Telomere Binding Protein Antagonists for Stem Cell Therapy

By

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Abstract

Understanding the mechanisms through which stem cells self-renew is important for the development of new therapies to repair and restore damaged tissue. This research explores possible mechanisms of telomere mimic action and seeks to validate a new assay for evaluating telomere binding antagonists and studying the effects of telomere binding antagonists on stem cells.

This project is important for screening potential drug properties for use in bone marrow therapy and identifying new reagents to modulate stem cell growth and differentiation. This study is designed to test the hypothesis that our labeled telomere 'mimics' will interact with telomere binding proteins to modify the binding profile within the nucleus. Modulation of telomere binding proteins at the telomere could potentially explain the observed enhancement of highly pluripotent stem cells following telomere mimic treatment.

The most likely targets for telomere binding protein antagonism are TRF1 and TRF2, proteins that bind directly to telomeres involved in controlling lengthening, regeneration and maintenance of telomere structure and function. There are no published reports and only preliminary experiments to demonstrate relationships between drug concentrations and telomere binding protein TRF2 concentrations in cell nuclei.

Project goal is to measure interactions between telomere binding protein antagonists and telomere binding proteins. There are no published reports and only preliminary experiments that demonstrate relationships between telomere mimic concentrations and telomere binding protein TRF2 concentrations in the nucleus, this research measured interactions between telomere binding protein antagonists and telomere binding proteins, finding that the TAG9_1 oligonucleotides had the highest intensity bands on a Western Blot, followed by TAG9_2 when treated with TRF2 primary antibody.

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INTRODUCTION

Understanding the mechanisms through which stem cells self-renew is important for the development of new therapies to repair and restore damaged tissue. This research explores the use of telomere binding protein antagonists to increase the pluripotency of hematopoietic stem cells to regenerate or repair tissue. An infusion of stem cells can lead to tissue regeneration, which shows promise in therapies for aging, cancer, bone marrow transplants, skin grafts, and general healing damaged tissue (Patel 2009).

Eukaryotic cells have repetitive DNA sequences, known as telomeres, with the sequence 5'-d$(TTAGGG)_n$-3' at each end of the chromosome. The DNA of telomeres are guanine-rich repeated sequences containing both double-stranded and single-stranded repeats decorated with a unique complex of proteins that form the "T-loop." The T-loop protects the telomeres from attrition through the actions of specific telomere binding proteins. These proteins play a role in maintenance of the normal cell phenotype, cellular self-renewal, aging, and diseases that include cancer. Tumor cells can express higher levels of telomerase (Shay and Bacchetti 1997), which results in the telomeres of cancer cells being maintained at a length that allows the cell to divide uncontrollably (Shay and Wright, 2006). Telomerase activity is partly dependent on access to the telomere, which is regulated by telomere binding proteins. However, much of the research to date has focused on telomerase rather than access to the T-loop.

The enzyme telomerase is crucial for maintenance of the telomere and is unregulated in 98% of cancers. This enzyme has been the focus of intense basic science and clinical research because inhibition of telomerase in cancer cells leads to apoptosis and tumor regression (Tamakawa et al. 2010). The leading telomerase inhibitor Imetelstat

(GRN163L) is currently being investigated in eight clinical trials for a variety of cancers including chronic lymphoproliferative diseases, solid tumors, multiple myeloma, non-small cell lung and breast cancer. Imetelstat is a potent and specific telomerase inhibitor and so far the only drug of its class in clinical trials (Röth et al. 2010). The use of short oligomers to target telomerase led to some critical observations that could lead to new methods to rejuvenate bone marrow and enhance pluripotency of stem cells (Mata et al. 2000).

During a study of pharmacokinetics of a 6-base telomerase inhibitor in swine (Mata and colleagues 1997) observed changes in bone marrow derived stem cell populations. This led to additional studies of this same oligomer in mice given the drug 5-fluoruracil to deplete circulating hematopoetic cells. This study revealed that the reduction of granulocyte and erythrocyte lineages during infusion of drug was not due to toxicity of the drug but rather because a more hematopoetic lineage, the highly pluripotent (HPP) bone marrow cells, was expanding and not differentiating into more defined cell types, specifically CFU-GM (colony forming unit, granulocyte macrophage) and BFU-E (Burst-forming unit-erythrocyte) (Mata 1997).

TRF, (Telomere Repeat-Binding Factor) proteins, also known as TERF, plays a role in controlling lengthening, regeneration and maintenance of telomeres. TRF1 is a negative regulator of telomere length (Her et al. 2009). It binds to the telomeric double-stranded TTAGGG repeat and is a component of the shelterin complex (Hanaoka et al. 2005). Shelterin associates with and protects the telomere ends. Without this activity, telomeres are accessible to the DNA damage surveillance machinery, and chromosome ends are inappropriately processed by DNA repair pathways (Ye J. et al. 2010)

In contrast to TRF1, the TRF2 protein is associated with control of telomere length (Kendellen et al. 2009). It binds to duplex TTAGGG repeats and is thought to protect chromosome ends by maintaining the correct structure at the terminal ends of the telomeres (Figure 1). Inhibition of TRF2 has been shown to result in apoptosis (cell death). TRF2 is over expressed in a number of human tumors and research has shown that the over expression of TRF2 can be oncogenic (leading to cancer) in a mouse model of carcinogenesis (Bombarde O et al. 2010).

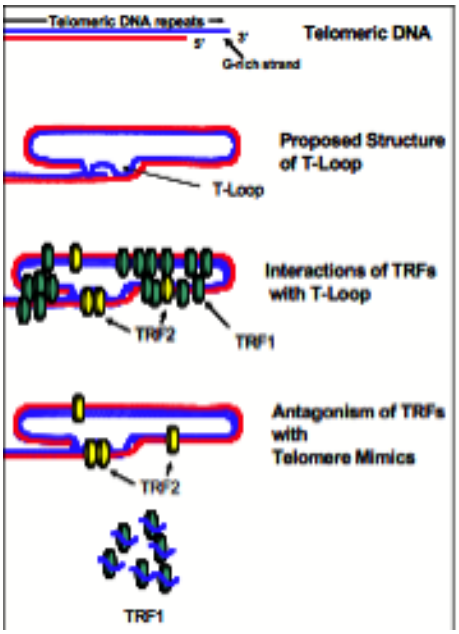


Figure 1. (Mata 1997)

Figure 1. Proposed model of TRF antagonism.

TRF1 and TRF2 are required for human telomere maintenance to protect the telomere from degradation (Broccoli et al. 1997). It has been previously shown that over expression of TRF1 in a telomerase-expressing cell line (any non-somatic cell line) leads to progressive telomere shortening, whereas inhibition of TRF1 increases the length of telomeres (Iwano et al. 2004).

Oligonucleotide based telomerase inhibitors are currently being evaluated in eight clinical trials for several types of cancer (Indiana University, Geron Corporation (six trials), Stanford University). GRN163L is an oligonucleotide, also called Imetelstat sodium, which has demonstrated potent telomerase inhibitory activity at very low concentrations in biochemical assays, various cellular systems and animal studies (Ginelle et al. 2006). This oligonucleotide binds directly to the template binding domain of the active or catalytic site of the telomerase reverse transcriptase. The high affinity binding to the RNA component results in direct, competitive inhibition of telomerase enzymatic activity. Clinical data from an ongoing trial showed that Imetelstat inhibits telomerase both in the bulk myeloma fraction (accumulation of malfunctioning or "cancerous" plasma cells) as well as the stem cell-containing fraction in bone marrow (Ginelle et al. 2006).

Although many of these studies are still in progress, there have been reports of transient effects on white blood cell fractions, specifically, bone-marrow-derived mesenchymal stem cells in rats (Tokcaer-Keskin et al. 2010). This observation is significant because early work with telomere mimics demonstrated similar transient effects in mice and swine (Mata et al. 1997, Mata et al. 2000). We hypothesize that the telomere mimics that inhibit telomerase activity as competitive inhibitors also compete for TRF binding with the T-loop, and that these mimics will interact with telomere binding proteins to enhance regeneration of highly pluripotent stem cells. Further, preliminary results suggest that effects of telomere binding protein antagonists may be distinct from the cellular responses from telomerase inhibition.

Telomere mimics used in this research were 9 base phosphothioate oligonucleotides. These are phosphorothioate oligonucleotides, which means that they consist of a sulfur group in place of a non-bridging oxygen in the phosphodiester backbone of the DNA, attached to two guanines. This substitution on the phosphate backbone makes the complex nuclease resistant relative to the diester (normal) DNA structure.

The objective of this research was to explore possible mechanisms of telomere mimic action that also might explain effects of Imetelstat observed in recent clinical trials. TRF (Telomere Repeat-Binding Factor) proteins, specifically TRF1 and TRF2, are the likely targets for telomere mimic antagonists. These proteins play a role in controlling lengthening, regeneration and maintenance of telomeres, and are believed to promote the formation of the closed state of a telomere, the T-loop (Griffith et al. 1999). We hypothesized that our labeled telomere mimics will interact with telomere binding proteins. Based on preliminary data, there is an expected sequence preference for TRF binding (Mata, unpublished). There are no published reports and only preliminary experiments that demonstrate relationships between telomere mimic concentrations and telomere binding protein TRF2 concentrations in the nucleus. Our work measured interactions between telomere binding protein antagonists and telomere binding proteins.

Materials and Methods:

Materials: The oligomers were made using recommended protocols for automated phophorothioate DNA synthesis (BioSynthesis, Lewisville, Tx). Each was synthesized as a 9-mer oligonucleotide with the following sequences and molecular weights:

TAG9_1: GGGTTAGGG, 66.67% CG content. 2962.87 MW.

TAG9_2: TTAGGGTTA, 33.33% CG content. 2896.85 MW.

Each compound was diluted to 10 mM stock concentrations in 1x Hanks (DPBS) buffered salt solution.

Collection of cells: Mouse bone marrow samples were collected from mouse femur by flushing the exposed marrow compartment with saline, using a 22 gauge needle. Cells were washed three times in 1X Hank's buffered salt solution and maintained in RPMI 1640 medium with L-Glutamine media bottles (BioWittaker; Walkersville, MD). Human aortic fibroblast cells and all other cell lines used were from American Type Tissue Culture (Washington DC).

Culturing cells: Cells were placed in flasks with media (RPMI 1640) containing 20% FBS (fetal bovine serum), 1% antibiotic-antimycotic (anti-anti; Penicillin, Streptomycin, Amphotericin B, stock concentration of 10 mL/L each) and 1% non-essential amino acids added to RPMI-1640

Encapsulating cells materials: Methods were adapted from Wittig et al. 1989.

To encapsulate the cells prior to disrupting the cell wall, human aortic fibroblast (AF) cells grown in RPMI-1640 medium for one to two weeks were detached from the flask with trypsin and washed twice with 50 mL Phosphate Buffered Saline (PBS; pH. 7.2) at room temperature. Cells were then suspended in 20 mL PBS to yield approximately 2.5 x 10^7 cells per mL. These cells were stored at 39° C.

In separate flasks, 5 mL of 2.5% (wt/wt) low melting agarose liquefied and cooled to 39° C. 50 mL liquid paraffin was warmed to 39° C. The warmed cells, paraffin and agarose were combined in a pre-warmed 300 mL Erlenmeyer flask in the order of liquid paraffin, cells, and agarose. Mixture was emulsified by shaking at 400 rpm in rotary shaker at 20° C for 30 seconds. Following shaking, the mixture was equilibrated in ice-water bath (0° C) for 5 minutes. 100 mL ice-cold PBS was added to mixture and flask was rotated manually to combine. Solution was quickly distributed to glass centrifuge tubes (80 mL capacity tubes) and centrifuged at 3,500 g at 0° C for 5 minutes. To collect the encapsulated cells, supernatant was removed by aspiration and the microbead pellet was washed three times with 60 mL of ice-cold PBS per wash, and centrifuging for 5 minutes at 4,800 g. Pellets were united resulting in 10-12 mL of microbead encapsulated metabolically active cells. This mixture was then subjected to an isotonic solution (pH. 7.6, Wittig et al. 1989) that ruptured the outer cell wall while maintaining the patency of the nuclear membrane. In order to visualize the nuclei within the agarose beads a 1 ml aliquot of the mixture was incubated with nuclear dye 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to A-T rich regions in DNA. Beads were

assessed under microscope at 40x under white light and fluorescent illumination (See figure 2A and 2B).

Alternatively, whole cells were used for Western Blot analyses using aortic fibroblast cells.

Isolation of metabolically active nuclei for Western Blots procedures:

Human aortic fibroblast cells were grown in media supplemented as described above. After three days of growth, medium was removed from flask, and cells were washed twice with 20 mL of 0.9% NaCl, then 2 mL trypsin to lift cells from adhering to flask. Trypsin was removed quickly and new medium (50 mL) was added. The cells were then split into three 12 well plates.

Treatment of cells: The cells were assigned treatment groups, four samples per group. The groups were treated with 10 μM TAG9_1 (GGGTTAGGG), TAG9_2 (TTAGGGTTA), or received no treatment (control). For this, old media was removed from each plate using a Pasteur pipette, 300 μl of fresh media were added, and 3 μl of selected oligo or vehicle control. Wells 1-4 received TAG9_1, wells 5-8 received TAG9_2, and wells 9-12 received no oligo (control). Plates were incubated for two hours in an incubator at 37° C with 5% CO_2. After two hours, cells were checked under microscope and media was removed from top using pasture pipette. 100 μl of Ripa buffer and protease (20mL Ripa + 20 μl protease inhibitor for stock solution) were added to each well. After five minutes, the solution in each well was pipette vigorously to disturb cells and samples were collected into labeled tubes to be analyzed by Western Blot assays for detection of TRF.

Time Course Study:

For the treatment of these cells, the same procedure listed above was used, and replicates were allowed to incubate for 1, 2, 4, 8, and 16 hour periods. Cells were then collected as described above.

Western Blots: Western blots were performed according to manufacturer's protocol using the Power Ease 500 ™ system (Invitrogen Carlsbad CA). Samples were prepared using stock buffers (NuPAGE LDS Sample Buffer 4X) and Reducing Agent (NuPAGE Sample Reducing Agent 10X).

In 0.5 ml vials, 15μl sample, 10μl sample buffer, and 6μl DI water were combined for a total volume of 30μl. Samples were mixed, and placed in a 70°C water bath for 10 minutes.

Gel buffers were prepared using 50 ml 10X MES SDS Running Buffer: (stock 20X) mixed with 950 ml ultra pure water. Protein transfer buffer was prepared with NuPAGE Transfer Buffer (stock 20X) by adding 50 ml stock transfer buffer, 100 ml methanol and 850 ml ultra pure water.

The acrylamide gels used in the study were NuPAGE 4-12% Bis-Tris Gel, 1mm x 12well and the gels were set up according to packaging directions. One well per gel contained 5μl ladder (Sea-Blue Pre-Stained Protein Standard), and 20 μl sample was added per well. Gels were run according to the manufacturer's directions. To transfer the protein from the gel to the membrane a standard 'sandwich' procedure was used according to manufacturer's directions and transfer was run for one hour.

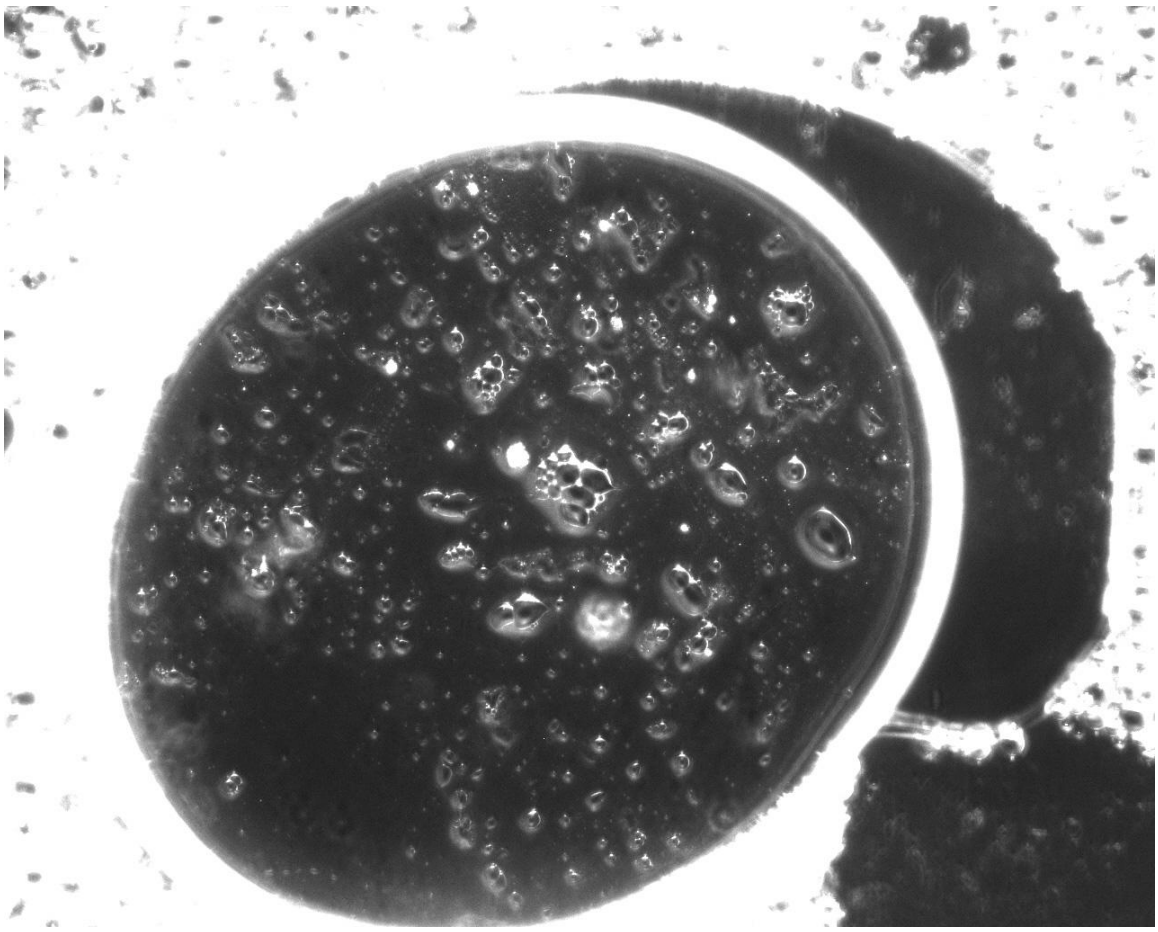
The membrane was then taken out of the apparatus and blocked in 5% non-fat dry milk (NFDM) in TBS with 0.05% Tween-20 for one hour at room temperature. Primary

antibody TRF2 monoclonal mouse (Abcam, Cambridge MA) at 1:5000 with 2.5% NFDM in Tween TBS solution was added to the blot, and incubated overnight at 4° C. Membrane was washed three times with 20 ml TBS-Tween for 10 minutes each time. Membrane was blocked in 10% NFDM in TBS-Tween for 10 minutes (20ml total volume), solution was removed and secondary antibody was added at 1:2000 concentration, (Goat anti-mouse LgG H&L DyLight 549, abcam) plus 2.5% NFDM in TBS-Tween for 60 minutes at room temperature, or overnight in refrigerator. Antibody was removed and saved, and membrane washed three times (20ml each wash) in TBS-Tween. Chemoluminescent reagents (Thermo Scientific, Austin TX) in a 1:1 ratio to 5ml volume were poured on membrane on acetate paper and protected from direct light exposure for 5 minutes, then excess was blotted with wipes, and blot was covered with second acetate on top. Film was exposed to blot for 30 minutes, then developed using an automated film developer.

Results:

Our objective was to examine interactions between telomere binding protein antagonists and telomere binding proteins. To do this, we first explored the methods for cell encapsulation. Figure 2 A and B show that nuclei were successfully encapsulated in agarose beads, that yielded pictures of metabolically isolated nuclei, fluoresced in picture.

Figure 2A



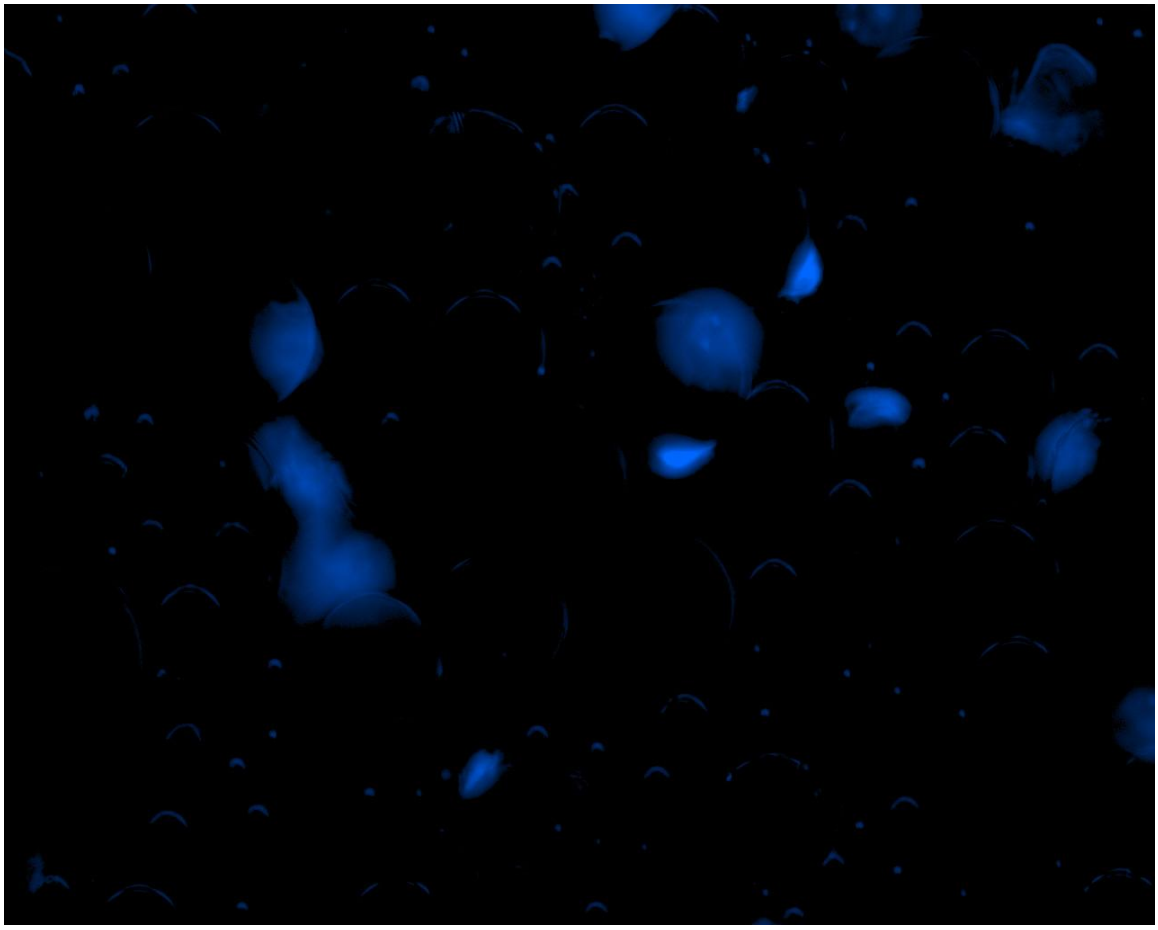


Figure 2 B

Figure 2 (A and B). Isolated metabolically active nuclei visualized at 40X under light (A) and (B) fluorescence illumination.

Western Blot procedures were performed multiple times, using both mouse bone marrow cell cultures and human aortic fibroblast cells. Despite adjusting procedures, cell amounts, washing methods, and secondary antibodies, only one blot showed distinct bands. However, we were unable to reproduce this blot to date.

A Western Blot using TRF2 showed bands with increased intensity and size for samples treated with TAG9_1, and decreased for samples treated with TAG9_2, and barely visible bands for untreated samples (Figure 3).

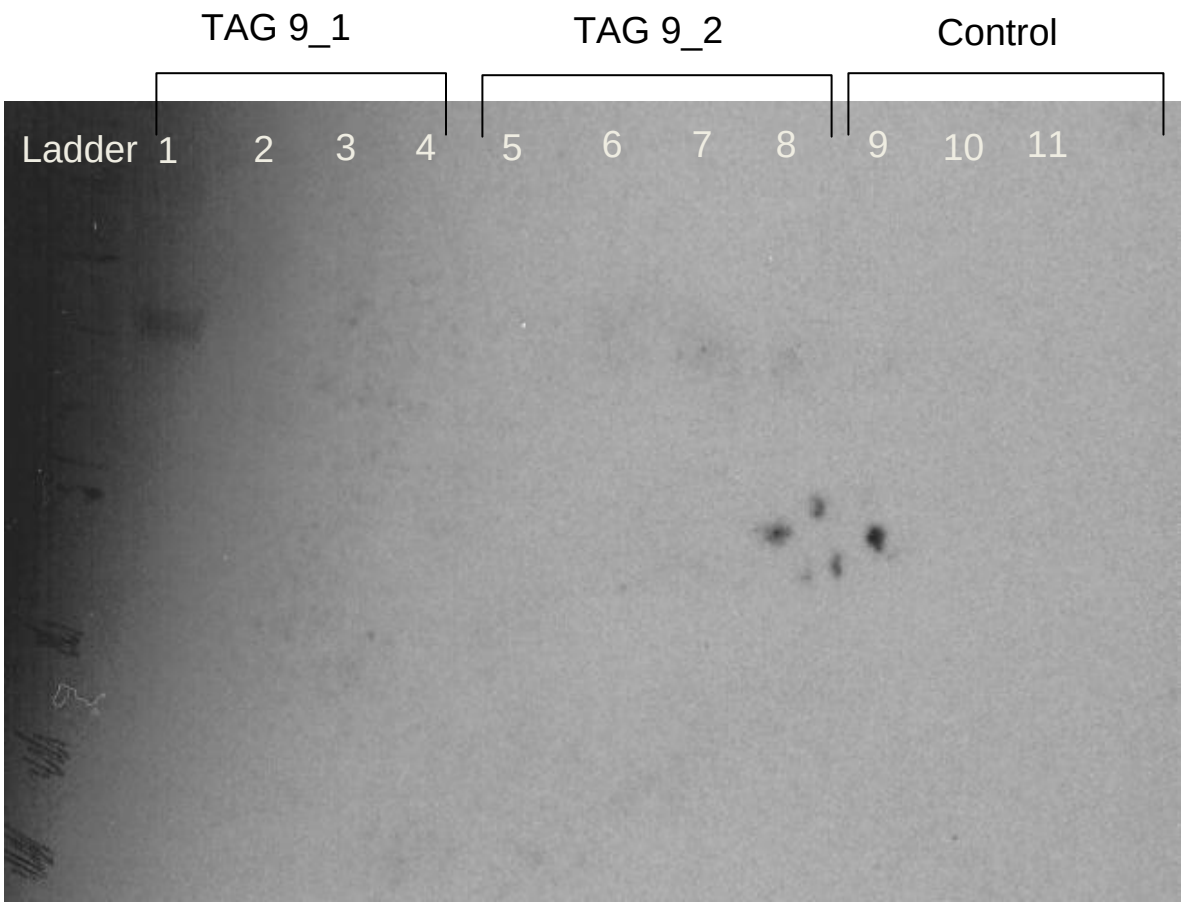


Figure 3. Lanes 1-4 treated with TAG9_1, lanes 5-8 treated with TAG9_2, lanes 9-11 received no treatment (control). Primary antibody was TRF2.

Later replications of the same procedures were tried, with no results. The same double secondary antibody application was tried, and still there were no bands when film was exposed. Different cell lines were also tried, again to no avail.

Discussion:

This experiment proved to be more difficult than planned. It was rather disheartening to start with one blot that showed bands, exactly as we wanted to see, but was not replicated after.

In the blot that showed bands, the strongest bands (widest, darkest) were found in samples treated with TAG9_1, second strongest with TAG9_2, and least strength in the control/no treatment samples. This would indicate that the TAG9_1 treatment group had the most TRF2 binding, indicating that the GGGTTAGGG (TAG9_1) sequence binds more readily than TTAGGGTTA (TAG9_2) sequence, which bound better the control.

In previous experiments, (Mata, unpublished) blots showed a direct correlation between concentration of telomere mimics and TRF2 (Figure 4).

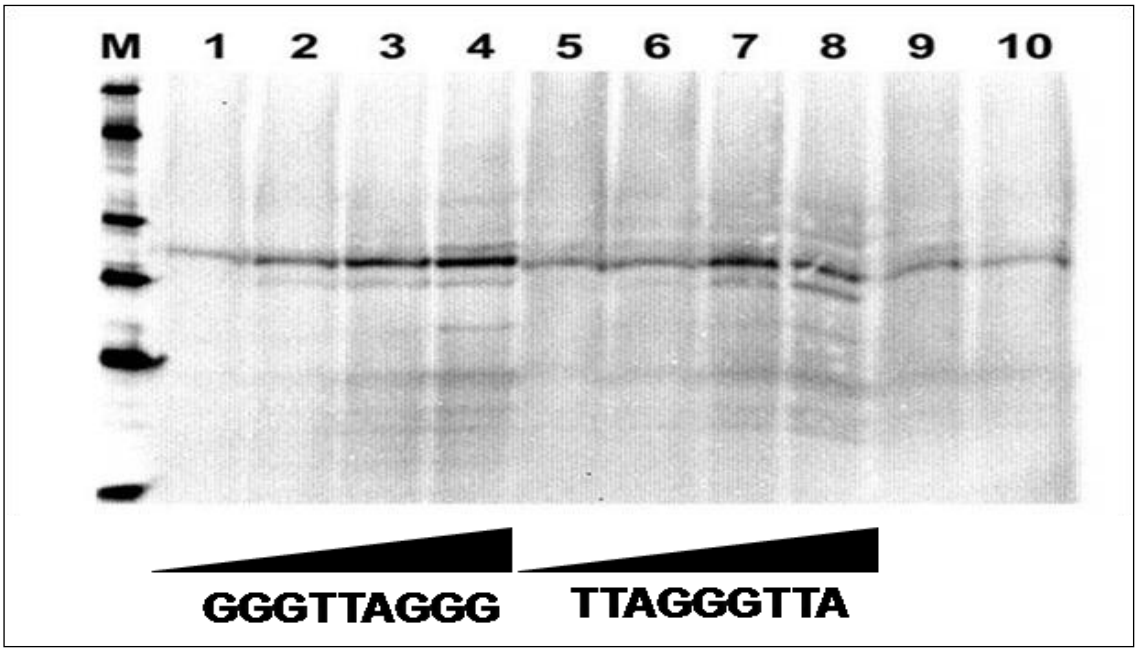


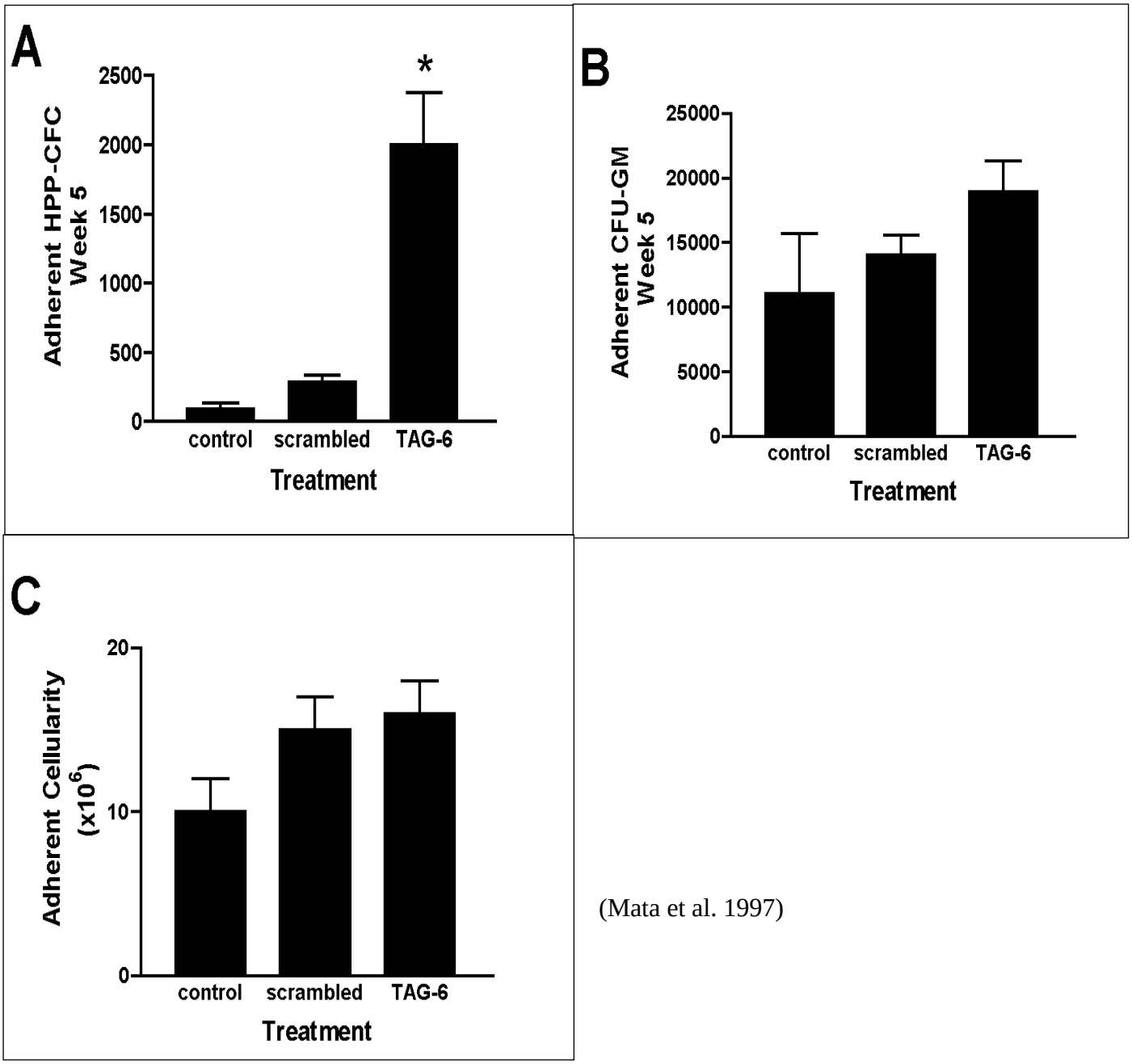
Figure 4. (Mata, unpublished data)

Figure 4. Typical analysis of TRF2 efflux from isolated active nuclei following co-incubation with increasing concentrations of telomere mimics (0.1, 0.3, 1.0 and 3.0μM). Lanes 9 and 10 are untreated.

The results of previous studies confirm that short synthetic telomeric DNA has selective effects on telomere binding protein TRF2. Had this research project been replicated, each blot should have shown similar bands as seen in Figure 3 and 4, showing that TRF2 showed the strongest bands with TAG9_1, with decreasing strength for samples treated with TAG9_2, and weakest bands for samples that received no oligonucleotide treatment.

In order to carry this project to its conclusion, our plan was to begin with a mechanistic study of telomere mimic antagonism presented above. It is unclear why only one Western Blot analysis worked; there were no changes in reagents, protocol, or equipment used. After many failed attempts, a new cell line was started, using human aortic fibroblasts (experiment began with mouse bone marrow cell samples). Cells were treated using the same protocol and reagents, and in a greater cellular concentration than before (in case it was lack of cell density causing no bands to show). These experiments also proved unsuccessful in showing bands. Because the very first Western showed bands, it's hard to say what the issue inhibiting later experiments was. If there was more time, protocols could be tested more to determine if it was a reagent issue.

Future studies will include measurements of competitive inhibition with TRF1 binding protein followed by additional work to extend previous work that demonstrates utility of telomere mimics. We are confident that this research will ultimately lead to new research tools and perhaps new options for treatments. For example (Figure 5), previous work by Dr. Mata demonstrates that a 6-base telomere mimic has a significant effect on the HPP bone marrow fraction in cultured swine bone marrow.



(Mata et al. 1997)

Figure 5. The effect of TAG-6 and scrambled control on adherent hematopoietic stem cell subpopulations HPP-CFC (A), CFU-GM (B), cultured to 5 weeks. Panel C shows relative cellularity between cultures.

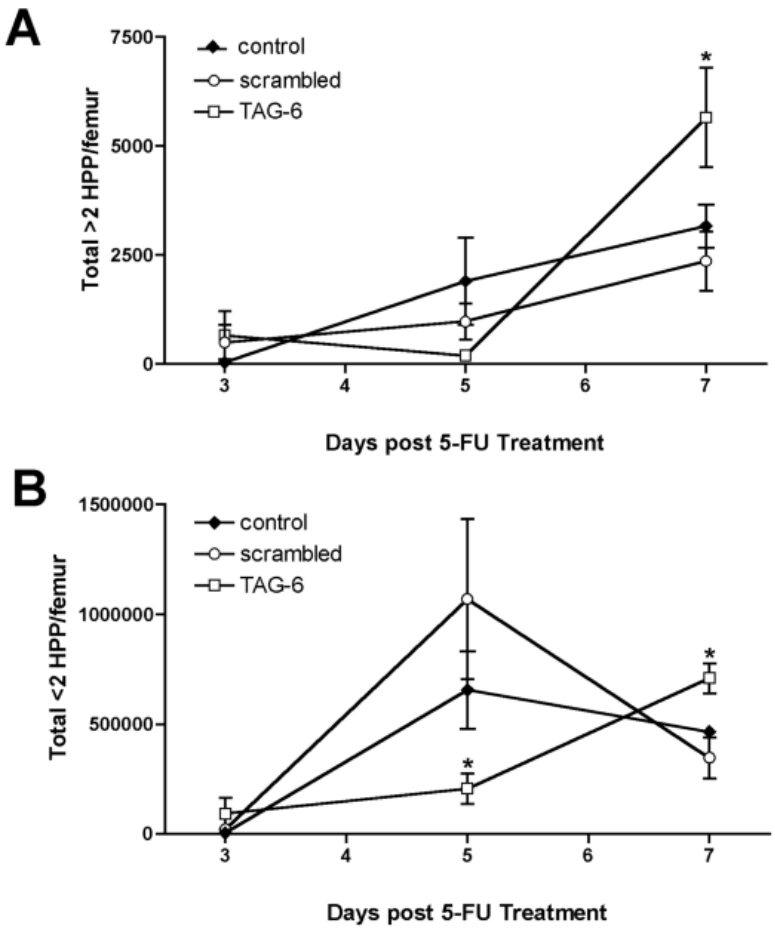


Figure 6. The effect of TAG-6 and scrambled control on the recovery of HPP-CFC in Murine bone marrow 3, 5, and 7 days following administration of a myelosuppressive dose of 5-FU. Panel A represents the number of colonies greater than 2 mm in diameter. Panel B represents the number of colonies greater than 1 mm and less than 2 mm in diameter (Mata 1997).

This effect was also seen in vivo in mice treated with this same compound. Mice in this study had been treated with 5-FluoroUracil to deplete their hematopoietic system and circulating cells. Groups of mice were then treated with TAG-6 by osmotic pump and bone marrow aspirates collected at the end of 3, 5 and 7 days were assessed for numbers of HPP (highly pluripotent) colony forming units. As seen in Figure 6, after seven days of

treatment the TAG-6 treated mouse femurs contained significantly higher proportions of HPP cells than scrambled sequence or untreated controls.

In conclusion, although much of what we wanted to accomplish did not work out as we intended, because of the limited success and our strong preliminary data, we believe that this project will lead to a more complete understanding of the role of telomere binding proteins in modulation of stem cells.

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