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THE EXPRESSION OF MKRN1, AN E3 UBIQUITIN LIGASE FOR TELOMERASE REVERSE TRANSCRIPTASE, IS INDUCED WITH DIFFERENTIATION THERAPY IN LEUKEMIA.

by

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B.S. University of Central Florida, 2004

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Molecular Biology and Microbiology in the Burnett School of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

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Major Professor: Mark T. Muller
ABSTRACT

Telomeres are important structural and functional components of chromosomes, serving to provide stability and enabling full replication of the chromosomes. However, a shortening of the telomeres occurs with each cell division that can be fixed by a polymerase activity provided by telomerase, preventing this loss which would otherwise eventually lead to chromosome end-to-end fusions, senescence and cell death. The telomerase activity is present in stem cells and germ line cells, but absent or barely noticeable in adult somatic cells. However, in approximately 80-90% of transformed somatic cells the telomerase activity is recovered, resulting in a “telomerase positive phenotype”. This phenotype has been a prime target in cancer research, and recently a novel mechanism for regulating telomerase levels has been uncovered. Makorin 1 RING finger protein (MKRN1) was found to be an E3 ubiquitin ligase for hTERT, the rate-limiting catalytic component of telomerase, leading to the ubiquitin-mediated 26s proteasomal degradation of hTERT and reduced telomerase activity. So, MKRN1 plays a role in telomere homeostasis.

In this study we looked at the expression of MKRN1 in numerous tumor cell lines (Hela, HCT116, HL60) and the normal diploid fibroblasts (WI-38). In the latter cell line, basal levels of MKRN1 were found to increase 6-fold when the cells were serum starved and arrested in G\textsubscript{i}/G\textsubscript{0}. In contrast, the cancer cell lines expressed MKRN1 at low levels or undetectable. This would indicate that MKRN1 is up-regulated in resting or G\textsubscript{i} arrested cells. In one cell line the promyelocytic leukemia, HL-60, showed no protein levels of MKRN1. This cell line is able to be terminally differentiated upon ATRA treatment, when cells are arrested at G\textsubscript{i}. In this model
system of cellular differentiation hTERT mRNA levels and telomerase activity decrease drastically and quickly. We hypothesized that the differentiation of HL-60 induced by ATRA would be accompanied by an increase in MKRN1 levels. MKRN1 mRNA and protein levels were strongly up-regulated during the ATRA-mediated differentiation of HL-60 cells. Although, a decrease in hTERT mRNA is a contributor to telomerase inhibition during cellular differentiation; our data indicate that the up-regulation of MKRN1 ensures the effective removal of residual telomerase activity by the ubiquitin-mediated degradation pathway at the proteasome.
I dedicate this thesis to my wife, Christy, for her patience and understanding; and for providing love and support, aiding me in this craziness of graduate school.
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CHAPTER ONE: INTRODUCTION

General Introduction

Human telomeres are nucleoprotein structures found at the ends of linear chromosomes, consisting of a repetitious hexameric DNA sequence (5’-TTAGGG-3’) and telomere-associated proteins. These structures preserve chromosomal stability by preventing end-to-end fusions, rearrangements, and nucleolytic degradation. If these events should occur, then either cell death or tumorigenic selectivity could result. Differentiated cells lack telomerase activity and due to the end replication problem, 150-300 base pairs of DNA are lost with each cell division, resulting in cellular senescence, and loss of cell proliferation. However, in highly replicative tissues, such as hematopoetic, germline and most human cancers, telomerase activity is a token phenotype required for the cell proliferation and survival of these cells.

Telomerase is a ribonucleoprotein consisting of human telomerase reverse transcriptase (hTERT, the catalytic protein subunit), and an RNA template, hTR, complementary to the hexameric repeat sequence. Telomerase is responsible for maintaining telomere length by adding to the 3’-end of the G-rich strand; thereby preventing erosion of the chromosome ends. Thus, telomere homeostasis is a pacing mechanism controlling cellular replication and senescence.

There are diverse mechanisms for the regulation of hTERT and telomerase activity. Transcriptional regulation of the hTERT gene is tightly controlled by numerous transcription factors and epigenetic mechanisms. Activators for hTERT transcription include c-MYC, Sp1 and estrogen response elements, and repressors include MAD1, p53, CTCF and WT1. The methylation status of the hTERT promoter has an effect on how the gene is regulated.
by affecting the binding of activators or repressors (18-22). Recent evidence in retinoid-differentiated human leukemic cells suggests that hTERT promoter methylation occurs after initial transcriptional repression, to further silence the gene (23). Alternative splicing has also been implicated in regulating hTERT expression and telomerase activity in human development (24). Although, these reports show a strong correlation between levels of hTERT transcripts and telomerase activity, there is evidence that protein interactions and post-translational modifications of the hTERT protein are involved as well. The phosphorylation status of hTERT has been shown to regulate telomerase activity (25-27), and in T-lymphocytes, it serves to regulate hTERT localization as well as activity (28). Some proteins, such as PinX1 act as inhibitors of telomerase (29), whereas others like KIP stimulate telomerase (30). The hTERT protein also interacts with and is stabilized by HSP90, allowing it to associate with hTR to form the functional telomerase holoenzyme (31,32). This interaction can be blocked by treatment with the chaperone inhibitor, geldanamycin (GA) (31). Recent studies show that GA treatment destabilizes hTERT and promotes its ubiquitination, which leads to proteasomal degradation efficient removal of telomerase activity (33). Furthermore, this proteolysis of hTERT could be reversed when proteasome inhibitors were used. In the same studies, the Makorin RING Finger Protein 1 (MKRN1) was found to interact with hTERT protein, serving as an E3 ubiquitin ligase, and having a role in hTERT proteolysis. The effects of this interaction enhanced hTERT ubiquitination and proteasome-mediated degradation, even in the absence of GA. In addition, stable expression of MKRN1 promoted the loss of telomerase activity and telomere length in telomerase positive cells, without affecting hTERT mRNA or hTR levels. Thus, a new mode of
telomerase regulation was introduced, with MKRN1 serving as a negative regulator of the catalytic component for telomerase activity.

In humans, MKRN1 is ubiquitously expressed in normal fetal and adult tissues, and well conserved orthologs have been found in plants, invertebrates, and vertebrates; suggesting an ancient origin and importance for this gene (34); however, little is known of its expression and activity in telomerase positive cells. It has also been shown to be an E3 ubiquitin ligase for p53 and p21(35). MKRN1 was shown to affect RNA polymerase II dependent transcription both positively and negatively (36). Here, we show that MKRN1 is expressed differently in telomerase positive human cancer cell lines and the normal diploid fibroblast cell line, WI-38. One cell line in particular, the HL-60 acute myeloblastic leukemia cells, had no detectable MKRN1 protein and very low mRNA levels. However, upon All-Trans Retinoic acid induced differentiation of these cells, MKRN1 expression is strongly activated leading to a concurrent decrease in telomerase activity. Differentiation of HL-60 cells has been established as a tractable model system for telomerase regulation during cellular differentiation.(23,37,38) It has been shown that hTERT gene transcription is shut off, and recently, post-translational modifications of hTERT have been suggested as a mechanism of down regulation(39); however, mechanistic details are unknown at this time. Our observations suggest that induction of MKRN1 expression contributes to the effective termination in telomerase activity and ensures effective removal of telomerase activity during cellular differentiation.

The specific objectives within this thesis were to : 1) characterize the levels of MKRN1 in different cancer cell lines and the normal diploid fibroblasts, WI-38, to test the hypothesis that
the expression of MKRN1 would be counter to that of hTERT and telomerase activity; 2) determine if the expression of MKRN1 is induced in terminally differentiating HL-60 cells, which are known to have telomerase activity and hTERT expression turned off.

Makorin1

The Makorin RING finger protein-1 gene is the founder of a family of 8 intronless mammalian genes that encode a new class of zinc finger (ZnF) proteins. This gene can be found in fungi, plants, C. elegans, fruit fly, chicken, mouse, humans and even in the poxviral genomes (34). For this, it has been deemed ancient and lauded as functionally important for development. The gene is highly transcribed in both humans and mice, and is found on chromosome 7q34 and 6A respectively. In humans, at the mRNA level it is found uniformly in the heart, brain, placenta, lung, liver, kidney, skeletal muscle, in both adult and fetal tissues. In mice its expression is heterogeneous with higher levels in the testis and brain, and two splice variants are present, a 2.0 kb and a 3.2 kb version, compared to just one ~3 kb form in humans. The 3.2 kb transcript is the predominant form in the brain, while the shorter form is expressed more in the embryo (34). The human cDNA was found to be 3026 bp with an open reading frame from +121 to +1567, entailing 8 exons. The first exon contains a CpG island, which also encompasses the first intron and the proximal promoter region. Towards the upstream region of the proximal promoter beginning at -630 bp are found a series of Alu repeats, implying that the functional components of said promoter are found between the -630 and +1 bp region (34)(Fig1). In both humans and mice pseudogenes have arisen, totaling 7 in humans. The hMKRN1 resembles the longer murine MKRN1 in both size and sites for expression.
The protein product of MKRN1 possesses a plethora of zinc fingers that are highly conserved, four of which are C$_3$H ZnF’s, with two of these found proximal to the N-terminus, another medial, and one more proximal to the C-terminus, aft of the C3HC4 RING (Really Interesting New Gene) zinc finger domain. There is also a unique Cys-His motif consisting of C$_2$H$_2$CH, in the central region just before the RING domain, which may be another class of Zinc Finger (ZnF). The human protein is 482 amino acids, weighing in at 53.3 kDa with an isolectric point of 4.95 (34)(Fig2). It has been shown in two separate yeast-two-hybrid screens to interact with hTERT, the catalytic portion of telomerase, and OIP2, a component of an exosome complex that is involved in degrading mRNA(40). While no role has been determined for its interaction with OIP2, MkRN1 has been shown to be a RING finger E3 ubiquitin ligase for hTERT, targeting it for ubiquitin/26s proteasomal degradation(33). hTERT levels and activity were shown to decrease in the human lung carcinoma cell line H1299 and the human fibrosarcoma cell line HT1080; and in the latter case the RNA levels for hTERT and hTR were unaffected. Furthermore, in the telomerase (-) human osteosarcoma cell line Saos-2, hTERT transfected in was degraded when co-transfected with MKRN1. Makorin 1 levels, it seems, are regulated by auto-ubiquitination as well, which concurs with what is known of other RING finger proteins(41). A mutant of MKRN1, where His 307 was changed to Glu, resulted in a non-functional protein(33). MKRN1 has been shown to also interact and function as an E3 ubiquitin ligase for p53 and p21.(35) In the case of p53 it only ubiquitinates p53 and mediates its degradation under normal conditions. Once p53 is triggered to a damage response like DNA damage, MKRN1 is unable to ubiquitinate p53. In contrast, p21 is regulated by MKRN1 by ubiquitination regardless of the cell state. MKRN1 has also shown to have an effect, both negative and positive, on RNA
polymerase II dependent transcription.(36). So, MKRN1 may be serving diverse roles in the cell.

**RING Finger Proteins**

RING finger proteins are a class of Ubiquitin E3 ligases that are capable of facilitating the transfer of ubiquitin to its substrate without covalently bonding to it, and auto-ubiquitination(41). There are two types depending on the residue in the fifth position of the zinc coordinating motif, RING-HC contain a Cys and RING-H2 have a His(41). The RING finger domain in MKRN1 is of the RING-HC variety. RING finger proteins are involved in regulating numerous cellular processes such as, the cell cycle, transcription, apoptosis, DNA repair, and signaling(41). Mutations in or irregular regulation of these proteins has implicated them in cancers(42,43). There have been mutations in the RING finger of BRCA1 that link it to familial breast and ovarian cancer(44). RING finger proteins are important for cellular functions and any misregulation can lead to a disease state(41,42).

**Ubiquitination and the 26s Proteasome**

Ubiquitin is a highly expressed and well conserved protein found in all eukaryotes that plays a role in many cellular processes by post-translationally modifying proteins involved in cell cycle progression, signal transduction, DNA repair, apoptosis, and transcriptional regulation. This 76 amino acid polypeptide covalently binds to the ε-amino group of a lysine (K) residue on protein substrates(44,45). There are different forms of ubiquitination; mono-,di-, multi-, and poly-ubiquitination. Poly-ubiquitination comes in two varieties, depending on how ubiquitins connect to each other. If the ubiquitin polymerize at K48 then it will signal for proteasome
degradation, but if K63 is used then a different signal is conveyed, such as relocalization (45).

The process of ubiquitinating a protein is carried out by a series of enzymes, denoted E1 (activating enzyme), E2 (conjugating enzyme) and E3 (ligase). The E1 enzyme activates an ubiquitin protein by forming a thiol-ester bond between a Cys residue and G76 on ubiquitin in ATP dependent fashion, prepping it for nucleophilic attack by a Cys residue in the E2 enzyme, forming another thiol-ester bond. Finally, E3 ligases recognize the substrates and mediate the transfer of ubiquitin from E2 to the substrate. For substrates with ubiquitin polymers of the K48 type, localization to the 26s proteasome follows (44,45).

The 26s proteasome consists of a proteolytic core made up of 4 alternating α and β heptameric rings in a barrel configuration responsible for degrading proteins in an ATP dependent fashion, and one or two 19s multisubunit complexes that binds to the ends of the proteasome and that recognize, de-ubiquitinate and unfold target proteins. This entire process is important for cellular homeostasis and irregularities in it have been implicated in cancers and hereditary diseases (45).

**Telomere and Telomerase biology**

Telomeres are unique heterochromatin found at the ends of chromosomes consisting of the sequence TTAGGG repeated one or two thousand times in humans, with this G-rich strand over-extending its partner strand (2). These telomeres are able to protect chromosomes in two ways. One being, the vast stretch of non-coding DNA serves as a buffering zone warding coding DNA from the end-replication problem arising from the faulty DNA duplication of the lagging strand. However, in telomeraseless somatic cells, this barrier lasts for only 60-70 divisions as approximately 50-100 bp are lost from the telomeres with each cell division (2,46). Once
telomeres are gone, it is thought that apoptosis and cell arrest take place, leading to the stoppage of cell growth or cellular senescence (2,46,47). This process is known as M2 (mortality stage 2) or crisis, and comes about when the shortened telomeres are no longer able to protect vital genes from chromosomal degradation or are subject to end-to-end joining with other chromosomal ends or double strand breaks(2,46). This leads to the second protective function of telomeres, end-capping. The repetitive nature of the telomere enables the single-stranded 3’ G-rich overhang to fold backward, displacing upstream regions and hybridizing with the opposite strand, forming a D-loop and T-loop respectively. There are various proteins that participate in end-capping, such as TRF1, involved in telomere coiling; TRF2, stabilizes T-loop; TIN2, promotes TRF1 function; Rap1, unknown activity in humans; TPP1, recruits POT1 to telomeres; and POT1, which binds to and protects single-stranded telomeres at the D-loop(2,3,46). Together these proteins have been dubbed, as a protein complex, Shelterin(3).

The telomere binding protein that has been the main focus of telomere biology is the ribonucleoprotein enzyme, telomerase. It consists of RNA, hTR, and a catalytic protein subunit, hTERT, and is a reverse transcriptase. It is able to maintain telomere length, not extend it, by adding bases using the hTR as a template(46). Telomerase is not present or active in somatic cells, but is, in stem cells, germ cells and transformed somatic cells. This provides 85-90% of tumors with the ability to forgo cellular senescence and grow indefinitely(11,46). The ability of telomerase to maintain chromosomal ends seems not to be its only role in tumorigenesis. It has been implicated in human mammary epithelial cells to directly induce growth-promoting genes(48). As a client protein of Hsp90 its stability is susceptible to geladanmycin which inhibits Hsp90 from binding proteins(32,49). This has provided an avenue of treatment against
Telomerase (+) cells. It has been found recently that hTERT is regulated by degradation via the 26s proteasome (33).

Telomerase activity is a highly regulated and important for cellular differentiation. During the development of blood cells, hematopoiesis, telomerase activity is expressed basally in primitive stem cells (50). When these cells are required to differentiate into different lineages of progenitor cells telomerase activity is ramped up to accommodate the increase in cellular proliferation to generate large pools of progenitor cells. Afterward, when these progenitor cells are signaled to terminally differentiate into mature cells telomerase activity is down-regulated to barely detectable levels or shut off completely (50). Since, telomerase possesses an enzymatic activity that could prove deleterious if left on.

**HL-60 cells as a model for tracking telomerase regulation in cellular differentiation**

The HL-60 cell line was derived from a single patient with acute promyelocytic leukemia (51). It has proved a useful tool as an in vitro model system for studying the cellular and molecular processes involved in differentiation and proliferation of both normal and leukemic cells of the monocyte and granulocyte lineage. These cells are blocked at an immature stage of differentiation with the potential to proliferate indefinitely (51,52). However, these cells can be differentiated with the treatment of certain chemicals to mature terminal cells. All-Trans Retinoic acid (ATRA) and dimethyl sulfoxide (DMSO) can induce these cells to differentiate to granulocytic neutrophil cells where upon they cease proliferating and eventually enter apoptosis. (51) Treatment with Vitamin D$_3$ and Sodium Butyrate will differentiate to monocytes, however there is little loss of proliferation for these cells (51). So, as a differentiation therapy, ATRA
factors as a better candidate. However, this form of therapy has not proven to be definite as relapses are resistant to further treatment. (53)

When HL-60 cells are treated with ATRA certain characteristic changes occur. They loose their round smooth appearance and begin to form aggregates, with these aggregates increasing in size and number as more cells differentiate.(37) Another characteristic change is a cessation in cellular proliferation as cells first enter in G\textsubscript{1} arrest, and finally apoptose. One marker for differentiation, cell differentiation maker 11b (CD11b) increases with exposure to ATRA. These events are not synchronous, with the cells first entering G\textsubscript{1} and proliferation ceasing, then CD11b expression is up-regulated, and aggregation occurring with increased CD11b expression.(37)

This model system has been very tractable in following telomerase regulation.(23,37,38) With ATRA treatment hTERT transcription is shut off early on, while telomerase activity is still present from newly translated transcripts that are still being made and by stable and active left over hTERT protein as transcripts disappear. Many suggest that the down-regulation and epigenetic silencing of hTERT transcription is solely responsible for this inhibition of telomerase activity(18,23,37). However, not much has been done to look at the regulation of the hTERT protein in this model. One study points to phosphorylation and a decrease of hTERT protein, but no clear mechanistic link has been made(39).
CHAPTER TWO: MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human cervical carcinoma cell line HEla was cultured in RPMI-1640 medium; the human diploid fibroblast cell line WI-38 in Minimum Essential Media; and the human colorectal carcinoma cell line HCT116, human embryonal kidney cell lines HEK293 and HEK293FT were cultured in Dulbecco’s modified Eagles medium supplemented with 10% fetal bovine serum (FBS), 100units/mL penicillin, and 100ug/mL streptomycin in 5% CO2 at 37 ºC. The human promyelocytic leukemia cell line HL-60 were acquired from ATCC (American Type Culture Collection, Manassas, VA) and cultured in suspension at 250,000 cells/mL with Iscove’s modified Dulbecco’s medium supplemented with 20% FBS, 100units/mL penicillin and 100ug/mL streptomycin in 5% CO2 at 37 ºC. To induce differentiation, HL-60 cells were seeded at 500,000 cells/mL and 10uM All-Trans Retinoic Acid (ATRA), purchased from Sigma (Sigma Chemical Company, St. Louis, MO).

Analysis of Proliferation

For the growth curve studies, HL-60 cells were counted using Trypan blue dye exclusion on a Neubauer Hemacytometer. Morphological changes of HL-60 cells were tracked using a Nikon Eclipse TE200 inverted microscope.

Fluorescence-Activated Cell Sorting (FACS)

Differentiation of HL-60 cells was determined by using Fluorescence-Activated Cell sorting (FACs) analysis of a differentiation marker for HL-60 cells, CD11b. Briefly, 1 x 10^6
cells were washed three times with ice cold wash buffer (PBS containing 1% BSA) at 300 g’s and 4ºC, and incubated with .05ug of Fluorescein Isothiocyanate (FITC) conjugated anti-CD11b monoclonal antibody (BD Biosciences, San Jose, CA) at 4ºC for 30 minutes in the dark. Then, cells were washed in wash buffer (1% BSA in 1 X PBS) at 300 g’s at 4ºC for 5 minutes, and analyzed immediately using a Becton Dickinson FACScalibur flow cytometer(Becton Dickinson, Franklin, NJ) and WinMDI 2.8 software (Joseph Trotter, Flow Cytometry Core Facility, Scripps Research Institute, http://facs.scripps.edu/software.html). For cell cycle analysis HL60 cells were harvested from 0-6 days, washed three times in 1mL of 1 X PBS, re-suspended in 1mL of PBS and added slowly to 9 mL of 70% ethanol at 4ºC to be fixed. Fixed cells were then treated with RNase and stained with propidium iodide at 20ºC for 30 minutes; and then analyzed using a Becton Dickinson FACSCalibur flow cytometer and WinMDI software.

**RNA Extraction**

Total RNA was extracted from the cell using TRIZOL reagent (Invitrogen.). Cells were centrifuged at 2000 r.p.m. for 2 minutes, resuspended in 1mL of TRIZOL reagent, and incubated at room temperature for 5 minutes. Then, Chloroform was added and shaken by hand for 15 seconds, and incubated for 3 minutes at room temperature. Next, the lysate was spun down at 12000 g’s for 10 minutes at 4ºC and the upper aqueous phase was saved. To the aqueous phase was added 500mL of isopropanol, then incubated at room temperature for 10 minutes, and spun down at 12000 g’s for 10 minutes at 4ºC. The pellet containing the RNA was washed in 75% ethanol, by vortexing and centrifuging at 7500 g’s for 5 minutes at 4ºC. Finally, the washed pellet was resuspended in 30ul of RNase free water and stored at -80ºC, until needed.
**Reverse Transcription and PCR Amplification**

Reverse transcription of, 2ug of RNA extracted as described above, was carried out using M-MLV Reverse Transcriptase and random primers (Promega,USA) for first-strand cDNA synthesis. The RNA(4ul) was mixed with 1ul of the random primers and 10ul of RNase free water, incubated at 70 °C for 5 minutes, and then put on ice. Next, 2ul of 5x M-MLV reaction buffer, 1.5ul of 10uM dNTPS, 1ul of RNase Inhibitor, 1ul M-MLV reverse transcriptase and 4.5ul of RNase free water were mixed together and added to the RNA/primer mixture. These were then incubated at 37 °C for 1 hour. Afterwards, the newly synthesized cDNA was stored at -20°C until needed. One microliter of the cDNA was then used as the template for PCR amplification using Accuprime Pfx polymerase (Invitrogen, USA). The products were visualized by a 1% agarose gel electrophoresis and staining of the gels with ethidium bromide. The images were captured using Syngene’s (Cambridge, UK) ChemiGenius Bio Imaging System and digitized using their GeneTools program. PCR was performed using the primers listed on Table 1. The conditions for PCR of each cDNA are listed in Table 2.

**Western Blots**

Whole Cell extracts were prepared by re-suspending cells in lysis buffer (1X PBS, pH7.5, 1% NP-40, .5% deoxycholate, .1% SDS, 10% glycerol and 1X protease inhibitor cocktail (Roche). Following incubation on ice for 20 minutes, lysates were centrifuged at 13000 rpm for 20 minutes at 4°C. The protein concentration was measured using the Bio-RAD Protein Assay (Bio-RAD, California) and a Tecan GENios 96-well microplate reader; using a BSA (1mg/ml) Standard curve. Then, the whole cell lysates were frozen at -80°C until needed. Samples were subjected to 10% SDS polyacrylamide gel electrophoresis, and proteins were transferred to a
nitrocellulose membrane. Anti-MKRN1 rabbit polyclonal antibody was prepared using a commercial antibody production service (Harlan Laboratories) and was raised against MKRN1 polypeptide. β-ACTIN antibody (Sigma) was used as a loading control. Immunoreactive bands were visualized using SuperSignal West Dura (Thermo Scientific), captured using Syngene’s (Cambridge, UK) ChemiGenius Bio Imaging System and digitized using their GeneTools program.

Telomeric Repeat Amplification Protocol Assay (TRAP)

For TRAP assays, cells were harvested and lysed in CHAPS lysis buffer (10mM Tris-HCl, pH 7.5, 1mM MgCl2, 1mM EGTA, 0.5% CHAPS, 10% glycerol, 0.1mM PMSF, and 5mM β-mercaptoethanol). After incubating on ice for 30 minutes, lysates were centrifuged at 13,000 rpm, 4°C for 20 minutes. CHAPS cell extracts (1ug) were added to telomerase extension reactions, consisting of 10 X TRAP reaction buffer (200mM TRIS-HCl,pH 8.3; 15mM MgCl2; 620mM KCl; 0.05% Tween-20; and 10mM EGTA), 10mM dNTPS, and 10uM HTS primers; and then incubated for 30 minutes at 27°C. PCR amplification of TRAP reaction products (5ul) was performed using: 10uM ACX and TS primers, 50mM MgCl2, 10mM dNTPS, and 2.5 units of Taq polymerase for 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C, and extension at 72°C for 30 seconds. 10uM NT and 1amol of TSNT primers were added to the PCR reactions to generate an internal telomerase assay standard (ITAS). Primer sequences for the TRAP assay are listed in Table 3. PCR products were resolved using a non-denaturing polyacrylamide gel consisting of 12.4% acrylamide/bis-acrylamide (29:1), .5 X TBE, APS and TEMED. The gel was run for 80 minutes at 120 V and then visualized by staining with SYBR
Gold (Molecular Probes, USA) using the ChemiGenius Bio-Imaging system with the Epi short wave UV light and Short wave band filter. The signal intensity was quantified with GeneTools.
CHAPTER THREE: RESULTS

Differential expression of MKRN1 mRNA and protein in cancer cell lines and WI-38 cells

Telomerase activity is correlated with 85-90% of all cancer cells. Since MKRN1 is a negative regulator of hTERT, we reasoned that MKRN1 expression may be counter to hTERT in model cancer cell lines. Using GAPDH normalized cDNAs and semiquantitative RT-PCR, we assessed MKRN1 mRNA levels in different human cancer cell lines and the normal diploid fibroblast cell line, WI-38 (Fig. 5). We were able to detect MKRN1 mRNA readily in all the cell lines, although HL-60 and Hela cell lines showed the lowest overall expression patterns relative to GAPDH. To verify that the changes in MKRN1 mRNA was not due to poor integrity of the RNA extracts used, we checked the RNA extracts on a 1.5% agarose gel and stained with ethidium bromide. The integrity of the 18s and 26s ribosomal RNA was of good quality (Fig. 4). We also analyzed MKRN1 protein expression using whole-cell lysates (normalized to β-Actin) and a MKRN1 polyclonal antibody. The data show that MKRN1 protein levels were highest in WI-38, HEK 293, Hek 293FT and HCT116 cells; low but detectable in in Hela, and undetectable in HL-60 (Fig. 6a). Interestingly, upon serum starvation of WI-38 cells for 48 hours, MKRN1 protein levels were 6-FOLD higher relative to exponentially growing (serum fed) WI-38 cells (Fig. 6b). This last observation shows that MKRN1 protein levels are higher in resting cells. This result also suggests that MKRN1 expression may be related to differentiation status. This was tested next.
Differentiation of HL-60 cells with ATRA as a model for MKRN1 influence on telomerase

HL-60 cells terminally differentiate into neutrophils after treatment with retinoic acid derivatives, such as ATRA. A number of alterations in morphology, gene expression and growth characteristics are known to occur (37). Our analysis of MKRN1 expression focused on differentiation events in the first 6 days following ATRA exposure. The number of live cells increased continuously in control cells, whereas in ATRA treated cultures cell growth was inhibited within 1 day. Cell proliferation remained flat over the next 6 days in the ATRA treated cultures (Fig. 8) and during this interval we detected increases in the fraction of dead cells, as the population terminally differentiated into neutrophils. We also observed that after 2 days of ATRA treatment HL-60 cells lost their smooth round appearance and formed clusters that grew in size and number with each subsequent day; such changes in morphology were not seen in the control cells (Fig. 7). The cell differentiation marker, CD11b, was expressed in approximately 7% of ATRA-treated cells at day 1 and 50% by day 6 (Fig. 9). Finally, we looked at the cell cycle and found that approximately 80% of the cells were in G1 after 3 days of ATRA treatment (Fig. 10). This last coincides with the above cessation of cell proliferation results. These data are similar to published results on cytodifferentiation of this promyelocytic leukemia cell line.

MKRN1 mRNA and protein levels in differentiating HL-60 cells

It is well documented that hTERT expression and telomerase activity are down-regulated upon ATRA-mediated differentiation of HL-60 cells and that telomerase activity is quite high in undifferentiated HL-60 cells (14,37,38). Since, MKRN1 has been shown to negatively regulate hTERT and telomerase activity and is elevated in resting cells (Fig. 6a), we predicted that differentiation of HL-60 cells would be attended by an increase in MKRN1 expression. We
compared MKRN1 and hTERT mRNA levels in differentiating HL-60 cells by RT-PCR and observed a slight increase in MKRN1 transcripts within 24 hr post ATRA treatment (Fig. 12). During this period, hTERT mRNA levels were significantly reduced and by day 3-4 completely absent (Fig. 12). To verify that the decrease in hTERT mRNA was not due to poor integrity of the RNA extracts used, we checked the RNA extracts on a 1.5% agarose gel and stained with ethidium bromide. The integrity of the 18s and 26s ribosomal RNA were of good quality (Fig. 11). In contrast to the sudden decrease in hTERT mRNA, the decrease in telomerase activity appears to be more gradual, dropping sharply after days 4-5 and barely detectable by day 6 (Fig. 14). These results indicate that hTERT protein is still stable and that telomerase activity persists as hTERT transcript levels display a precipitous decay. Under the same conditions of HL-60 differentiation, MKRN1 polypeptide levels are increasing with peak values around days 4-5, a time when telomerase activity is sharply decreased (Fig. 13).

To further investigate these findings, we examined changes in MKRN1 levels during short-term treatment of HL-60 cells with ATRA. MKRN1 mRNA levels did not change significantly in the first 12 hours and then doubled by 24 hours (Fig. 16). MKRN1 polypeptide levels were first detected at 24 hours (Fig. 17).

**Effects of MG132 on telomerase activity in differentiating HL-60 cells**

Previously, in H1299 cells, it was shown that the HSP90 inhibitor, GA, could destabilize telomerase, causing hTERT protein to be susceptible to ubiquitin-mediated proteasomal degradation and a correlative decrease in TRAP activity(33). This degradation could be abrogated by the proteasome inhibitors, ALLN or MG132. In the same studies, ectopically
expressed MKRN1 induced ubiquitylation of hTERT and MKRN1 stable over expression in HT1080 cells caused a loss of TRAP activity and telomere length, even in the absence of GA. Since MKRN1 polypeptide levels are up-regulated in differentiating HL-60 cells, we examined the effects of MG132 treatment on telomerase activity in control and differentiated cells. There was a 20% recovery of TRAP activity when day 3 differentiated HL-60 cells were treated with MG132 alone; indicative that a loss in TRAP activity is not only due to the depletion of hTERT transcripts, but also to the loss of functional telomerase enzymes through proteasomal degradation of hTERT (Fig. 15). These observations bolster the aforementioned model for telomerase regulation, in which MKRN1 destabilizes telomerase via ubiquitin-mediated proteolysis of hTERT, and place it in the context of a biological event, the granulocytic differentiation of HL-60 cells.
CHAPTER FOUR: DISCUSSION

In previous studies from this lab, MKRN1 was shown to induce the ubiquitin-mediated proteolysis of hTERT (33). In this study the following can be drawn up. Under physiological conditions hTERT interacts with HSP90 and moves into the nucleus to associate with hTR forming telomerase; and maintaining the telomeres. When GA is added to cells the association between hTERT and HSP90 is abrogated and hTERT ubiquitination is induced and it is localized to the 26s proteasome for degradation. Also, in the absence of GA, ectopic MKRN1 is able to mediate ubiquitination of hTERT mediating its proteasomal degradation (Fig. 3). To investigate this model of telomerase regulation under physiological conditions, we looked at the ATRA-mediated differentiation of HL-60 cells, in which much work has been done focusing on the inhibition of telomerase activity. In differentiated HL-60 cells hTR does not seem to be factor in the decrease in telomerase activity (54). The rate-limiting component appears to be hTERT. There is much evidence that links hTERT gene expression to telomerase activity in cellular differentiation (23,37,50,55,56). However, little work has been done looking at the regulation and turnover of hTERT at the protein level, and how this contributes to the inhibition of telomerase activity. Recently, studies point to post-translational modifications, such as phosphorylation, and a decrease in hTERT protein, as contributing (39,56). We report that MKRN1 expression is significantly activated during terminal differentiation of HL-60 cells, which can be detected as early as day 1 for MKRN1 protein and 12 hours for MKRN1 mRNA post ATRA treatment. The activity of MKRN1 on hTERT may be a necessary part in regulating telomerase activity in somatic cell differentiation based on these data. First, MKRN1 expression, both mRNA and protein, are up-regulated early on in differentiation as decreases in telomerase
activity are detected (Fig. 16, Fig.17). Even though hTERT transcription is shutoff early on, there is still telomerase activity present, either from de novo translation as transcription is being turned off, or from leftover stable and active hTERT protein. We also note that recent studies show hTERT protein disappearing after day 3 of ATRA treatment of HL-60 cells (39), when MKRN1 levels peak (Fig.13). Furthermore, with MG132 treatment, the recovery in telomerase activity is indicative that the inhibition of telomerase activity seen is not only from the exhaustion of hTERT transcripts, but also the ubiquitin-mediated degradation of hTERT protein. The up-regulation of MKRN1 by ATRA may play an important role in the cytodifferentiation of HL-60 cells; notably, the down-regulation of hTERT protein. Cells may have evolved this mechanism to prevent an enzymatic activity from performing a function, telomere elongation, that is no longer necessary in terminally differentiated cells. MKRN1 function would serve, then, to prevent irreversible extension of telomeres.

Looking at the data from the MKRN1 protein levels from the different cell lines suggests that there is an opposite relationship between MKRN1 and hTERT. Diploid fibroblasts have been reported to contain very low telomerase activity and hTERT protein (57). So, it’s of interest to find MKRN1, an E3 ubiquitin ligase for hTERT, in early passage WI-38 cells. This supports the findings of an active telomerase in normal diploid fibroblasts. MKRN1 is a protein that is up-regulated in cells that are undergoing G1 arrest or quiescence as evidenced here in serum starved resting WI-38 cells (Fig 6) and in G1 arrested differentiated HL-60 cells (compare Fig. 13 and Fig.10). This is expected considering that it targets hTERT, a protein necessary for active cellular proliferation. In immature, hematopoietic stem cells telomerase activity is expressed at basal levels, where upon activation of these cells to proliferate as progenitor cells, telomerase
activity is up-regulated. Once the signal to differentiate to mature cells is given telomerase is
down-regulated (50). The HL-60 cells represent a type of progenitor cell that is blocked in the
proliferative stage. But it can become terminally differentiated to a mature neutrophil with
ATRA. Recently, MKRN1 has been shown to be an E3 ubiquitin ligase for p53 and p21 (35),
both proteins important for proper cell cycle regulation and cellular development. The study
found that when U2OS cells were depleted for MKRN1, cell death decreased. Presumably, this
occurs by MKRN1 inhibiting the role of p21 in inhibiting apoptosis. HL-60 cells are p53 Null,
but p21 is expressed (58). Moreover, p21 levels are up-regulated upon ATRA-induced
granulocytic differentiation of HL-60 cells (59) Constitutively active, p21 can maintain cells in a
permanent cellular senescence (60). However, neutrophils are short-lived, lasting a few hours to
a few days. Although, p21 is present to maintain the cells in G1, MKRN1 is also up-regulated
and may be acting on p21 as well to help induce apoptosis, by ubiquitinating p21 and mediating
its proteasomal degradation. So, MKRN1 maybe involved in more than one role in the cellular
differentiation of HL-60 cells, given that MKRN1 is a well conserved gene emphasizing a role in
cell growth processes.

In summary, we provide an additional mechanism of regulating telomerase activity,
whereby MKRN1 promotes the ubiquitin-mediated proteolysis of hTERT to ensure the full
inhibition of telomerase activity during differentiation. In this scenario, in undifferentiated and
cycling HL-60 cells, hTERT is not degraded and is able to maintain the telomeres; however, with
differentiation therapy (ATRA) hTERT is ubiquitinated by MKRN1 localizing it to the 26s
proteasome for degradation(Fig.18). Importantly, we think MKRN1 expression could be used as
a marker indicative as to whether strategies targeting the stability and degradation of
hTERT/telomerase, such as GA or differentiation therapy, would be successful for the treatment of certain cancers. Differentiation therapy has been used with APL, however, there is an incidence of resistance to the treatment (53). Perhaps treatment with GA can provide another avenue either alone, or in combination with differentiation therapy, of treating leukemias, given that hTERT is necessary for proliferation and highly sensitive to degradation with GA treatment (33).
Figure 1 The structure of the MKRN1 gene
The MKRN1 gene is comprised of 8 exons, 7 introns and contains a CpG island that runs from the proximal promoter region and through the first exon and into the first intron.
Figure 2 The structure of the MKRN1 protein
Figure 2

The MKRN1 protein consists of 4 C3H zinc fingers, a RING zinc finger domain which possesses the E3 ligase activity, and the MKRN1 Family Cys-His domain which may be a novel type of zinc finger.
Figure 3.

hTERT interacts with HSP90 which stabilizes hTERT and allows it to associate with hTR in order to carry out telomere maintenance (Yellow arrows). When Geldenamycin, an HSP90 inhibitor, or MKRN1 are added hTERT ubiquitination is induced which localizes to the 26s proteasome to be degraded (Red arrows).
Figure 4  Electrophoresis analysis of total RNA displaying intact ribosomal RNA
Figure 4

Total RNA (1μg) was checked on an 1.5% agarose gel stained with Ethidium Bromide (.5μg/mL) for the cell lines indicated. The integrity of 18s and 28s ribosomes was shown to be intact, indicating that the RNA used for the cDNA synthesis and PCR was of good quality.
Figure 5. Differential expression of MKRN1 mRNA in tumor cell lines and WI-38 cells.
Figure 5

(A) Expression of MKRN1 mRNA in the indicated tumor cell lines and diploid fibroblast cell line WI-38. All cDNAs were normalized to GAPDH mRNA expression and analyzed using semi-quantitative RT-PCR. GAPDH was used as the internal control and relative MKRN1 expression levels to GAPDH are shown graphically (MKRN1/GAPDH). (B) The graphical values represent the mean and ± SD of three independent experiments.
Figure 6 Differential expression of MKRN1 protein in tumor cell lines and WI-38 cells.
Figure 6

(A) Whole cell lysates were normalized to β-ACTIN levels and MKRN1 polypeptide levels were determined using anti-MKRN1 polyclonal antibody. β-ACTIN was used as the internal control and the relative MKRN1 expression levels to β-ACTIN are shown graphically (MKRN1/ β-ACTIN). WI-38 cells were early cycling cells, WI-38(-) serum, are WI-38 cells fed only 0.2 % Serum for 2 days. (B) The graphical values are representative of three independent experiments.
Figure 7 Morphological changes to HL-60 cells treated with ATRA
Figure 7

ATRA affects the morphology and behavior of HL-60 cells. Cells were treated with 10uM ATRA and changes in the smooth round appearance and clumping of cells were observed as early as day 3 and increasing in prominence by day 6. Such morphology changes were not seen in the control cells.
Figure 8 Changes in Proliferation Rate of ATRA treated HL-60 cells
Figure 8

Cells were treated with 10uM ATRA for 6 days and the total number of live cells were counted daily (0-6 days). The cells were re-seeded every two days with fresh media and fresh ATRA.

Cells treated with ATRA stopped growing while control cells were unaffected and continued to proliferate. The values represent the mean and ±SD of three independent experiments.
Figure 9 CD11b expression in HL-60 cells treated with ATRA
Figure 9

CD11b expression, a marker of granulocytic differentiation, was measured after 10uM ATRA treatment of cells and incubated with a CD11b antibody conjugated to FITC. (A) HL-60 cells were incubated with 0.05ug of anti-CD11b conjugated to FITC. Control unstained and RA unstained were both used to set the gating threshold value (.3%). (B) The percentage values represent the proportion of CD11b-positive HL-60 cells as determined by Fluorescent Activated Cell Sorting (FACS). Con cd11b, untreated cells stained with FITC; con Unstained, untreated cells not stained with FITC; unstained, ATRA treated cells unstained with FITC. The values represent the mean and ±SD of three independent experiments.
Figure 10 Cell cycle distribution in HL-60 cells after induction by ATRA.
Figure 10

HL-60 cells were treated with 10uM ATRA from 0-6 days and incubated with Propidium Iodide to stain DNA. The DNA content was measured by FACS analysis. The percentage of cells in $G_1/G_0$ increased with induction of differentiation by ATRA. The values represent the mean and ±SD of three independent experiments.
Figure 11  Electrophoresis analysis of total RNA displaying intact ribosomal RNA
Figure 11

HL-60 cells were treated with 10uM ATRA for 0-4 days and the total RNA was extracted. Total RNA (1ug) was checked on a 1.5% agarose gel stained with Ethidium Bromide (.5ug/mL). The integrity of 18s and 28s ribosomes was shown to be intact, indicating that the RNA used for the cDNA synthesis and PCR was of good quality.
Figure 12  Expression of MKRN1 and hTERT mRNAs in HL-60 cells treated with ATRA
Figure 12

MKRN1 and hTERT mRNAs were detected using semi-quantitative Reverse Transcriptase to synthesize cDNA and then amplified using PCR from HL-60 cells treated with 10μM ATRA for 0-4 days. GAPDH was used as the internal control.
Figure 13 MKRN protein expression in HL60 cells treated with ATRA
Figure 13

(A) MKRN1 protein levels from cell extracts (30ug) were checked by Western blot using a rabbit polyclonal antibody (at a 1:2000 dilution) targeting MKRN1 from HL-60 cells treated with 10μM ATRA. B-ACTIN was used as an internal control. (B) The relative MKRN1 expression levels to β-ACTIN are shown graphically (MKRN1/ACTIN). The values represent the mean and ±SD of three independent experiments.
Figure 14 The telomerase activity of HL-60 cells treated with ATRA
Figure 14

HL-60 cells were treated with 10uM ATRA for 0-5 days, harvested and lysed with 1X CHAPS lysis buffer. The telomerase activity of these cells was measured using the TRAP assay.
Figure 15 The effect of MG132 on TRAP activity in HL-60 cells treated with ATRA.
Figure 15

HL-60 cells

Control cells were untreated HL-60 cells that were lysed in 1X CHAPS lysis buffer. RA3 were HL-60 cells that were treated 10uM ATRA for 3 days and lysed in 1X CHAPS lysis. RA3+MG132 were HL-60 cells treated for 3 days with 10uM ATRA and then co-treated with 10uM MG132, a proteasome inhibitor, for 8 hours. The telomerase activity of these cells were then measure using the TRAP assay.
Figure 16 MKRN1 and hTERT mRNA level during early differentiation in HL-60 cells
Figure 16

(A) MKRN1 and hTERT mRNA levels were checked using semi-quantitative Reverse Transcriptase and PCR in HL-60 cells treated with 10uM ATRA from 0-24 hours. GAPDH was used as the internal control. (B) The relative MKRN1 expression levels to GAPDH are shown graphically (MKRN1/GAPDH). The values represent the mean and ±SD of three independent experiments.
Figure 17 MKRN1 protein expression levels during the early differentiation of HL-60 cells
Figure 17

HL-60 cells were treated with 10uM ATRA for 0-24 hours. To see if MKRN1 protein was present at these early time points, a higher amount of protein (60ug) was used on a 10% SDS-PAGE gel. MKRN1 protein levels were checked using a Rabbit-polyclonal antibody targeting MKRN1 at a dilution of 1:500 by Western blot. β-ACTIN was used as the internal control.
Figure 18 MKRN1 mediated proteolysis of hTERT during HL-60 differentiation
Figure 18. A schematic of the mechanism for MKRN1-mediated proteolysis of hTERT during ATRA induced differentiation of HL-60 cells. Under cellular proliferation no MKRN1 is present so hTERT is able to form a stable telomerase enzyme and maintain the telomeres. When HL-60 cells are treated with ATRA, MKRN1 is up-regulated and is able to ubiquitinate hTERT, localizing it to the 26s proteasome for degradation.
Table 1 List of primers used for PCR

Oligo(dT) Primers were ordered through Integrated DNA Technology

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Table 2 The conditions used for PCR

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Table 3 Dilutions of antibodies used for Western Blots

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Table 4 Primers used for TRAP assay

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REFERENCES


