaking. Plasmids were prepared from these cultures using QIAprep Spin Miniprep kit (Qiagen, 27104) and digestion pattern was confirmed with SpeI (New England Biolabs, R0133S). Clones showing correct pattern were further amplified with second round of transformation and inoculation in 100 ml of LB broth, and prepared using Qiagen Plasmid Maxi Kit (Qiagen, 12162). Sequences of these plasmids were confirmed by Sanger sequencing. Primers used in sequencing reactions are shown in Table 2. Staden package software suite and Phred basecall software were used for examination of sequencing results. Resulting mutants as pCMV6-mTOR-S2448A, pCMV6-mTOR-S2448D, pCMV6-mTOR-S2448E, pCMV6-mTOR-S2481A, pCMV6-mTOR-S2481D, and pCMV6-mTOR-S2482E, respectively.

2.2 Preparation of Plasmids for lentiviral vector production

2.2.1 Digestion pCMV6-mTOR plasmid and pEntr11-Dual (Invitrogen, A10467) were digested separately using 0.5μl of SalI and 0.5μl of NotI
restriction enzymes (New England Biolabs, R0138S and R0189S) to every 0.5μg/μl of DNA. Reaction was incubated for two hours at 37°C. Small portion of reaction solution were analyzed by 0.8% agarose gel electrophoresis to confirm sufficient digestion. The plasmids were purified using QIAquick PCR Purification Kit (Qiagen, 28104).

2.2.2 Ligation Both digested plasmids were mixed in a 1:2 ratio (pEntr11: mTOR) with 1μl of T4 DNA ligase (New England Biolabs, M0202S) to every 10 μg of the mTOR plasmid. The reaction mixture was incubated at 16°C for one hour. Plasmids were then precipitated by ethanol precipitation method, adding 1/10 and twice the amount of DNA solution of sodium acetate (NaOAC) and 100% ethanol, respectively. Allowing the DNA to precipitate, the mixture was incubated on ice for 15 min then centrifuged for another 15 min on highest speed. Pellets were washed with 70% ethanol and then dissolved in distilled water.

Remaining parts of the vectors were digested to reduce the amount of undigested circular plasmids from preceding digestion reaction by adding 0.5μl of NgoMIV (New England Biolabs, R0564S) restriction enzyme,
incubated for one hour at 37°C. The reaction was stopped by incubating the reaction mix at 65°C for 10 min. Then the DNA was precipitated as described above.

2.2.3 Transformation Escherichia coli GeneHogs (Invitrogen, C80005) and Stbl3 (Invitrogen, C7373-03) strains were used as recipient strains in transformation experiments. GeneHogs Electrocompetent cells were mixed with ligation product and incubated for five minutes on ice, then transferred to a 1 mm gap-cuvette. Electroporation was conducted using BTX ECM 399 electroporation system (BTX) followed by immediate addition of 1 ml of SOC medium, and incubated at 37°C for one hour before spreading on a plate. Stbl3 chemically competent cells were mixed with ligation product and incubated for five minutes on ice, exposed to heat shock using 42°C water bath for 45 seconds, followed by addition of 1 ml of SOC medium and incubation at 37°C for one hour before spreading on a plate.

Transformants were selected on LB agar (Invitrogen, 22700-025) plates containing 100 mg/L kanamycin (Thermo Fisher scientific, BP906-5), and a
selection of the resulting colonies were inoculated in 10 ml of LB broth (Difco, 244620) overnight at 37°C with 300 rpm shaking. QIAprep Spin Miniprep kit (Qiagen, 27104) was used for DNA extraction and the insertion of the fragment containing mTOR ORF was checked by SalI and NotI digestion as described above.

2.2.4 LR Gateway reaction The mTOR gene in the the pENTR11-dual vector was transferred to pLenti6.3/V5-DEST Gateway destination vector (Invitrogen, V533-06) or pLenti7.3/V5-DEST Gateway destination vector (Invitrogen, V534-06) by recombination with Gateway LR clonase II (Invitrogen, 11791-100). Gateway LR recombination reaction was conducted according to manufacturer’s instruction. The reaction was stopped by addition of 1 μl of Proteinase K solution (Invitrogen, 11791-100). DNA was precipitated by ethanol precipitation and transformed into both E. coli GeneHogs and Stbl3 strains as explained before, but in inoculation, they were incubated at 30°C overnight to eliminate possible occurrence of undesirable homologous recombination within lentiviral plasmids.
2.3 Simple Transfection

2.3.1 cell lines

Human embryonic kidney 293FT cells (Invitrogen, R700-07) and CHO-K1 (Chinese Hamster Ovaries) cells (ATCC, CCL-61) were cultured in complete high-glucose Dulbecco modified Eagle medium (DMEM) with pyruvate (Invitrogen, 41966) supplemented with 10% fetal bovine serum (FBS; Gibco, 26140-079), 1% Glutamax (Gibco, 35050-061), 1% Non-essential amino acids solution (NEAA; Gibco, 11140-050), 1% penicillin-streptomycin (Thermo Fisher scientific, HyClone SV30010) in 37°C 5% CO2 humidified incubator. When the confluency reached 80-90% cells were trypsinized by 0.05% Trypsin-EDTA (Gibco, 25300-054) and split.

Human acute myeloid leukemia-derived HL-60 cells (ATCC, CCL-240) were grown in RPMI-1640 (Gibco, 52400) supplemented with 10 % FBS and 1% penicillin-streptomycin. They were maintained in a humidified atmosphere at 37°C with 5% CO2. The cell cultures were diluted every two to three days so that the cell density was maintained around $1 \times 10^6$ cells/ml.
2.3.2 Transfection

- **293FT cells**

293FT cells were transfected with pCMV6-mTOR plasmid using Lipofectamine2000 reagent (Invitrogen, 11668-019). Cells were seeded into 6-well plates one day prior to transfection as 1x10^6 cells per well in 2 ml complete medium without penicillin-streptomycin antibiotic. On the day of transfection medium was changed with fresh medium. For each well, 750 ng of the DNA was diluted in 250 μl of Opti-MEM I reduced serum medium (Gibco, 31985-062) without serum in one tube, and 4 μl of the reagent Lipofectamine 2000 in another 250 μl of OptiMEM I reduced serum medium, without serum, in another tube. Lipofectamine mix was incubated for 5 minutes, then was added to the DNA mix and incubated for additional 20 minutes. The transfecting mix was then added drop-wise to the cells, 500 μl to each well. Cells were incubated in same previous conditions, 37°C 5% CO2 humidified incubator. After 48 hours, cells were collected and lysed for western blotting.

- **CHO-K1 cells**

For six-well plate transfections, CHO-K1 cells were seeded at
0.2×10^6 cells/well in 2 mL of complete media one day before transfection. Cell density was adjusted to be 50~80% confluent on the day of transfection. For each well of cells to be transfected, 2.5 μg of DNA was diluted into 500μl of Opti-MEM I reduced serum medium without serum, then 8.75μl of Lipofectamine LTX (Invitrogen, 15338030) was added into the DNA solution and incubated for 25 minutes at room temperature. Before the DNA-Lipofectamine LTX complex was added to the cells, growth medium was removed from cells and replaced with fresh 2 ml of complete growth medium without penicillin-streptomycin antibiotics. Then 500μl of the DNA-Lipofectamine LTX complex was added drop-wise to each well. Cells were then incubated at 37°C in a CO2 incubator for 18-24 hours post-transfection.

- **HL-60 cells**

6-well plates were coated with Human Plasma Fibronectin (Gibco, 33016-015) then right before adding the cells to the wells, the PBS solution was removed and the cells diluted in RPMI-1640 media, without any additions, were added into wells as (0.75×10^6) cells per well in 2 ml of RPMI-1640 media. Transfection mix was prepared by diluting 4 μl of Lipofectamine
2000 into 250 μl Opti-MEM I reduced serum medium per well in a tube and incubated for five minutes. In another tube, 750 ng of the DNA was diluted in 250 μl Opti-MEM I. Then both solutions were mixed together and incubated for 20 minutes at RT. After incubation the mix was added to the cells drop-wise with shaking. The cells were then incubated in the humidified 37°C, CO2 incubator. After around five hours 10% of FBS is added to the wells. Cells were then collected after 18-24h post-transfection.

2.4 western blotting

- **Cell lysis**

After transfection, cells were harvested, pelleted by centrifugation, washed in ice-cold phosphate-buffered saline (PBS; Gibco, 14190), and repelleted. Cells were lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris (tris(hydroxymethyl)aminomethane)-HCl with Complete protease inhibitor cocktail, EDTA-free (Roche Molecular Biochemicals, 04693132001) for one hour at 4°C with continuous rotation. Lysate was then centrifuged
at 13,000 rpm for 30 minutes. The supernatant containing the desired proteins was collected for analysis.

- **Western Blotting**

Lysates were combined with the appropriate amount of NuPAGE LDS Sample Buffer (4 x; Invitrogen, NP0008) and β-mercaptoethanol (Sigma, 63689-100Ml-F), boiled on 95°C for five minutes, then loaded onto 4-20% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and separated by electrophoresis. Gels were transferred to nitrocellulose membranes, blocked with 5% dry milk solution diluted in TBST (Tris-buffered saline/0.05% Tween 20, Cell Signaling Technologies, #9997), for 1h in room temperature, incubated with primary antibodies (rabbit anti-mTOR polyclonal antibody, Cell Signaling Technologies, #2972; mouse anti-Histidine tag monoclonal antibody, AbD serotec, MCA1396; at 1:1000 dilution with TBST) for two hours at room temperature (RT), and washed three times for five minutes with PBST, and followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit Ig HRP, Southren Biotech, 40100; Immunopure goat anti-mouse IgG H+L, Thermo, 31430; at 1:10,000 dilution with TBST) incubated for one
hour at RT, and also washed three times for five minutes with PBST. Signals were detected with X-ray films using SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific, 34087).

Table 1. Primers used for mutagenesis.

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*Positions corresponding to mutations are shown in lower case and underlined.*
Table 2. Primers used for Sanger sequencing.

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3. Results

The following flow chart outlines the series of experiments that need to be achieved to ultimately transfect HL60 leukemic cells with the various mTOR constructs. Our first step is the preparation of the various phospho-mTOR mutants that are point-mutated at the two sites of phosphorylation: serine 2448 and serine 2481. The pCMV6-mTOR form the human wildtype was used as a parental plasmid to construct two phospho-mimic mutants for each of the serine sites replacing it with aspartic acid (E) or glutamic acid (D). An additional substitution was undertaken for each site as a control ‘no phosphorylation’ by replacing the serine with an alanine (A). After the production of the pCMV6-mTOR plasmid mutants, the mTOR gene of each mutant and wildtype were transferred into an entry vector, then into a lentiviral. These lentivirals were then transfected into a packaging cell line (here 293FT cells) to produce the viral particles containing the required plasmid. These particles are easier to transfect into the leukemic cell line HL60. The second option used here was to directly transfect the
pCMV6-mTOR plasmid, wildtype and mutants, into HL60 cells using transfecting reagents. In order to confirm that the transfection happened properly, the presence of the mTOR protein was analyzed by western blotting on the whole cell lysate and its quantity was compared to the amount of mTOR in the untransfected cells or by using an anti-Flag or anti-Myc antibody. This last method will be more accurate as we are sure that the antibodies used are specific to the transfected mTOR and will not recognize the endogenous mTOR.
Fig. 3.1 The flow diagram provides an overview of steps used to ultimately overexpress mTOR and various mutants into Leukemic cells.
3.1 Construction of mTOR Phosphomimic Mutants

To examine the role of mTOR in CD44-mediated signal transduction and specifically in the aspect of phosphorylation relevant to two distinct mTOR protein complexes, we employed phospho-mimic mutants at the two sites of phosphorylation, serine 2448 and serine 2481. We used pCMV6-mTOR as a parental plasmid harboring wildtype human mTOR gene under the control of a strong CMV promoter available for expression in mammalian cells. Based on this plasmid, we constructed two possible phospho-mimic mutants for each serine site where serine residue is replaced by aspartic acid (E) or glutamic acid (D). Alanine substitutions were also prepared to produce “no phosphorylation” mutants for each site, which could serve as a control in following experiments. Though it is expected that carboxy groups on the side chains of aspartic acid and glutamic acid could resemble the negative charge associated with the phosphorylation of serine residues, actual mimicry ability of these mutants is to be confirmed in future experiments. We successfully were able to construct each of the proposed
mutants outlined in Table 3 and the next step is to transfer these mutants and the wild-type form into appropriate expression vectors as outlined in the flow diagram above.

Table 3. mTOR phospho-mimic mutants

<table>
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<th>Mutant</th>
<th>Site of mutation</th>
<th>Substitution</th>
<th>Type of mutation</th>
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<td>S2481A</td>
<td>Serine 2481</td>
<td>Alanine (A)</td>
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3.2 Transferring mTOR gene into a gateway entry vector

In order to ultimately prepare viral particles to be used to convey the mTOR gene in HL60 cells, the first step is to insert the mTOR gene into an entry vector compatible with the Gateway (Invitrogen) system reactions. The mTOR gene was transferred from the pCMV6 vector (where it was originally cloned), into an entry vector, pEntr11-dual, using conventional DNA manipulation techniques utilizing restriction enzymes and DNA ligation reactions. Both plasmids, pCMV6-mTOR and pEntr11-dual, were digested separately using the same combination of restriction enzymes, SalI and NotI, to form compatible sticky ends. When mixing digested products of these plasmids in the following ligation process, each sticky ends of the DNA fragment containing mTOR gene overhang with the compatible ends of the vector backbone fragment derived from entry vector, pEntr11. Covalent bond will be formed at appropriate sites using DNA ligase resulting in the pEntr11-mTOR vector. A correct digestion pattern of the
plasmid was confirmed by agarose gel electrophoresis (put the figure number corresponding). Also, the correct sequence of the plasmid was further confirmed by sequencing analysis. This plasmid was then ready to undergo the LR gateway reaction, transferring the mTOR gene into the lentiviral backbone plasmids, pLenti6.3/v5-DEST and pLenti7.3/v5-DEST, for further preparation of lentiviral particle vectors to deliver the mTOR gene into the target cells.
**Fig. 3.2** Insertion of mTOR gene into pEntr11 entry vector. A. Schematic representation of pEntr11-mTOR plasmid construction. The restriction sites to be used for the construction of the final vector are SalI and NotI. The resulting fragments after digestion of the pEntr11 release a 1650 bp “stuffer” product and a backbone for which the mTOR gene will be ligated into following the digestion of the pCMV6-mTOR with SalI and NotI to release the ~ 9bp mTOR gene. B. Digestion patterns of parental and resultant constructs. Marker, 1 kb plus DNA Ladder (Invitrogen); lane 1, digested pEntr11; lane 2, digested pCMV6-mTOR; lane 3, the resultant plasmid pEntr11-mTOR, undigested; lane 4, pEntr11-mTOR, digested. Digestion in all cases was done by restriction enzymes: SalI and NotI. The insertion was confirmed by digestion as the last column represents the digested pEntr11-mTOR where the top band matches one of the mTOR bands, which contains the right size of ~9Kbps (marked with **). The middle band represents the undigested pEntr11-mTOR. Where the bottom band represent the part required from the pEntr11 vector to form the pEntr11-mTOR plasmid containing the LR gateway reaction site (marked with*).
3.3 Transferring the mTOR gene into lentivirals

It is known that the efficiency of gateway reaction decreases as the size of the inserted gene increases, e.g. more than 3 kb, even though it is reported to be possible to transfer genes of more than 10 kb in length. The large size of the pLenti plasmids, which is around 10 kb, also limits the efficiency of the reaction. Since the size of mTOR gene is more than 7 kbp, transferring the mTOR gene into the lentiviral by LR gateway reaction is a challenging task. To achieve this task several experiments were done by manipulating several parameters in order to optimize the conditions of the reaction, such as incubation time after transformation. Results showed that the number of transformant colonies was inadequately small in all experiments and no positive clones were obtained in these experiments, indicating necessity of further optimization of the reaction for the insertion of the mTOR gene in the lentiviral plasmid.
3.4 Leniviral transfection into 293FT cells

The gene of interest can be inserted into leukemic cells using viral particles produced from lentiviral particles that are formerly prepared by transfection into the 293T packaging cell lines. In this experiment, to establish and practice the procedure required for the preparation of viral particles by lentiviral transfection, 293FT cells were transfected with the lentiviral plasmid pLenti7.3/V5-GW/lacZ, containing a GFP expression cassette, using lipofectamine 2000 reagent (according to ViraPower Lentiviral Expression Systems User Manual provided by the vendor; invitrogen). Fluorescent microscopy was used to examine the expression of exogenous GFP as an indicator for insertion of the plasmid into cells.

As shown in Fig. 3.2, the cells were successfully transfected with the pLenti7.3/V5-GW/lacZ plasmid, and expression of GFP in the cells was observed. In the future experiments using pLenti plasmids with mTOR mutants and helper plasmids for lentiviral particle formation, viral particles
may be collected from the supernatant and further used to transfec
t leukemic cells according to the presently established procedure.

Fig. 3.2: Transfected 293FT cells with a pLenti7.3/V5-GW/lacZ (GFP+) plasmid. 293FT cells were transfected with pLenti7.3/V5-GW/lacZ plasmid that contains a GFP cassette using lipofectamine reagent. The green florescence observed indicates that the transfection was achieved and the GFP reporter gene was delivered into the cells and expressed successfully.
3.5 Transfecting adherent cell lines

In order to confirm that mTOR mutants could be expressed normally from constructed plasmids, we conducted transfection experiments using the adherent cell lines, 293FT cells and CHO-K1 cells. Cationic lipid-based reagent-mediated simple transfection was conducted to introduce the plasmids harboring mTOR mutants into these adherent cell lines. First, 293FT cells were transfected with the pCMV6-mTOR plasmid. The transfection efficiency and the insertion of the gene were confirmed by examining the presence of the mTOR protein by western blotting.

293FT cells were transfected using the Lipofectamine 2000 reagent. Cells were incubated for 48-72 hours in the humidified 37°C, 5% CO₂ incubator. The cells were then collected and lysed. Western blot analysis using 1) anti-mTOR antibody was undertaken to check the presence of the total mTOR, and 2) anti Myc and anti-Flag antibodies (as these tags were included as tags for mTOR; Fig. 3.3) to check the presence of transfected mTOR.
CHO-K1 cells were transfected using another transfecting reagent, Lipofectamine LTX. The cells were collected between 18-24 hours post-transfection. Western blot analysis using anti-mTOR antibody clearly showed that the amount of mTOR increased for the cells treated with the transfection reagent compared to the cells with the addition of the plasmid with no transfecting reagent (Figure 3.4). Anti-β-Actin was then applied on the membrane to ensure that the increase of intensity is not due to the increased amount of cells loaded to the well. As Fig. 3.4 illustrates, β-Actin has the same intensity of bands in all wells, proving that the same amount of cells are loaded into the gel, and the increase of intensity in bands of treated cells with anti-mTOR antibody is due to the increased amount of mTOR expressed by the plasmid (Fig. 3.4).
Fig. 3.3 293FT cells transfected with pCMV-mTOR. (A) The map of pCMV6-mTOR plasmid illustrating the sites of the Myc and Flag(DDK) binding proteins (The picture is supplied by vender; OriGene Technologies). Note that after the ORF of mTOR there is a 6 amino acid spacer followed by a 10aa Myc sequence, another a 6 aa spacer, then a 8aa Flag sequence and finally one amino acid residue and the stop codon. (B) Whole cell lysates of transfected 283FT cells were analyzed by Western blot for mTOR expression (lane 1), Myc expression (lane 2) and Flag expression (lane 3). The presence of the band when treated with anti-Myc and anti-Flag prove the successful insertion of the plasmid (pCMV6-mTOR) into the
Fig. 3.4 CHO-K1 cells transfected with pCMV6-mTOR plasmid and assayed for the expression of mTOR. Various transfection methods were used to introduce the pCMV6-mTOR plasmid to CHO-K1 cells: plasmid alone (Lane 1), lipofectamine LTX reagent (Lane 2), lipofectamine LTX transfecting reagent and PLUS reagent (Lane 3). Whole cell lysates from the various transfected cells were prepared and subjected to Western blot analysis for mTOR expression top using anti-mTOR antibody (top blot) or for β-Actin (housekeeping gene; bottom blot). It is evident from the blot that the addition of reagents, increased expression of mTOR protein in CHO-K1 cells.

To ensure that the transfection had occurred, the PVDF membrane needed to be treated with an antibody against one of the binding proteins. The membrane was subsequently stained using anti-Flag antibody. As shown in Fig. 3.5, the cells
that were treated with the transfecting reagent displayed bands at the right size of mTOR, while untreated cells did not show any band. Thus, the transfecting reagent clearly succeeded to insert the mTOR gene into the cells (Fig. 3.5)

**Fig. 3.5 Expression of mTOR via transfection of pCMV6-mTOR induces expression of exogenous mTOR as indicated by detection of the protein tag, Flag.** CHO-K1 cells were transfected with plasmid containing the mTOR gene (pCMV6-mTOR) using various methods [Lipofectamin LTX reagent or Lipofectamine LTX + Plus reagents, in the second and third lanes, respectively] or without any reagent in the first lane. Cell lysates were prepared from these cells and subjected to Western Blot analysis using an antibody specific to the Flag protein. Cells transfected with the plasmid using the transfecting reagent shows a band at the expected size. In contrast, the addition of the plasmid without the transfecting reagent does not show any band indicating that there is no endogenous “Flag” signal inherent in these cells.

In another experiment, CHO-K1 cells were also transfected with all the 6 mutants of the mTOR protein. Using
the same transfection reagent, Lipofectamine LTX, each well of cells transfected by a different plasmid (as discussed above in Table 3: S2448A, S2448D, S2448E, S2481A, S2481D, S2481E). All mutants were successfully transfected as the wild type mTOR (Fig. 3.6). Therefore, we confirmed that all 6 mutants are able to be transfected into the cells as well as the wildtype.

**Fig. 3.6** CHO-K1 cells transfected with the wild type mTOR and the six mutants of mTOR. CHO-K1 cells were transfected with the wildtype pCMV6 – mTOR plasmid then with each of the all six mutants of pCMV6 – mTOR plasmid (S2448A, S2448D, S2448E, S2481A, S2481D, S2481E), separately. The transfection was done using the Lipofectamine LTX and PLUS reagents. Whole cell lysates of transfected CHO-K1 cells were assayed by western blotting to detect the expression of the transfected mTOR. Anti-Flag antibody was used, as Flag is one of the Tag proteins to the mTOR gene in the pCMV6-mTOR plasmid. The first lane is the negative control, where cells had no additions to them, showing no signal corresponding to the Flag binding protein. The following lanes represent the transfected CHO-k1 cells with each of the wildtype pCMV6 – mTOR plasmid then with each of the all six mutants of pCMV6 – mTOR plasmid (S2448A, S2448D, S2448E, S2481A, S2481D, S2481E) respectively. The presence of the signal indicates the successful transfection of the pCMV6-mTOR plasmid with wildtype as well as all mutated forms created.
3.6 Transfection of HL-60 cells

Since HL-60 cells are generally known to be difficult to transfect, different conditions were tested for transfection.

First we used the same method as applied to CHO-K1 using Lipofectamine LTX and Plus reagent, and following transfection, the expression of m-TOR was determined by preparing a whole cell lysate and analyzing for mTOR protein using Western Blotting. Since mTOR is already endogenously expressed in HL60 cells even before transfection, we determined the increase in the expression of m-TOR through analysis of the intensity of the m-TOR band.

To make sure that the increase is due to the insertion and not an increased number of cells, we counted the cells before treatment as 0.5×10^6 cells were seeded into each well, and then counted again post-transfection, before lysing the cells for examination. Untreated cells were 0.48×10^6, where treated cells whether with Lipofectamine LTX, or PLUS reagent or both resulted in a more intense band for a lower number of cells.
loaded \((0.32 \times 10^6)\) for cells treated with Lipofectamine LTX, 
\(0.31 \times 10^6\) for cells treated with both Lipofectamine LTX and 
PLUS reagent and \(0.4 \times 10^6\) for the cells treated with the PLUS 
reagent alone) (Fig. 3.7). The increase of intensity in the m-TOR 
bands of transfected cells is a sign of successful transfection.

![Image of Western Blot](image)

**Fig. 3.7 Transfection of HL60 with the pCMV6-mTOR plasmid.** HL60 cells were transfected with the pCMV6-mTOR plasmid using various transfection reagents: Lane 1- cells alone; Lane 2- cells transfected with pCMV6-mTOR plasmid using the Lipofectamine LTX reagent; Lane 4- cells transfected with pCMV6-mTOR plasmid using PLUS reagent; and Lane 3- cells transfected with pCMV6-mTOR using both Lipofectamine LTX and PLUS reagents. Whole cell lysates of the transfected HL60 cells were prepared and stained with anti-mTOR to show the mTOR in the cells and compare the untreated cells with the treated ones. The blots show an increase in the intensity of the mTOR signal in the transfected cells, indicating that the increased amount of mTOR signal is due to the excess amount of mTOR translated by the transfected pCMV6-mTOR plasmid.

As done previously with CHO-K1 cells, we needed to 
confirm by applying an antibody against one of mTOR binding 
proteins (Myc) of the mTOR plasmid. Surprisingly, we could not
detect any band to confirm the transfection (data not shown). That is probably due to a degradation of Myc antibody at the time of analysis. This experiment needs to be repeated using a new anti-Myc antibody.

Next, we employed Fibronectin coating of the culture plates to enhance attachment of the cells to the bottom of the wells, which is reported to improve transfection efficiencies of certain types of cells and cell lines. Also, the timing of the addition of the serum to the wells was delayed to five hours post-transfection assuming that prolonged duration of exposure of cells to the DNA-containing medium could increase the chance of incorporation of DNA into cells, even though a longer exposure to the transfection reagent could increase cell toxicity. Unfortunately, both of these attempts did not work in our experiments and no significant signal was detected in western blotting (data not shown).
4. Discussion

It has been previously suggested that the PI3K/mTOR pathway could be involved in the aberrant proliferation of AML cells and that blocking the PI3K/Akt/mTOR pathway could be very effective in inhibiting proliferation of leukemia cells. Indeed novel strategies have been suggested and tested clinically such as the use of rapamycin: a specific inhibitor of the mTORC1 complex. Rapamycin-based cancer therapy has shown great promise as a potential cure for AML in clinical trials. But relapse often occurs and researchers attributed the inability of rapamycin to inhibit the second complex that mTOR is part of: mTORC2.

In our laboratory, we showed that triggering of CD44 using anti-CD44 mAbs is a promising therapeutic target to treat AML patients because it leads to a strong inhibition in blast proliferation and to reversal in their blockage in differentiation, and strongly inhibits both mTORC1 and mTORC2 complexes. This recent discovery by our laboratory that the inhibition of
proliferation /induction of differentiation in AML cells is correlated to a strong decrease in mTOR phosphorylation is a prime reason to consider CD44 triggering as an effective targeted therapy in AML.

However, the direct relationship between TORC 1 and mTORC2 complexes and the inhibition of proliferation/induction of differentiation is yet to be determined. This study aims to reveal the link between the decrease in mTOR phosphorylation and the inhibition of proliferation /induction of differentiation in AML leukemic cells. To this purpose, we sought to overexpress an mTOR mutant that is hyper-phosphorylated. The overexpression of this dominant will counterpart the decrease of phosphorylation induced by anti-CD44 mAbs. If anti-CD44 inhibition of proliferation and/or induction of differentiation is abrogated by the over-expression of this hyper-phosphorylated mTOR mutant, then we can conclude that mTOR dephosphorylation is necessary for CD44 to trigger inhibition of proliferation and/or
induction of differentiation.

Preparing mTOR phospho-mimic mutants was the first step in this investigation. To this purpose, each of the two important sites in mTOR phosphorylation, Ser 2448 and Ser 2481, underwent a single point mutation where the serine was replaced by negatively charged amino acids, glutamic acid or aspartic acid, to act as a “constitutively phosphorylated” mTOR. This form of mTOR will mimick the activated form of the kinase. Another point mutation was processed as a control, by replacing the serine amino acid with an alanine, which cannot get phosphorylated. The correct mutants were confirmed by plasmid digestion and sequencing.

After the mutants were prepared, inserting them into the leukemic cell line (HL60) was the following step. Inserting the plasmid containing the required gene into a cell can be done in different ways. In this study, two methods were mainly used to introduce the mutated mTOR plasmids into the cells, lentiviral and simple transfection (liposome-mediated or cationic lipid-
mediated transfection).

Preparing lentiviral plasmids immediately appeared to us like a good option, because the plasmid can be transfected into normal cells first and then the viral particles that are produced into the supernatant can be used to introduce the gene into carcinogenic cells (HL60 in this case) which are sensibly more difficult to transfect. In addition, we wanted the insertion to be stable and not transient. At this point, our gene of interest, mTOR, was in a pCMV vector. In order to transfer it to a lentiviral, the mutated mTOR gene needed to be transferred from the pCMV vector into an entry vector (pEntr11) that contains an LR reaction site then further transferred into the lentiviral vectors. By digesting both plasmids, pCMV-mTOR and pEntr11, we created matching sticky ends that were later ligated together to produce the pEntr11-mTOR plasmid. Consequently, we needed to transfer the gene into lentiviral plasmids, as pLenti6.3/V5-DEST and pLenti7.3/V5-DEST before transfecting them to the cells. Several trials and optimization
methods were tested to succeed this transfer, but the size of the gene, around ~ 9 Kbps, made this transfer challenging.

The second option that we used to insert the genes into the cells was by “simple transfection”. This method is based on transfecting the same plasmid that contains the targeted gene, the pCMV-mTOR plasmid, directly into the targeted cells (HL60) using transfecting reagents capable of increasing the transfection efficiency of the plasmid into the cell by forming liposomes that entrap the plasmid and help it to cross the cell membrane. As carcinogenic cells are known to be difficult to transfec, the method was first applied on other cell lines, like 293FT and CHO-K1. These cells were easier to transfec as they are non-carcinogenic plus they are adherent cells, making them perfect candidates for transfection. The insertion was successful in both cell lines, and confirmation was done by western blotting and treating the membranes with anti-mTOR, anti-Myc and anti-Flag (the last two are binding proteins encoded in the mTOR plasmid) showing positive results in all
cases. To ensure that all six mutants were also capable of transfection, the method was applied on all of them with positive results. After that, the same method was applied on HL60 cells and the first attempt seemed promising, as there was an indication of transfection. Examining the insertion by western blotting, and treating the membrane with the anti-mTOR antibody gave positive results. Nevertheless, when confirming the insertion using an antibody against Flag or Myc tag, we did not see any bands of the required size. Several optimizations were done to seek positive results, like the use of fibronectin coating and the delay of adding the serum into the media as they showed positive results in previous studies. Unfortunately, these trials showed no insertions of the gene in our case.

Another method that can be applied in future work is the use of foamy virus-based vectors to deliver the gene of interest into the cells. Foamy viruses belong to a viral subfamily Spumaretroviridae, which comprises the family Retroviridae
with the other member, Orthoretroviridae, which includes HIV-1 and MMLV viruses. One major characteristic exhibiting suitability for gene delivery vector of foamy viruses is their nonpathogenic nature (Erlwein and McClure, 2010). Recently, several groups have developed gene delivery vector system based on these viruses. The experimental procedures required to prepare foamy virus-based vectors are roughly similar to those for lentivirus-based vectors including preparation of a plasmid with gene of interest and necessary viral cis-elements, transfection of the plasmid into packaging cells (e.g. 293FT cells) with several helper plasmids, and collection of viral particles from the culture supernatant (Trobridge et al., 2002). This method seems as an attractive one in this study as it has the capacity to deliver large genes due to its intrinsic largest genome size as a retrovirus (Trobridge, 2009). It is also shown to have ability to transduce broad range of cells including difficult to transfect primary cells such as human CD34+ hematopoietic cells and mesenchymal stem cells. These characteristics meet the requirement of our current study: to
deliver genes to leukemic cells, and to deliver large (more than 7 kb ORF) mTOR gene into cells.

This preliminary work is very important to the investigation process. We are challenged by two major obstacles here, 1) the size of the mTOR gene, and 2) the difficulty to transfecting carcinogenic cells such as AML cells. After inserting the genes into the cells, it would be interesting to see the effect of the A3D8 mAbs on the HL60 cells with the hyper-phosphorylated mTOR mutant. In our case, we anticipate that the effects of anti-CD44 mAbs on the cell inhibition of proliferation and/or induction of differentiation will be abrogated or at least reduced compared to control, non transfected cells or cells transfected with empty vector.

My work will help determine the importance of mTOR not only as an actor in the CD44 signaling pathway but also as an key determinant in the over-activated proliferation and or in the blockage of differentiation of AML cells in particular and in cancer in general.
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