QUALIFICATION OF TELOMERIC MAINTANANCE AND ELONGATION DUE TO PULSED ELECTROMAGNETIC RESONANCE EXPOSURE

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Cell and Molecular Biology

By

Scott Charles Kelsey

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ABSTRACT

Electromagnetic fields produced by electromagnetic resonance are a natural byproduct of electricity moving through a circuit. The effects that these electromagnetic fields have on biological tissue are poorly understood at the molecular level. Electronic devices that emit low to high level electromagnetic frequencies are ubiquitous to most buildings and with the invention of the cell phone electromagnetic resonance is virtually inescapable. Since the 1970's research on the effects of electromagnetic resonance on biological tissues has focused primarily on the detrimental effects and very little on the prospect of therapies. Electromagnetic fields are currently speculated to interact with molecules such as protein or DNA as phonon energy introducing subtle vibrations that can either disrupt or stabilize the conformation of the molecule. An oscillating circuit that creates an electromagnetic field with frequencies between 54 and 78 GHz was used to determine the effects of electromagnetic resonance on telomeric regions of DNA. Astrocytoma from human astrocyte tumors and cell cultures from *Mus musculus* primary cell lines received electromagnetic resonance exposure for 30 minutes daily. DNA from the exposed cell lines was isolated, subjected to either singlplex QPCR or the southern blot technique for telomere length analysis and compared against DNA from negative control cells of identical lineage to determine any telomeric response to electromagnetic resonance exposure.

Keywords: Electromagnetic Resonance, Telomere, QPCR, Southern Blot

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INTRODUCTION

Most mammalian somatic cells will divide and eventually enter a senescent state. However, germ line and somatic stem-cells retain their ability to divide almost perpetually due to their ability to maintain their telomeres (Shay 2005). Telomeres are repetitive sequence that occur at the ends of linear chromosomes and act as molecular caps to protect important regions of DNA. During replication a small portion of telomeric DNA is lost and when telomeres become heavily eroded the cell will enter senescence. The activation of a telomere maintenance enzyme, telomerase, in somatic cells in mice leads to an increase in organ upkeep and even the ability for cells to revert from senescence to an actively dividing state (Cox & Mason 2010). In addition, electromagnetic field stimulation of cell lines in culture stimulates cell signaling pathways that lead to an array of transcriptional activation (Natarajan 2006). If telomerase can be activated electrically within the cell it could lead to an increase in cellular upkeep without the administration of drugs

Mammalian cells undergo a complex process of DNA replication with each division that inadvertently results in loss of telomeric repeats at the ends of the DNA. During replication DNA is separated by a series of proteins called helicases. The point at which the base pairing of DNA occurs that is separated so that replication can occur is called the replication fork. At the replication fork RNA primers are attached to the leading strand, the 5’ to 3’ strand, and the lagging strand, the 3’ to 5’ strand. DNA polymerase, the main protein associated with DNA replication is only able to replicate DNA in one direction, the 5’ to 3’ direction. Therefore, DNA polymerase is able to replicate the leading strand with relative ease only necessitating re-priming the polymerase temporarily loses fidelity

and falls off. The lagging strand also uses DNA polymerase to replicate, however, since DNA polymerase is only functional in the 5' to 3' direction RNA primers are subsequently placed along the newly expanded replication fork creating Okazaki fragments. The Okazaki fragments are joined together by ligase proteins and when RNA primers are removed they are replaced with DNA by DNA repair proteins. When RNA primers are removed from the ends the DNA repair proteins are unable to attach to the newly synthesized strand and small portions of the telomeric ends are lost (Hornsby 2007).

The telomere consists of specific repetitive DNA sequence as well as a series of telomere specific binding proteins that protect the free DNA ends from degradation. The sequence of mammalian telomeres consists of hexo-nucleotide repeats of TTAGGG. These long repeats are bound by two main protein complexes telomere repeat-binding factors (TRF) 1 and 2. TRF 1 is primarily involved in telomere length control and assists in telomeric DNA secondary structure and TRF 2 offers telomeric protection from DNA repair proteins that might recognize a chromosomal free end as DNA damage or exonucleases that might try to degrade the chromosome. The secondary structure of telomeres involves a 3' overhang, created during telomere synthesis, and a single stranded DNA binding protein called POT1 that is also a part of the TRF 1 protein complex. POT 1 binds upstream on the 3' strand from the 3' overhang creating a small opening in the DNA base pairing allowing the 3' overhang to insert and match up with consensus telomere sequence. This structure allows the telomeric regions to be tightly bound so as not to expose free chromosomal ends or the 3' overhang to degradation (Armanios 2009).

Telomeric length in mammalian cells differs between species and it is estimated that telomere loss is between 50 and 200 base pairs (bp) per division (Cong and Shay 2008). Human telomeres vary in length between individuals between 5Kb and 10Kb and telomeres in *Mus musculus,* the common lab mouse, average over 20 Kb. If a cell and subsequent generations of cells were to divide 10 times that would lead to a net telomere loss of 500 to 2000 bp. Depending on the individual that can be the equivalent of 5% to 20% of the total telomeric sequence. Depending on the organism and initial telomere lengths once telomeres reach a critical length the cell will enter senescence, a stage in which cells cease dividing and enter a dormant state and will eventually go through apoptosis (Aubert and Lansdrop 2008).

In order to maintain chromosomal integrity and the ability to replicate and restore aged or damaged tissues cells incorporate the telomerase holoenzyme to extend telomeric regions. Telomerase consists of a catalytic enzyme bound to an RNA template classifying it as an RNA dependant DNA polymerase. Most cells express the *hTR* gene for the RNA template incorporated into the functional telomerase holoenzyme but mainly only germ line cells express the *hTERT* gene responsible for the catalytic portion of the holoenzyme. The RNA template embedded within the telomerase holoenzyme shares consensus sequence with telomeric repeats and therefore when expressed is able to bind the 3' end of the chromosome and elongate the strand using the RNA sequence within the holoenzyme as a primer for DNA synthesis. Once telomerase has elongated the 3' end of the chromosome RNA primers can be attached to elongate the complimentary strand utilizing DNA polymerase (Wai 2004).

When telomerase is not expressed and telomeric regions become eroded a cell will generally enter senescence, however, if a cell is to bypass the senescence checkpoint it can prove fatal to the cell. Once Telomeres have reached a critical length cell cycle checkpoints trigger the cell to enter a stage of senescence or halted growth. Oncogenes, when expressed, can cause a cell to bypass the senescence checkpoint allowing the cell to continue dividing and telomeres to continue to erode. Cells enter senescence when telomeres are still relatively long perhaps as a defense mechanism to allow cell cycle check points to stop the cell from dividing or to allow time for an immune meditated response. When telomeres become eroded to a point where they are virtually non-existent DNA alterations may occur. Depending on the circumstances, a cells natural DNA repair mechanisms may recognize the free ends of a chromosome as DNA damage and ligate one chromosome to another creating dicentric chromosome. Occasionally, during metaphase when sister chromatids are aligned at the equator of the cell, the free sticky ends of chromosomes will stick together creating dicentric chromosomes. When the chromatids are pulled apart during anaphase the chromosomes may break apart creating translocations and altered morphology of chromosomes that will allow improper pairing of sister chromatids in subsequent divisions and subsequent bridge fusion break cycles. If sister chromatids fuse during metaphase and are unable to separate then anaphase bridges occur that may persist causing internuclear chromatin bridges that link two daughter cells together (Weinberg 2007).

Cells that are able to bypass senescence and survive may develop alternative mechanisms for telomere elongation (ALT) and become immortalized. Copy choice ALT telomere elongation involves the DNA polymerase enzyme and a neighboring strand of DNA.

Telomeric sequence from a neighboring strand of replicating DNA may attach to a second strand of neighboring DNA and thus acts as the new template. The newly synthesized strand would contain any sequence downstream on the strand that was acting as the new template. This mechanism has been described to account for cells elongating telomeres in the absence of telomerase as well as the large number of translocations found in cancer cells. The more common of the two is the de-repressed expression of the *hTERT* gene. Most prevalent in cancer cells, when the *hTERT* gene is activated and allowed to be transcribed unhindered, telomere length will reach unnaturally long lengths, greater than 30 Kb (Blasco 2005). Although over expression of *hTERT* performed on healthy cell lines does not exhibit the same effect, cells that gain the ability to bypass senescence and de-repress *hTERT* generally become immortalized and can form neoplasms (Weinberg 2007).

The main role of telomerase in cells is to maintain telomeric length to ensure the progeny of the cell maintain genetic stability but telomerase is also able to restore senescent cells to an actively dividing state (Cox & Mason 2010). Mice that have a knock-out telomerase gene exhibit little phenotypical changes within their lifetime. Subsequent generations, by the 4^{th} generation almost across the board, exhibit phenotypes of aged mice even when they are still pups. The latency in response is most likely due to the unusually long telomeres of the common lab mouse. However, mice that express a knock-in telomerase gene, such as a telomerase gene inducible with 4-hydroxytamoxifen**,** even after expressing the aged phenotype as pups when telomerase is turned back on show an increase in organ upkeep and a return to a healthier physical state (Jaskelioff 2011).

Recent investigation has indicated that telomerase may have a second function aiding in the induction of mitochondrial mediated apoptosis. The hTERT protein of the telomerase holoenzyme responsible for catalyzing the polymerization of new telomeric DNA also has a 20 amino acid sequence at its N-terminus that targets the hTERT protein to mitochondrial DNA (mtDNA). This process has been shown to be ubiquitous to all cell types and is hypothesized to play a role in mitochondrial mediated cellular apoptosis. When telomerase is absent and telomeres erode to a point of senescence, cell cycle arrest occurs, and apoptosis proceeds in non-neoplastic cells but telomerase deficient cells that experience oxidative stress are less likely to engage in apoptosis. This indicates that telomerase plays a role in apoptosis that is specific to mitochondrial induced apoptosis (Cong & Shay 2008).

The hTERT protein is not only involved in telomere repair and mitochondrial induced cellular apoptosis but is also involved in the Wnt pathway of cell proliferation. The Wnt pathway is an important pathway in cellular proliferation of stem cells and has also been linked to several cancers in the epidermal, intestinal and hematopoietic systems (Frank 2004). The Wnt pathway involves the stimulation of the signal cascade by the Wnt ligand to frizzled receptor. Upon activation, frizzled activates deshevelled which in turn inactivates GSK-3β which is bound in complex with axin, APC and a phosphorylated β-catenin. Inactivation GSK-3β causes de-phosphorylation of β-catenin and a subsequent release of β-catenin from the complex. Once freed, β-catenin travels to the nucleus to act in conjunction with BRG1 and hTERT to act as transcription factors for several proliferative factors such as CyclinD-1 and c-myc as well as anti-apoptotic factors like Bcl-x (Park et al. 2009).

Although the Wnt pathway is traditionally thought of to be exclusively activated by the Wnt ligand, it has been demonstrated that the Wnt pathway can also be activated by subtle waveform energies such as low frequency electromagnetic resonance. Electromagnetic fields at 50 Hz stimulate Wnt pathways without addition of ligand and promote the up-regulation of occludin, β-catenin, and cadherin. Occludin and cadherin are products of the Wnt pathway that are involved with tight junctions and adherin molecules in cell to cell adhesion and communication. β-catenin as mentioned earlier is involved in cell cycle progression and proliferation (Somosy et al. 2004).

Waveform energies stem from a plethora of sources and have varying effects on cells. Perhaps the most understood waveform energy in regard to tissue interaction is ionizing radiation. Ionizing radiation occurs on the right side of the electromagnetic spectrum and begins at UV light (approximately 10^{16} Hz) and ends at the far right of the spectrum at gamma radiation. High waveform energies such as these are well known to cause damage not only at the level of tissues, burns for instance, but also at a molecular level such as crosslinking of DNA. The more subtle waveform energies occur primarily on the left side of the electromagnetic spectrum consisting of energies less than UV light (approximately 10^{16} Hz) and extend to the far left past long wave radio waves. The effects of subtle waveform energies are less understood than higher waveform energies as their effects are understated in comparison (Pidwirney 2006). Within the visible light spectrum waveform energies at 10^{15} Hz with wavelengths around 280 nm can activate cellular DNA repair mechanisms such as the photolyase protein PHR1 (Berrocal-Tito et al. 2007). Waveform energies as low as 50 Hz have been shown to activate cellular signal cascades in the absence of a ligand in cascades that typically begin with a ligand binding a receptor.

Higher energy frequencies such as those created by cell phones, 900MHz, can induce changes in glucose levels in the brain by yet to be discovered mechanisms (Lai and Hardell 2011).

Several subtle forces arise from electronic sources that interact with cells due to the intrinsic electrical nature of the cell. Electric fields (EF), the force surrounding electrically charged particles, magnetic fields (MF), a field created by the force of moving electrical charges, and electromagnetic fields (EMF), a combination of the static electric field and the motion of the magnetic field are forces able to act on the cell. Ion concentration differences between inside of the cell and the extra cellular space create low energy electric fields. The movement of electrical charges as ions travel through a membrane or diffuse through extracellular spaces induce low energy magnetic fields. The combination of the electric and magnetic fields yield an electromagnetic field that envelopes cells within a radius depending on the amount of energy generated by the ions involved in creating the fields. Depending on the ion concentrations around or action potential of a cell alternating fields arise generally in the extremely low EMF range (Caging et al. 2007). Exogenous extremely low EMFs (around 1GHz) from environmental or industrial sources are able to influence fields created by ions surrounding the cell and have effects on ion movement through membranes or within extracellular spaces. Extremely low EMFs are not only able to act on ion concentrations via electromagnetic forces but at varying frequencies, depending on the shape of the channel and amplitude of the frequency, can act as a gate to control the movement of ions through a channel (McLeod et al. 1992).

Forces created by extremely low EMFs may also interact with the subtle forces that dictate protein structure and conformational changes. It is well-known that energy can be measured as either a particle or a wave. When measured as a particle, higher order energies are described as a photon, a particle of energy that can interact directly with matter. More subtle energies are described as a phonon and behave as neither a particle nor wave but rather a vibration. Phonons are currently understood to be the subtle energies that interact with proteins on an atomic scale allowing them to maintain native confirmations and undergo confirmation changes in response to ligands or ATP hydrolysis. When ATP is hydrolyzed by a protein energy is released as phonons that vibrate down the amino acid backbone of a protein to either fortify or disrupt the subtle atomic energies such as hydrogen bonding, Van der Walls forces or ionic forces that hold proteins together (Pouthier 2010). Phonon energy vibrations have also been described as the driving force behind microtubule assembly and stability in the cytoskeleton (Prodan & Prodan 2009).

The circuits that make up the array of mechanical devices located within homes, cars, computers, etc. consist of an array of inductors, capacitors, and resistors to deliver the right amount of energy to the device and as a byproduct produce electromagnetic fields. Oscillating circuits, like the circuit in Figure 1, are circuits that exhibit currents and voltage that oscillate or vary in frequency. All telecommunication devices such as radios, televisions, and telephones utilize oscillating circuits. When an AC power is supplied to the circuit the current the capacitor discharges until the current is at a maximum within the circuit. The current will reach the inductor and the electrical energy from the current will be converted into magnetic energy within the inductor. Upon leaving the inductor the

magnetic energy is converted back into electrical energy and in this case would enter the resistor causing electrical decay. The remaining electrical energy would then leave the resistor and reach the opposite side of the capacitor causing an opposite charge on the capacitor. Once the capacitor becomes fully charged the current again travels out of the capacitor in a reverse motion. The electrical current travels through the resistor undergoing electrical decay again reducing the amount of energy in the system and is converted back into magnetic energy within the inductor leaving the inductor as electrical energy until it reaches and charges the conductor and leaves yet again in the opposite direction. This process continues until power ceases being supplied to the circuit and all residual power is dissipated by the resistor. The oscillating motion creates a sinusoidal pattern of energy due to the fluctuation of current between the capacitor and inductor. The sinusoidal pattern represents varying frequencies of a magnetic field created by the flow of electricity through the circuit (Knight 2002).

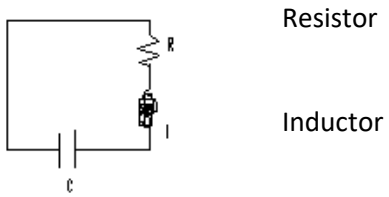

Figure 1. Oscillating circuit. AC power is supplied to the circuit causing electricity to build at the capacitor (C) until the current is at a maximum within the circuit. The current then travels to the inductor (I) where it is momentarilly converted into magnetic energy and back to electrical energy before entering the resistor (R). A small amount of electrical energy is lost at the resistor due to electrical decay an the remainder of th electrical energy travels to the capacitor charging it once again only to be released in the opposite direction undergoing the same changes at each strructure in the circuit. This process continues until the elctric current has disappated due to electrical decay at the resistor. The conversion of electrical energy to magnetic energy elicits an electromagnetic field and the fluctuating current between the capacitor and the inductor result in the oscillating current producing electromagnetci fields of varying frequencies.

Incorporating a device with a user controlled electrical output, such as a heavy duty spark tester, and a simple conductor allows for inducible electromagnetic fields at frequencies that can be controlled by simply adjusting the power input. Heavy duty spark testers are commercially available and are primarily used to test electrical flow through or to test capacitance of a circuit. Applying heavy duty spark to a conductor over a 1 mm gap creates an oscillating circuit similar to that described above. Relative electromagnetic field frequency can be measured using a signal analyzer to determine the power input necessary to generate an electromagnetic field of a desired frequency. Isolating oscillating frequencies allows for the detection of alterations to a cell specific to that range of frequencies removing ambiguity associated with the highly variable oscillating frequencies that originate from most electronic sources (Hansson 2009).

The device described above is optimal for describing the effects of a specific range of electromagnetic resonance on cells in culture. Due to the radial nature of electromagnetic fields cells in culture can be placed in the vicinity or directly under a conductor to receive direct exposure to the resonating electromagnetic field. This, in addition to controlled time intervals, allows the determination of the effects of varying exposures (Rahman & Salman 2009).

Cells in culture, when used to test cellular changes in response to a stimulus, offer relatively quick sample generation as well as low coefficients of variance. According to cell theory every cell is a genetic copy of the previous cell, meaning that the variance between cell lines that share a common lineage is very close to zero. Cells in culture are also more cost effective to maintain than whole organisms and require less lab space for

maintenance. Utilizing cells in culture also allows the differentiation of effects of a single stimulus on different tissues types as variables are more easily controlled in a lab setting from cultures originating from the same parent culture. (Akbarsha & Pereira 2010).

Elucidating the change in telomere length post exposure to electromagnetic resonance can be performed in a variety of ways. The most well known and perhaps most reliable technique for telomere length analysis is the Southern blot analysis. The Southern blot analysis allows for visual examination of telomere lengths against a molecular standard and is currently the most well known and considered the most reliable technique in telomere length measurement (Kimura et al. 2010). An emerging technique in telomere length assessment utilizes real time polymerase chain reaction (qPCR) that requires a PCR primer for telomeric regions as well as a primer for a single copy gene copy for relative calculation of the frequency in the telomere repeats (O'callaghan & Fenech 2011).

The Southern blot analysis is the oldest of the blotting technique and is widely accepted as a reliable test for isolating DNA of particular sequence but has a few drawbacks. Although the Southern blot is reliable it is a costly technique that takes a relatively long time to complete, often involve radioactive nucleotide labeling and requires large amounts of DNA (upwards from 20 µg). New Southern blot kits available from companies such as Qiagen offer a Southern blot technique that involves non-radioactive labeling of nucleotides but these kits can prove to be expensive when large numbers of samples need to be assessed. Southern blot kits are also currently available that offer telomere specific measurements and come included with restriction enzymes specific to

sub-telomeric regions and molecular markers homologous to telomeric repeats (Kimura et al. 2010).

Real time PCR (qPCR) is an emerging technique in telomere length analysis that is less expensive and time consuming than the Southern blot but requires more skill in completing the technique. The technique of qPCR involves all of the components of traditional PCR but adds an intercalating dye (usually SYBR green) for quantification between replication phases. PCR also necessitates the design of primers to anneal with consensus sequence within a DNA sample for amplification. The two qPCR methods utilized for telomere length analysis are singlplex and multiplex qPCR. Singleplex qPCR involves quantifying telomeric regions in a separate sample from the single copy gene copy and therefore creates a variable in regards to the amount of DNA in the separate sample. Therefore acquiring the same amount of DNA in the two separate samples is virtually impossible. Multiplex qPCR, however, combines both the single copy gene copy and telomere primers in one sample ultimately obtaining a value far more reliable than singleplex qPCR. Primers must be carefully designed for multiplex qPCR so that the single copy gene copy products melt at a higher temperature than the telomeric products allowing two separate signal acquisitions to occur. Then the process simply involves subtracting the telomere signal from the single copy gene copy signal to obtain the singe copy gene copy quantification and then dividing the telomeric signal from the single copy gene copy signal to get relative telomeric length against the single copy gene copy product (Cawthon 2002).

Primer design, annealing temperatures and melting temperatures of amplicon products are the most challenging aspect of multiplex qPCR. Primers must be carefully designed

so that their annealing temperatures are close enough to allow replication in the same cycle but their melting temperatures must be far enough apart to allow quantification of the amplicons separately. One technique is to attach a GC clamp to the single copy gene copy primers. A GC clamp is a six to ten nucleotide repeat of CG that, due to C and G nucleotides ability to form 3 hydrogen bonds, allows single copy gene copy products to have a higher melting point. This slightly elevated melting point allows both telomeric and single copy gene copy amplicons to form simultaneously and a signal acquisition taken of the combined amplicons. After the first acquisition the temperature and be raised slightly facilitating the melting of telomeric products and another signal to be taken of just the single copy gene copy products that contain the GC clamp. Relative telomeric lengths can then be extrapolated using simple arithmetic and values for telomeric length can be calculated with accuracy to Southern blot data of over 92% (Cawthon 2009).

Telomeres in cells exposed to resonating electromagnetic fields can be measured with any of the previously mentioned techniques. Acquiring cells in culture from isolated primary cell lines negates variations between treated cells and non-treated control cells. This allows for the direct quantification of the effect of electromagnetic resonance on telomeric maintenance. Utilizing several techniques to perform telomere length analysis also facilitates comparison of data between the separate analyses to support and validate the measurements. This research project is focused on identifying changes to telomeric maintenance due to electromagnetic resonance exposure in primary mouse cell culture utilizing several techniques for telomere length analysis.

Materials & Methods

Primary Cell Isolation

Primary cells were isolated from tissue for the purpose of establishing a primary cell line using the cold trypsinization technique. Kidneys from tg2576b *Mus musculus* were obtained from freshly sacrificed specimen and two lines were started from one kidney of each mouse. Once excised each kidney was placed in a sterile Petri plate containing 10 mL Hanks Balanced Salt Solution (HBSS), Cellgro, Manassas VA, and 1 X Penicillin, streptomycin and fungizone (PSF), Hyclone, Barrington IL, diluted from a 100 X stock solution. Kidneys were rinsed in the HBSS and PSF to remove excess debris before being transferred to a second sterile Petri plate containing 10 mL HBSS and 1X PSF and a sterile scalpel was used to cut away excess fat from the tissue. Each kidney was transferred to a third pre-weighed Petri plate containing 10 mL HBSS and 1X PSF and weighed again to obtain the mass of tissue. The tissue was cut carefully with a sterile scalpel to pieces approximately 3 mm^2.

Once the tissue had been thoroughly rinsed and cut into small pieces the trypsinization disbursement process began. Tissue pieces were transferred to a sterile 15 mL conical tube with a 25 mL serological pipette and allowed to settle before the HBSS 1X PSF was removed. The tissue pieces were rinsed in 5 mL HBSS 1 X PSF and supernatant removed twice. A final 5 mL HBSS was added to the 15 mL tissue and the volume was transferred to a chilled glass test tube on ice using a 25 mL serological pipette. Once the tissue pieces settled the supernatant was removed and replaced with 1

mL cold trypsin:EDTA, Gibco, Carisbad CA with 1 X PSF for every 100 mg tissue. The chilled glass tube and cold trypsin allowed the trypsin to become uniformly distributed within the tissue pieces and allowed for a controlled digestion. The tubes were then put on ice and stored at 4°C for 20 hours in the cold room.

The supernatant was removed leaving only residual trypsin and tissue pieces, the tissues were then incubated at 37°C for 15 min in an Aquabath 18002A water bath, St Loius MO. Tissue digestion was halted by addition of 6 mL Dulbecco's Modified Eagles Medium (DMEM), ZenBio, Research Triangle Park NC, with 25% Newborn Calf Serum (NCS), Sigma, St Loius MO, and 1X PSF mixed via pipetting and allowed to settle. Once large tissue pieces had settled to the bottom of the tube, aliquots of 1 mL suspension in DMEM was used to seed each of two T25 flasks. Cells were incubated in Thermo 800WJ incubator, Barrington IL, which electronically regulates temperature and CO_2 concentration at 37°C and 5.0% CO_2.

Electromagnetic Resonance Exposure of Cell Lines

The effect that the resonance exposure has on telomeric length was studied by designating one of the samples from each cell line to be exposed to oscillating frequencies of electromagnetic resonance for 30 minutes a day and a second sample from the same cell line as a negative control for comparison. Cells that received treatment were placed in the NuAire Wa 425-600, Plymouth MN, sterile tissue hood under a copper conductor that received an electric current over a 1 mm gap produced by a BD-50 Heavy Duty Spark Tester (HDS), ETP, Durham NH, for 30 minutes a day. The HDS, Figure 2, created an oscillating electromagnetic field between 54 and 78 GHz at 50 to 75 decibels.

A. B.

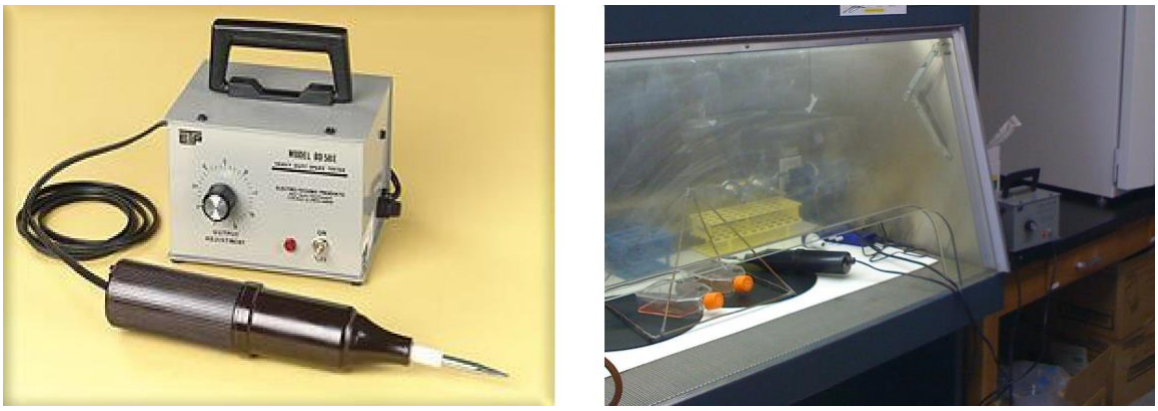

Figure 2. Multiple Wave Oscillator. A) BD-50 Heavy duty Spark Tester. B) Cells in culture exposed to multiple wave electromagnetic resonance. The heavy duty spark tester was applied to the copper conductor over a 1 mm gap. Cells were treated under sterile tissue hood for 30 min/day.

Cells in the non-treated control that did not receive treatment and were placed in a NuAire Class II Type A2 sterile tissue hood in an adjacent lab for 30 minutes per day.

Cell Line Maintenance

To maintain viable cells, the cultures were monitored closely and viewed under an Olympus CKX41 phase contrast optics, St Loius MO, every other day to estimate confluence and to ensure contaminants were not present. All cell culture work was performed under a NuAire Wa 425-600 sterile tissue hood. Culture media, DMEM with 25% NCS and 1 X PSF, was replaced 1 week after initial seeding to remove excess cellular debris from cells that had not adhered to the flask. When cell confluence was approximately 80%, cells were subculteured. The old media was removed and cells were rinsed in 2 mL Phosphate buffered saline (PBS), GibcoCarisbad CA. The PBS was removed and 1 mL trypsin was added and the cells were incubated at room temperature for 2 minutes to digest matrix and dislodge cells adhering to the flask. Trypsinization was halted by adding 1 mL DMEM with 25% NCS and 1XPSF. The cells were seeded to

another flask by calculating the volume in which 20% of the total cells resided and transferring that volume to a new flask and bringing the total volume of the new flask up to 4mL with DMEM, 25% NCS and 1XPSF. The volume transferred was calculated by visually estimating the confluence of the original cell culture and removing an appropriate amount of volume from the cells now diluted in 2 mL of trypsin and PBS. The remaining cells in solution were preserved for later analysis.

Cell Sample Preservation

The Southern blot Assay is both time consuming and expensive so it is best to perform it with multiple samples at one time. To preserve the cells they were first spun at 300 X G for 5 minutes in a Spectrafuge 16M centrifuge, Sigma St Louis MO, in 1.5 mL microfuge tubes and the supernatant was removed. The cells were re-suspended in 1 mL PBS and placed in a Sanyo VIP series freezer at -86°C.

Genomic DNA Isolation

Once enough samples had been collected to fill an entire Southern blot Gel, 50 samples total, the genomic DNA from each sample was isolated using a DNA miniprep kit, Qiagen, Valencia CA. The Qiagen kit included a lysis buffer AL buffer, mini spin columns, 1.5 mL collection tubes, wash buffers, AW1 and AW2, and an elution buffer, AE. Thawed cells were centrifuged at 300 X G in a Spectrafuge 16M centrifuge in 1.5 mL microfuge tubes for 5 min and supernatant was removed and cells were re-suspended in 200 µl PBS. Suspended cells were subjected to lysis and protein degradation with the addition of 20 µl protease K and 200 µl AL Buffer. The solution was pulse vortexed for 15 s and centrifuged briefly to collect droplets at bottom of tube and the solution was

incubated at 56°C for 10 min. After incubation 200 µl Ethanol was added und to rinse the DNA bound to the column and the solution was pulsed vortexed for 15 s. The solution was then placed in a mini spin column and centrifuged for 1 min at 600 X G. After centrifuging 500 µl Buffer AW1 was added and spun for 1 min at 6,000 X G in a fresh collecting tube. After centrifuging 500 µl of Buffer AW2 was added to the column and spun for 3 min at 20,000 X G in a fresh collecting tube and spun again at full speed for 1 min. The mini spin column was transferred to a 1.5 mL microfuge tube and 200 µl Buffer AE was added to column and incubated at room temperature for 5 min and centrifuged at 6,000 X G for 1 min. The procedure for genomic DNA isolation was supplied by the mini prep spin kit. Purified genomic DNA was quantified via NanoDrop and applied directly to quantative polymerase chain reaction (QPCR), Southern blot Assay or placed at -20° for later use.

NanoDrop 2000 Spectrophotometer

The NanoDrop 2000 spectrophotometer, Thermo Scientific, Barrington IL, allows for quick and conservative quantification of DNA in solution. The process can be completed in a fraction of the time as a traditional spectrophotometer and requires only 1 µL of solution. The NanoDrop 2000 interfaces through the supplied software called. In order to obtain quantification of DNA samples the NanoDrop 2000 software was opened and the Nucleic Acids program was selected from the start screen. The first step in quantification is to acquire a blank reading using the solvent that the DNA is dissolved in. To blank the instrument 1 µL of buffer AE from the Qiagen mini prep kit was placed on the reading platform of the NanoDrop 2000 and blank was selected on the NanoDrop 2000 software.

Samples were then quantified by placing 1 μL sample on the reading platform. The values were then recorded for each sample and are located in the results section.

Ethanol Precipitation of DNA

After a genomic DNA isolation, if the resulting DNA yield is too low to perform a desired technique ethanol precipitation can be performed to concentrate the DNA into a smaller volume of solution. Ethanol precipitation was performed by first adding 1/10 the volume of 3M sodium acetate at PH 5.2 along with 2X the volume of 100% ethanol and stored at 4 °C in the cold room for 24 hours. The DNA was then centrifuged at 6,000 X G for 30 seconds and the supernatant was removed. The DNA was then rinsed twice in 100 mL 70% ethanol centrifuging and removing the supernatant each step. After the second ethanol rinse and the supernatant was removed the tubes were inverted on a paper towel and allowed to dry for five minutes. After drying 10 μL buffer AE from the Qiagen miniprep kit was added to dissolve the DNA in solution.

Determining Cell Volume Necessary for Southern blot

The amount of DNA necessary to perform quantification techniques can very therefore, its important to establish the amount of cells needed in order to generate the necessary amount of DNA. In order to determine approximately the number of cells needed to yield enough genomic DNA to run a Southern blot three Astrocytoma cultures were allowed to grow to approximately 80% confluence in T25 flasks. The three cultures were then trypsinized as described earlier and 0.5 mL was taken from the first culture, 1.0 mL from the second and 1.5 mL from the third. The cell numbers in each volume were then quantified by adding 20 μL of cells in suspension to 20 μL 0.4% trypan blue stain by

Invitrogen Eugene, Oregon of which 12 μL were placed in a counting chamber slide also by Invitrogen. The counting chamber slide was then placed in a Countess Automated Cell Counter by Invitrogen and cells were counted using the astrocytoma quantification program. Each volume of cells was then subjected to genomic DNA isolation and the DNA concentrations were analyzed using the NanoDrop 2000.

Singleplex qPCR

The singleplex quantative polymerase reaction (qPCR) technique is not an accurate tool for quantifying mean telomeric length, however, it is an effective tool for qualifying relative changes in telomeric length. The inhibiting factor in the quantification in singleplex qPCR is that in order for singleplex PCR to work accurately each sample must contain as close to the same amount of DNA as possible. To obtain equal amounts of DNA the values obtained from the NanoDrop 2000 were used to calculate the amount of each sample to be used in the final volume of 20 μg DNA. The single copy gene copy was a primer set received from Renee Ehrenstrom of Dr Zimmerman's lab designed to amplify actin B17 used for a reference to calculate relative mean telomere length. Forward primers for the actin single copy gene copy were designed as 5'-ACC CAC ACT GTG CCC ATC TA-3' and reverse primers were designed as 5'-CGC AAC CGA TCA TTG CC-3'.

Telomeric regions consist of tandem repeats and therefore primer design is very difficult. The telomere primers were constructed as descried by Cawthon to be mismatched every sixth base pair so as to prevent annealing of primers creating primer dimer products. The forward primers were designed as 5'-ACA CTA AGG TTT GGG

TTT GGG TTT GGG TTT GGG TTA GTG T-3’ and the reverse primers as 5’-TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA-3’. This design, although not completely complimentary to the template strand, offers three times the affinity for the template compared to the reverse primer (Cawthon 2009).

The qPCR reaction was performed by adding 10 µl SsoFast EvaGreen supermix BIO-RAD, Hercules CA, 1.5 µl forward and reverse primers and variable volumes of RNase/DNase free water, BIORAD, Hercules CA, and 10 ng of DNA template depending on the concentration of DNA in the template equaling a total volume of 20 µl to a qPCR tube. SssoFast EvaGreen, and most qPCR supermixes, contain a DNA double strand intercalating dye called SYBR green. SYBR green is a sophisticated dye that has an excitation wavelength of 284 nm and a maximum excitation wavelength of 497 nm and fluorescence emission at 520 nm. All qPCR reactions were performed within a MiniOpticon MJ Mini personal thermal light cycler, BIORAD. The run information for the singleplex qPCR was based on the information supplied with the SsoFast EvaGreen supermix and was as follows: cycle 1 for 2 min at 98 °C, cycle 2 for 5 s at 98 °C, cycle 3 for 30 s at 60.3°C and a fluorescence reading, cycle 4 repeat cycles 2 and 3 39 more times and cycle 5 melt curve for 10 s at 5 °C intervals between 60°C and 95° C. Data obtained during the qPCR reaction was compiled using the BIO-RAD CFX Manager and the end point RFU data from the telomere products were divided by the end point RFUs of the actin products and normalized against the negative control baselines for each organ. The data from two separate reactions was then averaged and plotted in Microsoft Excel with error bars indicating standard deviation.

To further support the formation of telomere products each PCR product was added to an agarose gel and separated via electrophoresis. A large 13 cm X 25 cm gel of 0.8% agarose was prepared by diluting an appropriate amount of powdered agarose in 1X TAE (tris-acetate EDTA) buffer. A 10 pronged well comb was placed at the top and mid section of the gel to make enough wells for all 16 samples leaving 2 wells for the 1 Kb ladders placed in wells flanking the samples on both top and bottom. The 1 Kb ladder, New England BioLabs, Ipswich MA, and consisted of DNA fragments of ten sizes at 1 Kb, 0.8 Kb, 0.6 Kb, 0.5 Kb, 0.4 Kb, 0.3 Kb, 0.2 Kb, 0.15 Kb, 0.1 Kb and 0.05 Kb. Electrophoresis was performed for 45 min at 80 V until the bromophenol blue band had migrated about three fourths of the way from the top wells to the bottom row of wells. After electrophoresis the gel was submerged in a solution of 1/1,000 w/v of ethidium bromide in 100 mL 1 X TAE buffer for 5 minutes and then destained in 500 mL de-ionized water (DI water) for 5 minutes before viewing in the Gel Logic 200 Imaging System manufactured by Kodak. The image was then captured using the KODAK MI imaging software and exported as a JPEG file.

Monochromatic Multiplex qPCR

Monochromatic multiplex qPCR refers to the ability to amplify two target sequences within a sample and acquire the two amplicon quantitations separately using only one intercalating dye in the solution. This process was pioneered by Dr. Richard Cawthon with human leukocytes to facilitate a less taxing and more cost effective way to determine mean telomere length. Telomere primers were designed as described for singleplex and the single copy gene copy primers were designed for the acidic ribosomal phosphoprotein PO (36B4), a gene with sequence occurring only once within the genome

(Calicott & Womack 2006). The forward primer sequence for 36B4 was designed as 5'-CGG CGG CGG GCG GCG CGG GCT GGG CGG ACT GGT CTA GGA CCC GAG AAG-3' and reverse primer sequence was designed as 5'-GCC CGG CCC GCC GCG CCC GTC CCG CCG TCA ATG GTG CCT CTG GAG ATT-3'. The GC rich area at the head of the primers act as a GC clamp creating amplicons with high melting points allowing for an initial signal acquisition to be taken at a low temperature signifying both amplicons and a subsequent signal acquisition to be taken at a higher temp signifying only the single copy gene copy amplicon. This is the author's variation of the Cawthon protocol for human DNA modified to facilitate the measurement of mouse DNA, a protocol that has yet to be published (Cawthon 2009).

The qPCR reaction solutions were prepared via the authors variation of the Cawthon protocol by adding 10 μL Synnergy Brands (SYBR) Green ssofast mastermix, Qiagen, 0.6 μL of each forward and reverse primers for both telomere amplicons and 36B4 amplicons equaling 900 nM of the total volume, enough DNA solution to equal 25 ηg DNA and filled to a volume of 20 μL with nuclease free water, Qiagen. The following protocol was used for the multiplex qPCR Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and Stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition, 10 s at 84°C, 15 s at 88°C with signal acquisition. The first signal acquisition taken at 74°C included both amplicon products and at 84°C the telomere amplicon products melt away and the signal acquisition taken at 88°C represents only the 36B4 amplicon products. To calculate the telomere signal the second 36B4 signal was subtracted from the initial signal of both amplicons. The mean telomere

length is then calculated by dividing the telomere amplicon signal by the 36B4 amplicon signal to obtain the relative length against the 36B4 of known size.

Southern blot Analysis

Southern blot analysis is still considered the most effective and reliable form of telomere length assay and was performed using a TELOTAGG telomere assay kit, Roche, Tucson AZ (Kimura & Stone 2010). Before beginning the reaction a series of wash and transfer solutions were prepared for the Southern blot. Solution 1, TAE buffer, 500 mLs made of 0.04 M tris-acetate and 0.001 M EDTA in DI water. The TAE solution was adjusted to the correct PH, 8.0, by the addition of concentrated HCl (17.34 N). Solution 2, HCl solution, 0.25 M solution of HCL by diluting 17.34 N concentrated HCl in DI water. Solution 3, the denaturing solution, 0.5 M NaOH and 1.5 M NaCl solution in 500 mL of DI water. The neutralizing solution, solution 4, 0.5 M tris-HCl and 1.5 M NaCl in 500 mL of DI water. The PH adjusted to 7.5, by the addition of concentrated HCl (17.34 N). Solution 5, 20X SSC to be used for blot transfer, 3 M NaCl and 0.3 M sodium acetate solution in 500 mL of DI water. The solution was adjusted to PH, 7.0, by the addition of NAOH (8N). A 2X SSC solution, solution 6, 20X SSC solution 1 in 10 with DI water to a volume of 500 mL.

To hybridize the blot and allow for proper visualization of DNA banding a series of hybridization and wash reagents were prepared. The digioxigenin (DIG) easy hybridization solution used for hybridization of the blot and pre-hybridization of the blot, solution 7, incubated DIG easy hyb granules, supplied with kit, to 37 °C and mixed with

64 mL of pre-warmed DI water. The stringent wash buffer I, solution 8, 10 % SDS diluted to a concentration of 0.1 % in 100 mL 2X SSC. The stringent wash buffer II, solution 9, 2 X SSC diluted to a concentration of 0.2X and 10 % SDS to a concentration of 0.1% in 100 mL DI water. The blot washing buffer, solution 10, supplied 10 X wash buffer diluted to a concentration of 1X in 500 mL DI water. Solution 12, the maleic acid buffer used to make the blocking buffer, supplied 10X maleic acid buffer diluted to a concentration of 1X in 200 mL DI water. The blocking buffer to act as the blocking buffer for the blot and as the buffer to dilute the anti-DIG –AP probe for telomere fluorescence, solution 11, supplied 10 X blocking buffer diluted to a concentration of 1 X by in 100 mL 1 X maleic acid. The anti-DAG-AP solution, solution 13, supplied anti-digioxigenin-alkaline phosphatase (anti-DIG-AP) marker diluted 1 in 10,000 in 100 mL of blocking buffer. The detection buffer for detection of the marker, solution 14, supplied 10 X detection buffer diluted in 500 mL DI water.

To perform the Southern blot, volumes between 10 and 15 µL of four DNA solutions isolated from mouse cells and one DNA solution isolated from Astocytoma cells containing approximately 1.5 µg purified DNA were added to a variable amount of nuclease free water to total 17 µL in each reaction vial. Two positive control DNA samples, included in the TELOTAGG kit, were placed in two separate reaction vials, 15 µL each totaling 1.5 µg DNA, and brought up to a volume of 17 µL by the addition of 2 µL nuclease free water. Each sample was combined with 2 µl digestion buffer and 1 µl Hinf1/RSA1 enzyme mixture and incubated for 2 hr at 37°C. This digestion reaction digests subtelomeric and most genomic DNA into fragment sizes that migrate away from telomere fragments allowing for quantification of telomeric regions. The reaction was

halted by the addition of 5 µl 5X electrophoresis loading buffer. The 8 cm by 10 cm agarose gel was prepared at 0.8% agarose in 1X TAE Buffer and cast in a gel electrophoresis rig. The molecular weight marker was prepared by mixing 4 µl of the DIG molecular weight marker, 12 µl nuclease free water and 4 µl 5X loading buffer.

Once the gel had solidified and the apparatus had been assembled approximately 1.5 µg DNA of each digested sample was loaded into the wells of the gel, 10 µl of the diluted DIG molecular weight marker was added to the far left well of the gel, and 20 µL of a 1 Kb DNA, New England Biolabs, was placed in the far right well. Control DNA from the TELOTAGG kit samples were placed in wells flanking either side of the mouse and Astrocytoma DNA. The gel was run at 5V/cm, 50 V, in 1X TAE buffer until the bromophenol blue band was 10 cm from the starting wells, approximately 3 hours. After the gel had finished running an ethidium bromide staining solution was prepared by diluting ethidium bromide 1/1,000 in 100 mL 1 X TAE buffer and the gel was soaked for 5 min. After staining the gel was destained for 5 min by rinsing with DI water and viewed in the Gel Logic 200 Imaging System, Kodak, Rochester NY, and the image was processed using the KODAK MI imaging software.

Agarose gel electrophoresis separates the digested chromosomal DNA fragments from the preserved telomeric regions. This allows for hybridization of telomere probes and distinguishing relative size after blotting. The gel was submerged completely in solution 2, HCl solution, under agitation on a Multi-Purpose Rotator, Barnstead Lab-Lines, Barrington IL, for 5-10 min at room temperature until the bromophenol changed from blue to light green. The gel was rinsed twice in DI water and submerged in Solution 3, denaturing solution, two times for 15 min at room temperature. The gel was rinsed

again in water and submerged in Solution 3, neutralizing solution, twice for 15 min at room temperature. During the final submersions the blot apparatus was assembled. The blot apparatus consisted of an inclined surface placed in a small basin with a long thin strip the width of the gel overlaying the surface with both ends submerged in 20X SSC in the basin. The gel was placed on top of the filter paper well side up and a Nytran SuPerCharge positively charged nylon membrane, Schleicher & Schuell BioScience, Keene NH, was cut to fit the gel and placed directly on top. In order to absorb the 20 X SSC five extra thick blot paper squares, BIORAD, were cut to fit the nylon membrane and placed on top. Capillary transfer was performed in solution 5, 20X SSC, at room temperature overnight.

The blot had transferred from the agarose gel to the nylon membrane and the DNA was then affixed to the wet membrane with UV light in a CL-1000 Ultraviolet Crosslinker, Ultra-Viole Products, Upland CA. The crosslinking instrument was set at the preset conditions at 120,000 μJ and ran for approximately 13 seconds. The blotting membrane was washed two times with Solution 6, 2 X SSC, and during the wash 25 mL of Solution 7, DIG easy hyb, was warmed to 42°C. The blot was submerged in 18 mL of warm DIG easy hyb, solution 7 the pre-hybridization solution, and incubated for 45 min at 42°C. All incubations at 42°C and above with agitation were performed in an SI-600R shaking incubator, Lab Companion, Woburn MA. The pre-hybridization solution, solution 7, was discarded and the hybridization solution was added to the blot immediately and incubated for 3 hrs at 42 °C with gentle agitation. The blot was rinsed twice with Solution 8, stringent wash buffer I, for 5 min at room temperature with gentle agitation. All incubations at room temperature with gentle agitation were performed on a

Multi-Purpose Rotator manufactured by Barnstead Lab-Lines. The blot was again rinsed twice with Solution 9, astringent wash buffer II, in shaking incubator for 15min at 50°C with gentle agitation. The membrane was washed in 100 mL of Solution 10, 1 X washing buffer, for 5 min at 20°C with gentle agitation and subsequently incubated in 100 mL freshly prepared Solution 11, 1 X blocking solution, for 30 min at 20°C with gentle agitation.

The membrane had been prepared and was ready for hybridization of the telomere probes. Solution 13, the anti-DIG-AP working solution, was prepared fresh before use and the anti-DIG-AP working solution the vial was first spun at 13,000 rpm to separate probes that had congregated while in solution and diluted one in one thousand with 1 X blocking solution to a final volume of 100mL in which the membrane was incubated for 30 min at room temperature with gentle agitation. The working solution was removed by two washings in 100 mL washing buffer for 15 min at room temperature. After all of the working solution had been removed the membrane was incubated in 100 mL of Solution 14, detection buffer, for 5 min at room temperature with gentle agitation and immediately placed DNA side up in a hybridization bag and 40 drops of Solution 15, substrate solution, was added directly to the membrane and then immediately covered with the second sheet of the hybridization bag, carefully homogenizing the substrate solution over the membrane. The entire bag was then incubated for 5 min at room temperature and excess substrate solution was carefully squeezed out of bag and edges of bag were sealed by folding over the adhesive ends.

To detect the telomere sequence bands on the membrane, the membrane was removed from the hybridization bag and placed between 2 sheets of AF4300 Write-on

transparency, 3M, St Louis MO, and placed in an ElectroPhoresis Systems Autoradiography Cassette, Fischer Biotech, Rochester NY. The cassette was taken to the dark room and a sheet of BioMax XAR film, Kodak, was placed behind the fluorescing blot and exposed for 20 min. The film was developed by inserting the exposed sheet into a CP1000 film, Afga, Mortsel Belgum. The mean length of each smear was determined by measuring the length of the smear with a metric ruler until the intensity matched that of the background and finding the mid-point of the smear. This point was then compared against the DIG molecular marker for relative estimation of TRFL.

RESULTS

Primary Cell Isolation

The first round of primary cells were isolated from a male mouse born on 3/20/2009 and was euthanized the day of isolation 2/26/2010. Primary cells were isolated from the liver and kidneys. The subsequent cells in culture were divided into two groups from each organ to obtain a culture to receive treatment and another to act as a negative control (non-treated). The cultures were exposed daily to the range of electromagnetic frequencies mentioned in the materials and methods and the cultures were maintained according to the techniques mentioned in the materials and methods. After 12 weeks the first genomic DNA sample was taken and three subsequent samples were taken every two weeks until the cultures reached 20 weeks of age. At 20 weeks of age all cultures acquired yeast contamination and were disposed of by autoclaving all tissue culture flasks.

The second set of primary cell isolations was performed several months later by excising 10 kidneys from 10 female mice from the same litter born on 6/17/2010. Tissue excision and cell isolations were performed on 10/25/2010 and each kidney excised was used to create one culture that would be divided into an experimental group and a negative control equaling 20 cultures total. After transferring cells into T25 flasks and allowing incubation for 48 hours the cultures were viewed using phase contrast optics to inspect adherence, morphology and health. The cells in culture had very little adherence and appeared to also have bacterial contamination. The cultures were rinsed with 3 mL PBS containing 1X PSF six times until the bacterial contamination had appeared to have

disappeared. One week later the cells had only grown to 15 to 20% confluence and the bacterial contamination had reappeared. The cells were again washed with 3 mL PBS containing 1X PSF 6 times until the bacterial contamination had disappeared. The following week the bacterial contamination had returned and cell confluence had diminished in all cultures. The cultures were again disposed of by autoclaving.

Another round of primary cell isolations was performed on 12/22/2010 on 7 mice originating from different litters. Again the kidneys were excised for the fibroblast primary cells. Included in the samples were 3 male mice born on 4/11/2010, two females born on 2/23/2010, and 2 males born on 3/13/2010. After plating into T25 flasks and incubated for 48 hours minor contamination was present in all cells in culture. Twice a week for 3 weeks all cultures were rinsed in 3 mL PBS with 1 X PSF but the contamination prevented proper adherence of the primary fibroblast cells and hindered cell culture division until the contamination had overgrown the cultures. All cultures were disposed of by autoclaving.

A final set of primary cell isolations was performed on 1/4/2011 and included 2 male mice born on 3/11/2010, 2 male mice born on 2/23/2010, 4 female mice born on 2/23/2010 and 2 female mice born on 3/11/2010. After plating in T25 flasks and incubating for 48 hours all cultures again contained low levels of bacterial contamination verified by phase contrast optics. The cells were rinsed twice a week for 2 weeks with 3 mL PBS with 1 X PSF. Many of the cultures exhibited decent adherence and after two weeks all cultures were split into 2 separate flasks using the technique described above with trypsin also containing 1 X PSF. Upon plating into new T25 flasks several of the cultures exhibited very low adherence. After incubating for 48 hours to allow adherence

all experimental cultures began daily treatment with electromagnetic resonance and were viewed regularly to assess confluence and contamination. After 3 weeks of treatment and bi-weekly rinsing in 3 mL PSF with 1 X PSF confluence was still low in all cultures and bacterial contamination was still present. All cells in culture were disposed of by autoclaving.

Genomic DNA Isolation

Genomic DNA isolations were performed to isolate DNA for singleplex qPCR, multiplex qPCR, and the Southern blot analysis. All cells from the first round of cultured cells were preserved via the Cell Sample Preservation protocol described previously and were taken from the freezer at -86°C, Sanyo VIP series, Osaka Japan, and allowed to slow thaw on ice prior to isolation. During this first try of isolating genomic DNA samples were centrifuged in a Spectrafuge 16M centrifuge at 20,000 X G. The yield for each sample after genomic DNA isolation was taken with the NanoDrop 2000 and recorded in Table 1. Subsequent genomic DNA isolations were performed as described in the materials and methods so as to obtain a higher yield of DNA.

Table 1. Initial NanoDrop 2000 readings of genomic DNA isolations from primary cell culture.

Date of isolation	Liver isolation treated group	Liver isolation negative control	Kidney isolation treated group	Kidney isolation negative control
5/12/2010 (9 weeks old)	2.6 ηg/μL	1.5 ηg/μL	1.3 ηg/μL	0.5 ηg/μL
5/26/2010 (11 weeks old)	1.4 ηg/μL	1.6 ηg/μL	1.8 ηg/μL	1.3 ηg/μL
6/3/2010 (13 weeks old)	1.9 ηg/μL	1.4 ηg/μL	1.4 ηg/μL	1.4 ηg/μL
6/17/2010 (15 weeks old)	1.5 ηg/μL	1.5 ηg/μL	1.5 ηg/μL	1.5 ηg/μL

Most PCR reactions require a final volume between 20 and 25 μL. Concentrations at the level in Table 1 would require more volume than would fit in most thermal cycler PCR tubes; therefore, isoprpanol precipitation was used to concentrate the DNA samples. Post ethanol precipitation genomic DNA concentrations were taken again with the NanoDrop 2000 and recorded in Table 2.

All subsequent cells in culture after the first set of isolations became contaminated; therefore, genomic DNA was acquired from Renee Ehrenstrom, graduate student in Dr. Zimmerma's lab. The DNA acquired originated from the tail tip of a several mice from the same litter born on 3/23/2010. The genomic DNA was quantified with the NanoDrop 2000 and is recorded in Table 3. The DNA in these solutions is too dilute to perform the Southern blot analysis so sodium acetate/ethanol precipitation was used to concentrate the DNA samples. The resulting DNA concentrations are also recorded in Table 3. One sample was left in the diluted state to perform the monochromatic multiplex qPCR.

Table 2. NanoDrop 2000 readings taken after ethanol precipitation to concentrate DNA from primary cell culture.

Date of isolation	Liver isolation treated group	Liver isolation negative control	Kidney isolation treated group	Kidney isolation negative control
5/12/2010 (9 weeks old)	39.8 ηg/μL	32.1 ηg/μL	18.0 ηg/μL	24.0 ηg/μL
5/26/2010 (11 weeks old)	32.1 ηg/μL	30.5 ηg/μL	11.0 ηg/μL	45.1 ηg/μL
6/3/2010 (13 weeks old)	12.7 ηg/μL	30.7 ηg/μL	55.0 ηg/μL	35.1 ηg/μL
6/17/2010 (15 weeks old)	35.5 ηg/μL	35.5 ηg/μL	35.5 ηg/μL	32.0 ηg/μL

Table 3. Nanodrop 2000 readings for genomic DNA acquired from Renee Ehrenstrom in Dr Zimmerman's lab.

	Multiplex qPCR Sample 1	Southern blot Sample 1	Southern blot Sample 2	Southern blot Sample 3	Southern blot Sample 4	Southern blot Sample 5
Initial genomic DNA ηg/μL[1]	58.3	32.5	86.7	92.3	77.6	53.2
Concentrated genomic DNA ηg/μL[2]	NA	120	258	415	389	119

[1]Represents the values before the ethanol precipitation; [2]Represents the values after the ethanol precipitation.

Determining the Cell Volume Necessary to run the Southern blot

Using a hemocytometer or an automated cell counter the number and volume of cells needed to acquire a specific amount of DNA can be determined. Table 4 contains the number of cells present in several volumes of astrocytoma cells grown to a confluence of 80% in a T-25 flask. Table 4 also contains the genomic DNA concentrations acquired from each volume of cells. In order to perform the Southern blot technique large amounts of DNA, between 1 and 2 μg, are necessary and therefore a volume and estimation on the number of cells to run a Southern blot needed to be established.

Table 4.Countess Automated Cell Counter and NanoDrop 2000 readings volumes of homogenized astrocytoma cells post genomic DNA isolations.

Cell Volume	0.5 mL	1.0 mL	1.5 mL
Cell number/mL	2.2X10^5	7.6X10^5	9.7X10^5
Genomic DNA concentration ηg/μL	17.3	56.3	105.0

Singleplex qPCR

Singlplex qPCR involves quantifying unknown quantities of a particular gene or sequence within an isolated sample of DNA by comparing the gene or sequence of unknown quantity to a reference gene of known quantity. The singleplex qPCR reaction included each sample plated in duplicate and ran simultaneously in separate wells containing either the actin primers or telomere primers with 10 ng of genomic DNA. Once the reaction had finished the endpoint relative fluorescent unit (RFU) data was combined from two separate reactions and the RFUs of the telomere products divided by the RFUs of the actin products were normalized against the baseline negative control telomere RFUs and the average of the two reactions was plotted in a Microsoft Excel spreadsheet and are shown in Figure 3. The error bars indicate the standard deviation between the two reactions.

The qPCR, much like traditional PCR, generates DNA products through the subsequent polymerization reactions. Separating these products via electrophoresis allows for the confirmation of the appropriate size of PCR products as well as allows the verification of no primer dimer product formation. Figures 4 and 5 illustrate the PCR products formed during the qPCR reaction. The 1KB ladder was included in the gel to allow for comparison between amplicon product sizes and known DNA standards for relative estimation of size against the standard DNA bands.

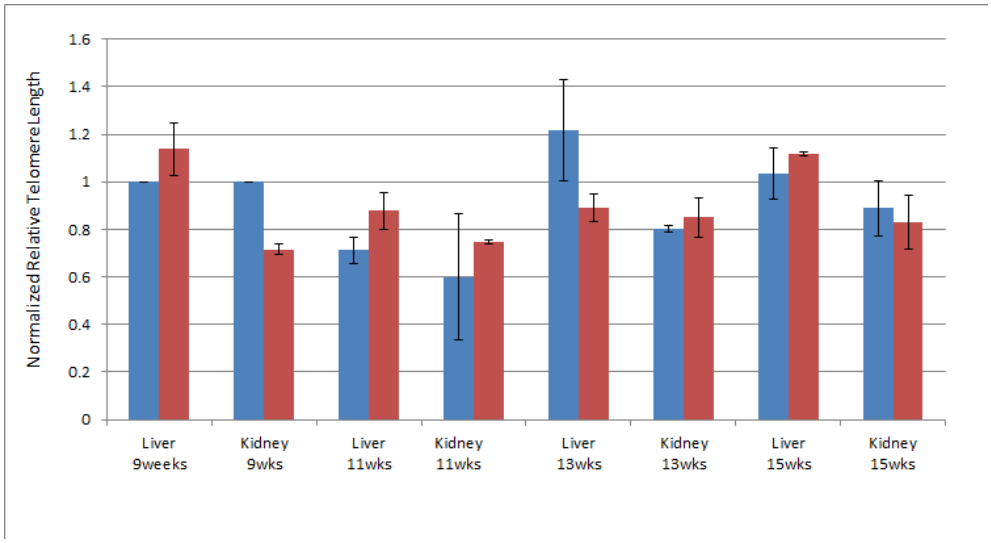

Figure 3. Singleplex qPCR of telomeric length. Two separate reactions with the average RFUs relative telomeric product lengths to actin normalized against the negative control baseline telomere readings. The error bars indicate standard deviation. Primary cell culture Negative controls cells (blue) paired with treated cells (Red) originating from specific organs (liver and kidney).

A. **B.**

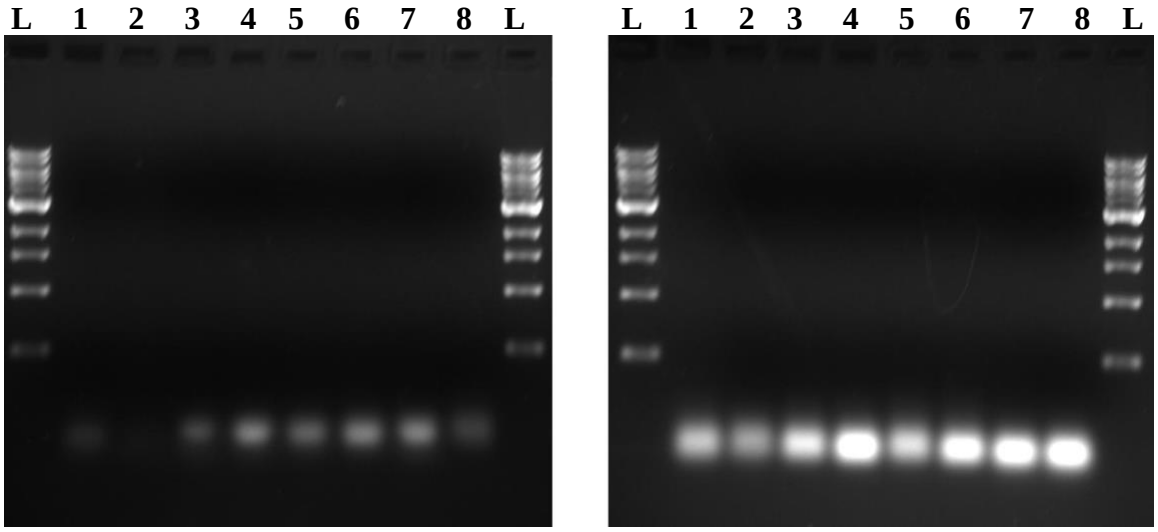

Figure 4. Agarose Gel Electrophoresis of Singleplex qPCR telomere products. A) Lanes L, NEB 1 kb size standard the band that traveled the farthest represents DNA 500 bp in length. Lanes 1 and 2, liver cell isolates treated and negative control, respectively, after 9 weeks. Lanes 3 and 4, kidney cell isolates treated and negative control after 9 weeks. Lanes 5 and 6, liver cell isolates treated and negative control after 11 weeks. Lanes 7 and 8, kidney cell isolates treated and negative control after 11 weeks. B) Lanes L, NEB 1 kb size standard. Lanes 1 and 2, liver cell isolates treated and negative control, respectively, after 13 weeks. Lanes 3 and 4, kidney cell isolates treated and negative control after 13 weeks. Lanes 5 and 6, liver cell isolates treated and negative control after 15 weeks. Lanes 7 and 8, kidney cell isolates treated and negative control after 15 weeks. PCR analyses were performed on 0.8% (w/v) agarose gel in 1X TAE

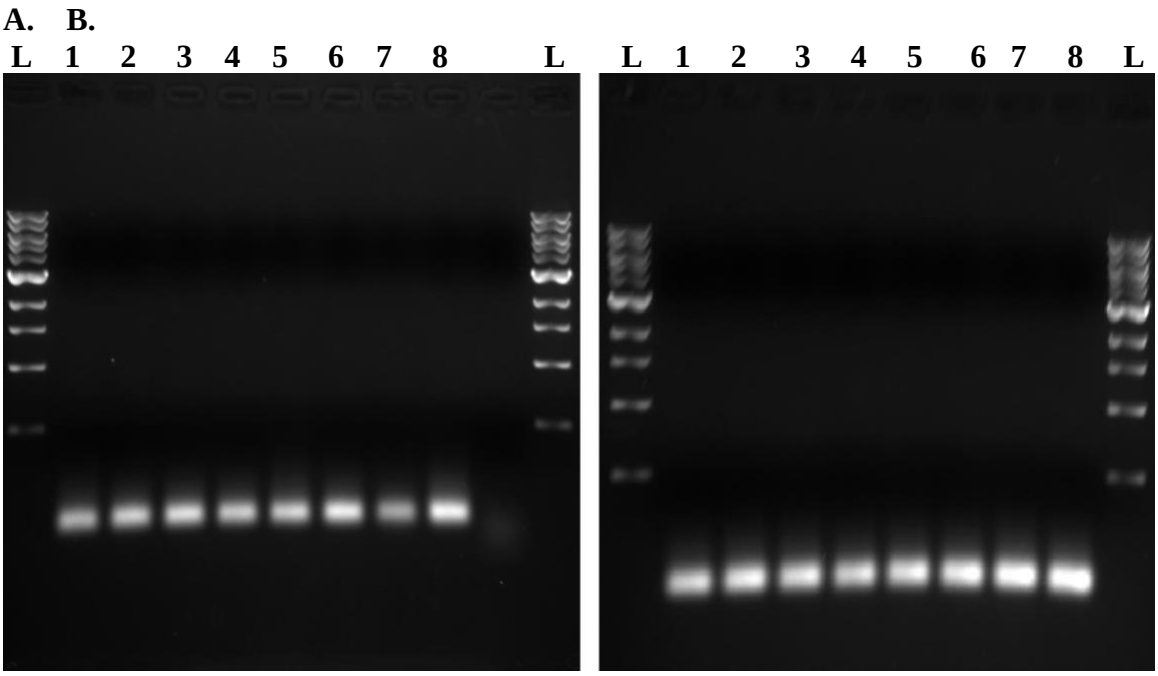

Figure 5. Agarose Gel Electrophoresis of Singleplex qPCR actin products. A) Lanes L, NEB 1 kb size standard the band that traveled the farthest represents DNA 500 bp in length. Lanes 1 and 2, liver cell isolates treated and negative control, respectively, after 9 weeks. Lanes 3 and 4, kidney cell isolates treated and negative control after 9 weeks. Lanes 5 and 6, liver cell isolates treated and negative control after 11 weeks. Lanes 7 and 8, kidney cell isolates treated and negative control after 11 weeks. B) Lanes L, NEB 1 kb size standard. Lanes 1 and 2, liver cell isolates treated and negative control, respectively, after 13 weeks. Lanes 3 and 4, kidney cell isolates treated and negative control after 13 weeks. Lanes 5 and 6, liver cell isolates treated and negative control after 15 weeks. Lanes 7 and 8, kidney cell isolates treated and negative control after 15 weeks. PCR analyses were performed on 0.8% (w/v) agarose gel in 1X TAE.

Multiplex qPCR

Multiplex qPCR is a relatively new technique that allows for accurate quantification of amplicon products with very low cost and high throughput. Several samples were run to test the possibility of performing multiplex qPCR using mouse DNA and the designed primers mentioned in the materials and methods. The first attempt included the 36B4 primers in a well alone, the telomere primers in a well alone and a combination of both sets of primers in a third well. The melting temperature for the telomere products alone was 77 °C, the melting temperature for the 36B4 products was 89 °C and the melting

temperatures for each product were the same when formed together in the same reaction. The melting temps of the products from each well are included in Figure 6. The quantification of each well is presented in Table 5.

Figure 6. Multiplex qPCR melting temperatures. The 36B4 products are plotted in pink, the telomere products are plotted in blue and the combination products are plotted in green. The melting temperature for the telomere products alone was 77 °C, the melting temperature for the 36B4 products was 89 °C and the melting temperatures for each product 77 The melting temperature for the telomere products alone was 77 °C, the melting temperature for the 36B4 products was 89 °C and the melting temperatures for each product were 77 °C and 89 °C, respectively, when formed together in the same reaction.

Table 5. Multiplex QPCR end RFU results.

Well	Fluor	Content	Sample	End RFU
A1	SYBR	36B4	1	0.62
A2	SYBR	Telomere+36B4	2	0.48
A3	SYBR	Telomere	3	0.29

The data acquired from the BIORAD CFX manager does not differentiate between RFUs of either product when both are ran together so products from two separate qPCR reactions were placed in either a 0.8% or 2.0% agarose gel to separate products formed during the Multiplex qPCR reaction. The gels were also run with a DNA ladder to illustrate a standard to determine relative product length and are shown in Figure 7.

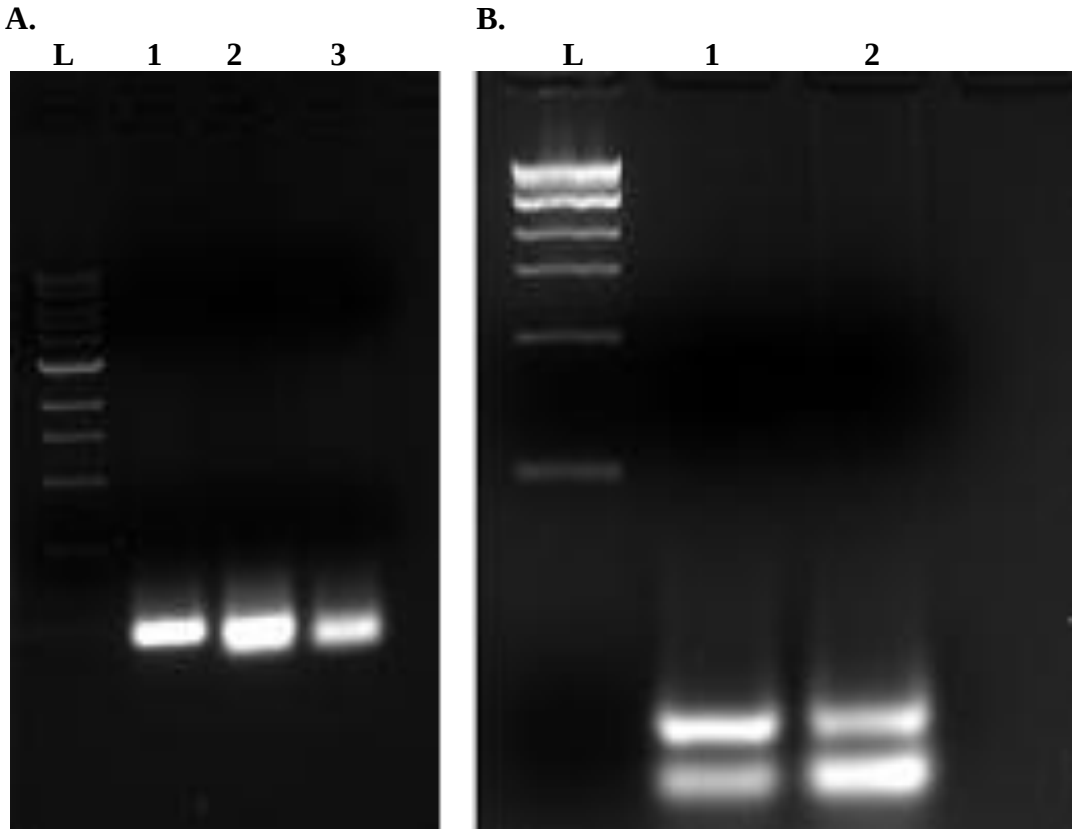

Figure 7. Agarose Gel Electrophoresis of Multiplex qPCR amplicon products. A) Amplicon products in a 0.8% agarose gel in 1X TAE buffer. Lane L, NEB 1 kb size standard the band that traveled the farthest represents DNA 500 bp in length. Lane 1, amplicon products of 36B4 primers. Lane 2, amplicon products of combined telomere and 36B4 primers. Lane 3 amplicon products of telomere primers. B) Amplicon products in a 2.0% agarose gel in 1X TAE buffer. Lane L, NEB 1 kb size standard. Lanes 1 and 2, amplicon products of combined telomere and 36B4 primers.

Southern blot Analysis

In order to determine mean telomere fragment lengths, sub-telomeric DNA must be digested enough to separate from telomeric DNA in an agarose gel. Once the telomeric DNA has substantially separated a blot may be performed and hybridized with fluorescent probes that mark telomeric bands. The 0.8% agarose gel was loaded so that the 1kb ladder was in the first well and subsequent wells contained: control DNA, mouse sample 1, mouse sample 2, mouse sample 3, mouse sample 4, mouse sample 5, Astrocytoma sample, control DNA and the DNA molecular marker. After performing gel electrophoresis the produced gel was stained in an ethidium bromide solution and viewed under the Kodak gel imager (Figure 8).

To perform the Southern blot analysis and identify telomeric length in the samples the digested DNA products must be transferred to a nylon membrane. This was performed as described in the materials and methods as was the blotting process. The resulting blot was developed and appears in Figure 9. The wells of the blot are still clearly visible and are congruent to those in the agarose gel (Figure 8). The DNA ladder does not appear in the blot even though it was visible in the gel of digested DNA. The standard DNA does not contain telomeric DNA repeats not allowing the telomeric DNA probe to bind.

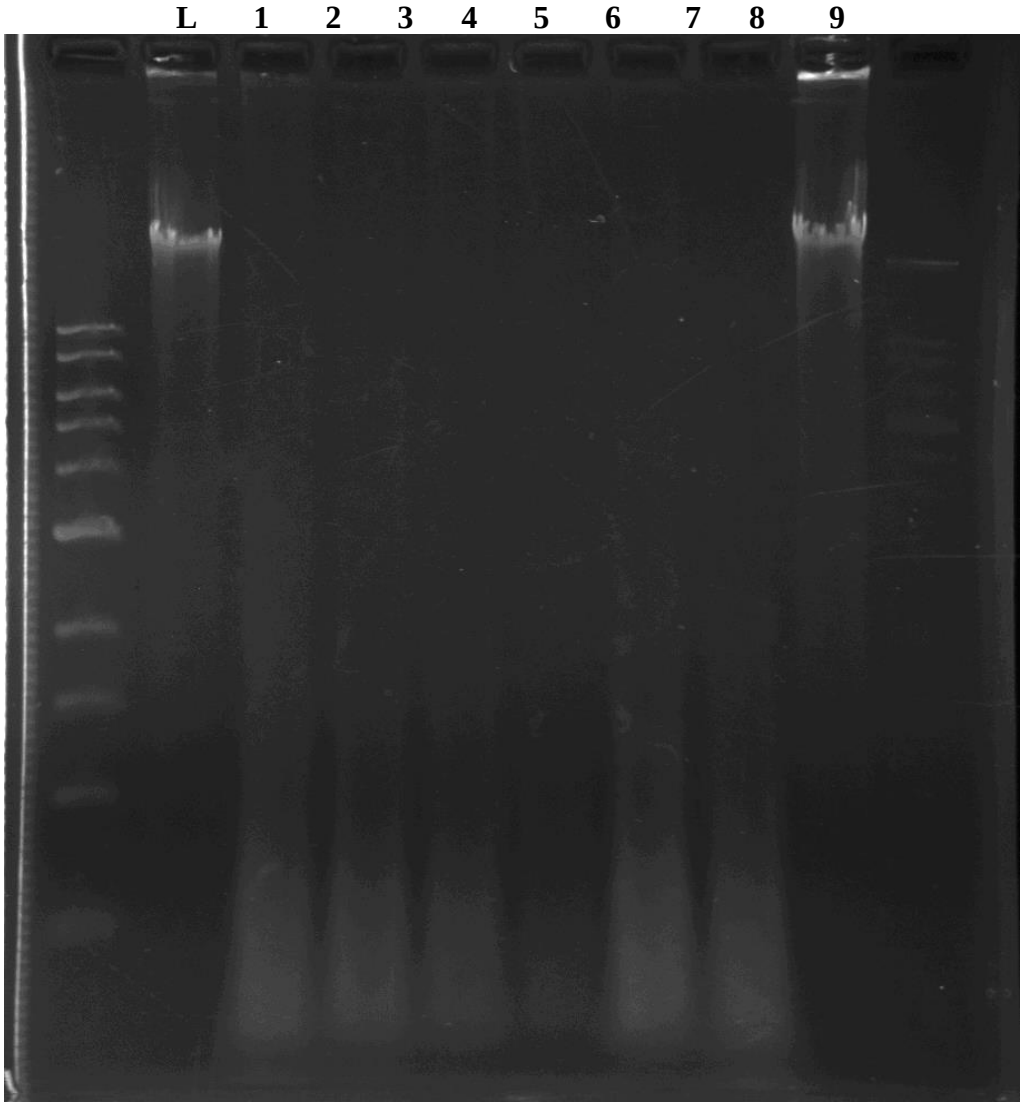

Figure 8. Ethidium Bromide stained Agarose Gel of digested genomic DNA. Lane L, NEB 1 kb size standard. Lane 2 control DNA sample. Lane 3, mouse DNA sample 1. Lane 4, mouse DNA sample 2. Lane 5, mouse DNA sample 3. Lane 6, mouse DNA sample 4 Lane 7, mouse DNA sample 5. Lane 8, control DNA sample. Lane 9, DIG molecular marker. Southern blot digestion analysis performed on 0.8% (w/v) agarose gel in 1X TAE.

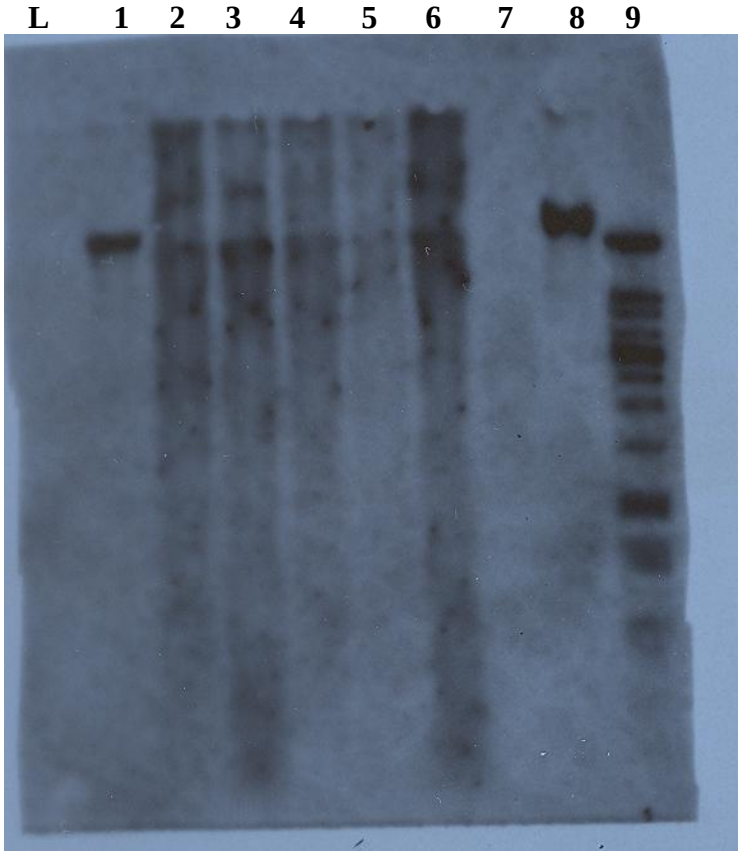

Figure 9. Southern blot on nylon membrane. Lane L, NEB 1 kb size standard does not appear. Lane 1 control DNA sample. Lane 2, mouse DNA sample 1. Lane 3, mouse DNA sample 2. Lane 4, mouse DNA sample 3. Lane 5, mouse DNA sample 4. Lane 6, mouse DNA sample 5. Lane 7, astrocytoma DNA sample. Lane 8, control DNA sample. Lane 9, DIG molecular marker The marker includes TRFLs of 21.2 Kb at the first band, 8.6Kb at the second, 7.4 Kb at the third, 6.1 at the fourth, 5.1 Kb, 5.0 Kb, and 4.9 Kb at the fifth, 4.2 Kb at the sixth, 3.6 Kb and 3.5 Kb at the seventh, 2.7 Kb at the eighth, 2.0 Kb and 1.9 Kb at the ninth, 1.6 Kb and 1.5 Kb at the tenth, 1.4 Kb and 1.3 Kb at the eleventh, 1.1 at the twelfth and 0.9 Kb and 0.8 Kb at the thirteenth.. Southern blot analysis performed on Nytran SuPerCharge positively charged nylon membrane.

Mean telomere restriction fragment length (TRFL) can be obtained visually by comparing the mean size of the telomere sequence smear to the molecular weight marker. Figure 10 shows the blot with the mean size of each smear measured with a metric ruler marked with a cross. This allows for estimation of relative TRFL against the distance traveled of each band in the DIG molecular marker.

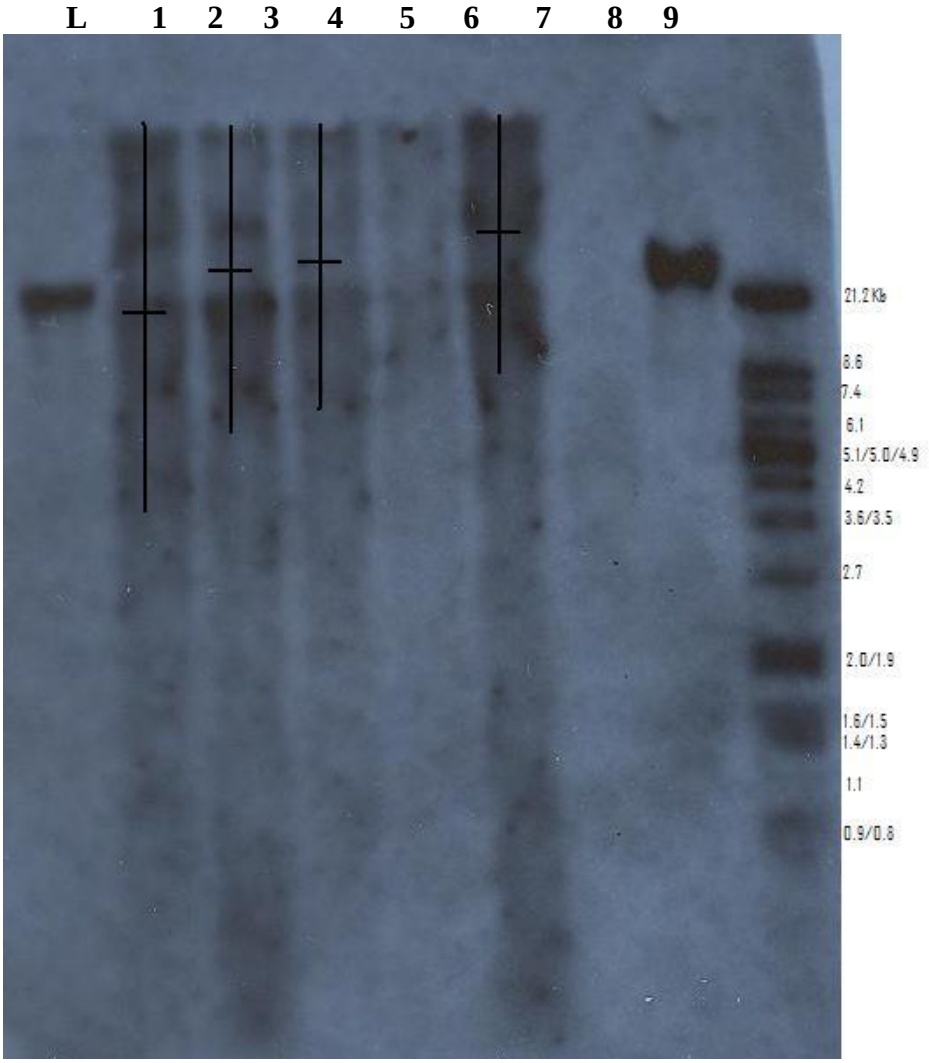

Figure 10. Southern blot mean TRFL. Lane L, NEB 1 kb size standard does not appear. Lane 1 control DNA sample. Lane 2, mouse DNA sample 1. Lane 3, mouse DNA sample 2. Lane 4, mouse DNA sample 3. Lane 5, mouse DNA sample 4. Lane 6, mouse DNA sample 5. Lane 7, astrocytoma DNA sample. Lane 8, control DNA sample. Lane 9, DIG molecular marker with band sizes displayed to the right of each band. The black crosses in each lane indicate the mean smear length. Lane 1 has a mean smear length just below the 21.2 Kb molecular marker indicating a TRFL of less than 21.2 Kb but more than 8.6 Kb. Lanes 2, 3 and 4 have mean smear lengths above the 21.2 Kb marker as to be expected from mouse DNA that generally exhibits telomere length over 24 Kb. Southern blot performed on Nytran SuPerCharge positively charged nylon membrane.

Discussion

Research performed on telomeres and telomerase aims to understand the regulation of these cellular processes that could lead to treatment of disease or improving the quality of life in the elderly. Diseases such as cancer or dyskeratosis congenita involve telomerase and its ability to maintain telomeres and "aging" results from large scale cellular senescence and death. Therefore, telomerase is a great target for treatment of these conditions. Cancer cells become immortalized when they regain the ability to extend their DNA and are then able to bypass senescence checkpoints indefinitely. Dyskeratosis congenita involves a mutated form of telomerase that is unable to elongate telomeres therefore resulting in premature aging of tissues and organs. The condition commonly referred to as "aging" is simple a manifestation of large amounts of cells experiencing telomere erosion leading to organ and tissue failure bringing about poor health. Developing treatments targeting telomeres and telomerase could lead to the treatment of or perhaps even reversing damage caused by these diseases.

Generally, in western medicine, treatment of a disease or syndrome often involves drugs; however, due to the recently discovered effects of electromagnetic resonance on tissue, electromagnetic therapy may be another option. Newly developed drugs are often offered at a very high price due to the rigorous testing of the FDA. Electronic circuits that elicit electromagnetic fields can be produced at low cost and are easily manipulated to oscillate at varying frequencies. In addition electromagnetic resonance may also be focused when paired with the right equipment allowing for exposure of specific areas on the body. However, in order to determine the possibility of electromagnet resonance as a therapy

and the safety of electromagnetic resonance therapy far more research needs to be performed.

This experiment was designed to determine if electromagnetic resonance exposure could affect the rate at which telomeres erode or elongate. The objectives of the experiments were to: 1) isolate primary cell lines to begin cells in culture, 2) engage the produced cells in culture in daily electromagnetic resonance exposure, 3) isolate and quantify genomic DNA from the cells in culture, and 4) compare the genomic DNA of the treated cells in culture against non-treated control cells in culture using singlplex qPCR, monochromatic multiplex qPCR and the Southern blot analysis techniques. Although the experiment did not work as planned it has set the foundation for further work involving studying the effects of this particular range of electromagnetic resonance on cells in culture.

Cell Culture Isolations

The establishment of cells in culture from primary cell lines allows for accurate comparison between non-treated control samples and samples from a group treated with a specific dosage of EMR. Unlike comparison of two different animals or organisms, cells in culture originate from identical lineage and share identical genotypes. Therefore, when comparing the effects of a stimulus on tissue, cell culture is ideal.

The first round of primary cell isolations worked well and the cells in culture adhered well within the first 48 hours of plating in T25 flasks. Most of the cells in culture grew to 80% confluence in 2 week intervals and were split into fresh T25 flasks. The cultures that did not grow all the way to 80% within the two week intervals had reached at least 60%

so in those cases a larger volume of cells was used to seed the new cultures. After the cells had reached 9 weeks old and had received ample treatments with the oscillating electromagnetic resonance cell samples were taken for analysis. This was done every 2 weeks by seeding approximately 25% of the cells into a new T25 flask and preserving the remainder in PBS as described in the cell sample preservation portion of the materials and methods. Shortly after the cells in culture had reached 15 weeks of age each culture began to form a thick white film within the media that appeared to be budding yeast cells when visualized under the phase contrast microscope. As a result all cells in culture were autoclaved and discarded.

The preserved cell culture samples were frozen prior to infection and were subjected to genomic DNA isolation. Unfortunately, the DNA yield was much lower than expected. According to the Qiagen Mini Prep Kit handbook approximately 6 μg genomic DNA should be present in the final 200 μL volume of eluent buffer after the final step. In all 16 samples from the cells in culture genomic DNA in each 200 μL volume of eluent buffer was less than 500 μg. In all 16 samples during each step of the isolation vials were spun at maximum speed in the Spectrafuge 16 M centrifuge which could explain the unexpected low yield. In further attempts at genomic DNA isolations, the protocol was modified so as to include the correct speeds for each step in the isolation and recorded in the materials and methods section. This allowed for higher genomic DNA yield as indicated by the isolated DNA concentrations of the astrocytoma cells in Figure 4.

The purified DNA acquired from the isolation was not enough to perform the Southern blot analysis but was enough to run singleplex qPCR (Figure 3) The telomere to actin RFU ratio normalized against the initial sample acting as a baseline was graphed. Under

normal circumstances the graph would be expected to exhibit steady decay over the course of several weeks in cell culture as telomere length begins to decrease with each cell division. However, the data is rather erratic and does not appear to follow a pattern. Also, according to the data the non-treated control group is not exhibiting decay either and experiences peaks of telomere length greater than those of the treated. If the electromagnetic resonance exposure is eliciting a response within the cell that is triggering telomere maintenance this could be explained by the positioning of the instrument emitting the electromagnetic fields and the non-treated controls. The non-treated control cultures were placed in an adjacent hood less than two meters from the hood where the experimental cells in culture were being treated. If the non-treated control cultures were also experiencing exposure to the electromagnetic fields then they too could display a response in telomere maintenance. In order to determine the extent of the electromagnetic fields produced by the multiple wave oscillator a high frequency signal analyzer would be needed to set up a perimeter of exposure. In order to alleviate exposure of negative control cells in culture later negative controls were moved to another lab during exposure times.

Although the generated data from the first round of cells in culture is interesting it is not enough to support any claims about telomere maintenance in response to electromagnetic resonance exposure. In order to generate more data several primary cell isolations were attempted but did not last in culture. Throughout subsequent primary cell isolations contamination occurred and cells in culture were discarded. After the first set of primary cells developed contamination the source of contamination was investigated ranging from

sterilizing all instruments and tissue hoods to inspecting the mice from which the tissue cultures originated.

Cells in culture originating from primary cell lines can be difficult to keep alive in culture and great care is required to prevent contamination. Unlike purchased immortalized cell lines that have been transformed with plasmids containing genes for antibiotic resistance, primary cell isolation originating cultures do not possess antibiotic resistance and therefore must not only be exposed to sterile equipment but also cell media may only contain low concentrations of antimicrobial agents. Therefore, minute levels of microbial contamination are able to grow quickly in nutrient rich media and overgrow the culture within the flask. In order to prevent microbial contamination it is very important to maintain sterile equipment and facilities.

In response to the unusually high levels of contamination in the primary cell isolates Dr. Zimmerman's lab, the lab that the mice were received from, was informed in case the contamination was resulting from an infection within the colony. Dr. Zimmerman's lab reported back a negative for colony infection. Therefore, cell culture media and ingredients were subjected to analysis.

It was discovered that the cell media mixture used in the cells in culture, when placed alone in a T-25 flask in the incubator, grew contamination. In order to determine which ingredient housed the contamination DMEM and NCS were placed in separate T-25 flasks without PSF overnight. Upon viewing through phase contrast optics it was apparent that the NCS at some point had become contaminated. Several samples were pulled from the common stock and all appeared to have the same contamination. The

common stock NCS was autoclaved and discarded. For future work, in order to exclude contamination all cell culture ingredients will be purchased new and filtered prior to use and common stock ingredients will be avoided if possible. In addition, all utensils, tools and water will continue to be autoclaved prior to use and all hoods will be sterilized as well.

Cell line maintenance

Maintaining the cell lines proved to be very difficult due to the high amounts of contamination experienced by every culture subsequent to the first. The first cell line was isolated during the summer months when no other labs were performing tissue culture work and a majority of the students had left for vacation. This could have been another contributing factor to the levels of contamination in the cells. Although contamination was imminent in the NCS it may have been at low enough levels to allow for the growth of the tissue cultures in the summer. When summer passed and more students and researchers began to enter and leave the cell culture lab keeping the amount of contamination under control quickly became futile. In the future it would be wise to advise against anyone entering the cell culture lab that is not working on cells in culture and also to schedule weekly sterilizations of the lab and equipment. Also, it may be prudent to offer classes on the sterile technique to beginning researchers so as to assure that contamination is kept at a minimum.

Electromagnetic Exposure of Cell Lines

In the first cell lines exposed to electromagnetic resonance, both treated cells and negative control cells more than likely received electromagnetic exposure. After

reviewing the data in Figure 3 it was taken into account that perhaps the electromagnetic field may have extended beyond the sterile tissue hood where the treated group was located and into the adjacent sterile tissue hood housing the negative control group. In order to correct this all further treatments happened in a separate lab from the sterile tissue hood where the non-treated control groups were placed. The separate lab was separated by several rooms, the distance and insulation of the building ensured absorption and dissipation of the electromagnetic fields.

Cell Sample Preservation

The preservation of the cell lines technique can be useful in minimizing the time and resources necessary to perform costly and time consuming techniques. The samples in the qPCR techniques can be prepared separately, however, the amplification process takes several hours and it is best to run samples at the same time to reduce variables. Running samples together also allows for easy comparison between amplicon RFUs and melting points within the BIORAD CFX Manager software. The Southern blot analysis requires the use of expensive reagents and preparation of several solutions that have a relatively short shelf life. Preserving several samples for the Southern blot is optimal in that it allows for the utilization of every well in a gel and the most efficient use of costly reagents. When comparing samples within an experiment running multiple samples on the same blot also allows for direct comparison as well as mathematical analysis.

Genomic DNA Isolation

When isolating genomic DNA, even when using a specialized kit, it is important to use care at each step so as not to lose the DNA in the process. The genomic DNA isolation

performed on the first set of cells in culture, DNA quantification results in Table 1, resulted in the loss of much of the DNA from each sample due to poor technique. The samples were spun at maximum speed throughout each step of the protocol and ultimately resulted in very low yield. Subsequent DNA isolations were successful in achieving higher DNA yields. The technique was tweaked by adjusting the centrifuge speeds at each step keeping the DNA from each sample adhered to the minispin columns until the elution step. The data collected from the subsequent successful DNA isolations is represented in Table 4. The DNA yield was much higher than before indicating that reducing the centrifuge spin allowed for the proper elution of DNA in the collection step and prevented premature loss of purified DNA.

NanoDrop 2000 Spectrophotometer

The NanoDrop 2000 allows for rapid and reliable quantification of DNA and requires little sample. Since the process only requires 1 μL to quantify the DNA within the entire solution even small samples, like the 10 μL samples in the singleplex qPCR reaction, can be quantified with plenty of DNA left over for the technique that needs to be performed. Since there is no dilution involved in the NanoDrop protocol, dilution calculations are not necessary. Also, the NanoDrop 2000 software program calculates DNA concentrations reducing the chance of mathematical mistakes in an OD calculation.

Ethanol Precipitation

If a problem occurs during a DNA isolation procedure that results in low yield of purified DNA resulting in a concentration of DNA that is too low to perform a desired technique the ethanol precipitation procedure can condense the concentration of DNA enough to

allow completion of the desired technique without repeating an entire experiment. The DNA concentrations acquired after the first run of genomic DNA isolations, quantification data in Table 1, were too low to even perform PCR. After ethanol precipitation, quantification data in Table 2, the concentration of DNA became high enough to quantify the sample and run several rounds of qPCR. Likewise, in the mouse DNA samples, the DNA concentrations were too low to run the Southern blot. After performing the ethanol precipitation on the mouse DNA samples, data for before and after concentrations in Table 3, the DNA concentrations in each sample were high enough to run this DNA demanding technique. Unfortunately, there was not enough DNA left in every sample after running the Southern blot to run Monochromatic Multiplex qPCR (MMqPCR). In future work it would be best to grow cell cultures in a T75 flask as opposed to a T25 flask so as to allow collection of larger cell samples and more DNA.

Determining the Cell volume Necessary to run the Southern blot

Counting cells with a Hemocytometer can be an effective tool for estimating the total number of cells in a sample. However, utilizing an automatic cell counter can be easier and can be a more effective method. According to the cell numbers and subsequent genomic concentrations obtained from the cell volumes it is apparent that the more cells that can be obtained the better. The highest volume of cells, 1.5 mL, was just enough to generate enough DNA, 1.5 μg in 10 μL, to run the Southern blot. Although the smaller volumes contained enough DNA to run the Southern blot, ethanol precipitation would need to be performed to concentrate the DNA into a small enough volume to run the Southern blot.

Singleplex Quantative PCR

With the aid of intercalating dyes such as SYBR Green the polymerase chain reaction can be quantified at the end of each cycle of replication. This allows for relative quantification of DNA associated with genes of interest or sequence of interest from a DNA extract. The telomeric regions of samples subjected to qPCR in this experiment were compared to an actin gene known to occur only one time within the mouse genome to obtain a relative quantification of the telomeric sequence.

Figure 3 illustrates the data acquired from the singleplex reaction with each average from two separate reactions of the telomeric product RFUs divided by actin RFUs and normalized against the baseline RFUs for the negative control of each tissue type. The standard deviation was calculated between the two reactions and used to incorporate error bars on the graph. The error bars show that there is a high correlation between the two reactions. This indicates that the experiment is repeatable with a low margin of error. According Figure 3 it appears that the telomeric regions of the cell samples was being maintained throughout subsequent generations of cells. Although this is what was expected to be seen in the treated group the negative control group should exhibit a gradual decrease in telomeric length. As mentioned earlier this could be a product of the negative control group receiving treatment due to proximity to the electromagnetic resonance. However, according to the singleplex data there is an obvious maintenance of telomeric length.

In qPCR reactions primers can dimerize and create polymerase products that are not a part of the desired product but elicit an RFU nevertheless. To ascertain that a primer

dimer product is not present the amplicon products are run in an agarose gel to be separated and viewed under UV light to detect the number of products formed. If the primers are designed well they will share a high affinity for the DNA template strand and low to no affinity for each other. If the primers are not designed well they will share an affinity for both the template strand and each other and several products of varying size can form. Figures 2 and 3 show that only one product is forming indicating that the primers do indeed share a much higher affinity for the template and unwanted amplicon products are not incorporated into the end RFUs.

Each culture was split every two weeks since seeding from isolation and approximately 20% of each culture was used to begin a subsequent flask. Therefore, each culture had experienced approximately 30-50 divisions before becoming infected with contamination. At this point it would be anticipated that a noticeable decline would occur in telomeric length. According to the singleplex telomere length is being maintained in both treated cells and negative controls. There are minor fluctuations in telomere length but average length is maintained throughout the duration of the life of the cells well above baseline. Although this data is not enough to make any definite inferences, it does justify further research under more controlled circumstances with more sensitive techniques.

Under normal circumstances cells lose telomeric sequence with each cell division and eventually reach a critical point which triggers the cells to enter senescence. Since it appears that these cells are maintaining their telomeric length it would have been optimal if they would not have been contaminated and had continued to proliferate so that more data could be collected and analyzed. The cell culture would have been subjected to

further telomere analysis and tested to see if the cells had become immortalized, similar to the way cancer cells become immortalized. If the cells had been maintaining their telomeres and not become immortalized lines this technique would hold promise for treatment in other organisms. However, if the cells had become an immortal cell lines and exhibited characteristics similar to those of cancer cells this technique would not be best used on other organisms and would best be utilized for creating immortalized cell lines for research purposes.

Multiplex qPCR

Multiplex qPCR, according to published literature, has not yet been performed on mouse DNA. The technique described above is a novel technique for quantifying mouse DNA using a multiplex reaction. Unfortunately, the BIORAD CFX manager supplied by BIORAD with the MiniOpticon qPCR system appears to be unable to separate quantification steps performed in the same cycle. Table 5 contains the end RFU values for each sample subjected to multiplex qPCR, but does not discriminate between the two quantities of products produced at different stages of each cycle. The MiniOpticon system, however, allows for multiple quantification steps within each cycle.

Due to the fact that the MiniOpticon system offers multiple quantification steps within each cycle it is likely that the BIORAD CFX manager is able to separate the quantities when compiling the data. BIORAD was called directly on several occasions so as to speak with product support. The protocol data file and data sets were sent to representatives at BIORAD for analysis. BIORAD responded indicating that the reaction was set up correctly and that the parameters on the machine were correct. Unfortunately,

even after correspondence with BIORAD, the results were the same with subsequent experiments and the quantification values continued to be combined by the BIORAD CFX manager software.

Due to the inability to discriminate quantification values within the BIORAD CFX manager, a melting point analysis and separation of amplicon products on agarose gel was performed. Figure 6 contains the melting point data acquired by the BIORAD CFX manager of each product from three separate tests of the multiplex reaction. The first well of the multiplex reaction contained the 36B4 primers, the second well contained the 36B4 and telomere primers and the third well contained the telomere primers. The peak for the melting point of the 36B4 primers is shown in peak and occurs at a high temperature, about 88°C, just as expected due to the GC clamp design within the primers. The telomere primer products, shown in blue, have a much lower peak indicating a melting point at about 77°C. The two primer products combined, shown in green, have two separate melting points that line up with the same temperatures as the primer products alone. Both products are being formed in the combined reaction indicating that the multiplex reaction is working as predicted.

To further support the formation of the desired primer products and not primer dimers each reaction product was subjected to gel electrophoresis. Figure 7 contains two gels containing the amplicon products from two of the multiplex reactions. Part A of Figure 7 contains the 1Kb NEB standard along with the amplicon products of the 36B4 primers in lane 1, the combined amplicon products of the 36B4 primers and telomere primers in lane 2 and the amplicon products of the telomere primers in lane 3. It appears that in lanes 1 and 3 only one product is being formed as expected. Lane 2 appears to have two products

but it is hard to tell since the two products did not separate very well on the 0.8% agarose gel in TAE buffer. A second gel was cast at a concentration of 2.0% agarose in TAE buffer in an attempt to further separate the two products formed. Lane L contained the 1 Kb NEB standard and lanes 2 and 3 contained amplicon products from both the 36B4 primers and the telomere primers. This gel does not contain amplicon products from the primers alone. The higher concentration agarose gel performed better at separating the products and two distinct bands are visible further supporting that two product are being formed in the multiplex reaction.

According to the data it appears that the multiplex reaction is working as predicted. However the BIORAD CFX manager is unable to distinguish between the quantification of the two amplicons separately. Since this technique is far less expensive and time consuming than the Southern blot and more reliable than the singlplex qPCR reaction, further work will be performed on the BIORAD CFX manager in an attempt to extrapolate the two values separately. The product support team at BIORAD indicated that the BIORAD CFX manager is able to separate signal acquisitions performed within the same cycle, therefore, the product support team will be contacted again for further assistance configuring the BIORAD CFX manger.

Southern blot analysis

The Southern blot technique is the oldest and most accepted technique for quantifying telomeric length. However, due to the cost and time necessary to run the Southern blot, developing a multiplex qPCR technique to measure telomeric regions would be optimal. Figure 8 illustrates the banding patterns of the digested DNA samples after being

separated by gel electrophoresis in a 0.8% agarose gel in 1X TAE. Lane L of the gel contains the 1Kb NEB standard, lane 1 control DNA, lanes 2 through 6 digested mouse DNA, lane 7 astrocytoma DNA, lane 8 control DNA and lane 9 the DIG molecular marker. The banding of all 8 digested samples along with the standard are clearly visible with well pronounced smears in all of lanes 2 through 7 except for the light smear in lane 5. The smearing that occurs toward the bottom of the gel is expected as the restriction digest is designed to digest all but telomeric and subtelomeric DNA. According to the banding of the gel, the presence of the control DNA bands and the molecular weight marker it appears that the digest was a success.

The resulting image from the photograph taken of the completed blot is shown in Figure 9. The dark background of the blot indicates a high amount of background luminescence. When the nylon membrane was placed on the gel for transfer the membrane was applied dry. This could account for the high amount of background. Next time, wetting the blot before application to the gel should correct this. The 1 Kb NEB standard does not appear on the blot which is to be expected since the standard does not contain the telomeric repeats specific to the probe. Although the astrocytoma DNA bands appear in the gel the probe on the blot does not show anything as far as telomeric DNA. This could be due to a mistake in calculating the amount of DNA digested, too little DNA would result in little to no telomeric signal. It also appears that the telomere smears continue far down the gel. This could be caused by a wash step at too low of temperature. The stringent wash steps are crucial for removing elements that can give false signal and in the future will be performed for double the recommended time.

The five mouse DNA samples, the control DNA and the molecular weight marker all appear on the blot indicating that the blot and probe of the telomeric DNA was successful. The TRFL can be estimated visually thanks to the DIG molecular marker supplied with the TELOTAGGG assay kit. Figure 10 illustrates the blot with the mean smear length marked by a black cross. The mean smear length can be used to estimate the relative TRFL by visual comparison against the DIG molecular marker. Lanes 2, 3 and 4 all appear to have smear means well over the top band, 21.2 Kb, DIG molecular marker. This appears to be correct since all four samples came from the same litter of young mice and mice, at least the common lab mouse, express very long telomere lengths, greater than 20 Kb. The first band, however, falls just below the top band, 21.2 Kb, and well above the second band, 8.6 Kb. Unfortunately, with this molecular weight marker, visual estimation of TRFL over 21.2 Kb is not possible and the sensitivity in the high Kb range is not good enough to distinguish between telomere lengths in between 8 and 20 Kb. In future experiments it would be wise to use cells originating from a mammal that has shorter telomeres that would be more easily measured.

Several techniques are currently well known to allow the estimation and quantification of DNA and other less expensive and time consuming techniques are being developed. Although the Southern blot currently considered the most reliable and accurate telomere length assay it is very time consuming to perform, involves costly reagents and requires large amounts of DNA. Developing methods such as multiplex qPCR allows for quicker, less effective telomere length assays using a fraction of the DNA needed for a Southern blot. Further experimentation needs to be performed utilizing both the Southern blot analysis and multiplex qPCR technique to obtain the percent

consensus between the two techniques. If the two techniques prove to be similar in quantification of identical DNA samples it would be safe to say that the multiplex qPCR reaction is sufficient in quantifying telomere length.

Validating the multiplex qPCR technique would also assist in the further investigation of the effects of electromagnetic resonance on telomeric regions of DNA. Utilizing a fast and cost effective technique such as multiplex qPCR would allow for more data collected from each sample and more consistent values. With the amount of DNA necessary to complete the Southern blot if a mistake or accident were to occur there would not be enough DNA to attempt the experiment again. However, using the multiplex qPCR reaction, even if the experiment were to fail several times there would be plenty of DNA to repeat the experiment.

Although the data collected is not enough to make any direct correlations between electromagnetic resonance exposure and telomere length, it does warrant further investigations to the effects of electromagnetic fields on cells. According to the singleplex qPCR data it appears that telomeric DNA is maintained in cells that receive daily electromagnetic exposures. Given that electromagnetic fields are produced by all electronic instruments that are ubiquitous in developed countries, it stands to reason that more research needs to be performed on the biological responses that these fields elicit. If the resonating fields utilized in this experiment promote telomeric elongation they would be a great therapy for individuals suffering from telomere loss such as those with dyskaratosis congenita or even individuals suffering from old age. However, if the resonating fields are inducing immortalized cell lines then it should be considered that

these fields could prove dangerous to organisms and should only be used to induce immortalization in cell lines used for research.

Many of the expected outcomes of this research project were not observed, however, a few inferences can be made from the results. Monochromatic multiplex qPCR appears to be able to be used to quantify telomeric regions of DNA, once the software issues have been resolved. According to the generated data from the singleplex qPCR reaction, resonating electromagnetic may impact cell lines and elicit a cellular response that may regulate telomere length. Hopefully, in the near future, another project will be performed using the same electromagnetic resonance along with perfected multiplex qPCR and Southern blotting techniques. The generation of more data will help to indicate the kinds of effects that electromagnetic resonance has on telomeric regulation of DNA.

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