UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Site of Action of PTHrP's Proliferative Inhibition in Non-Small Cell Lung Carcinoma

A Thesis submitted in partial satisfaction of the requirements for the degree

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in

Biology

by

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Professor Raffi V. Aroian

2010

The Thesis of Ryan Peter Vander Werff is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

The Site of Action of PTHrP's Proliferative Inhibition in Non-Small Cell Lung Carcinoma

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Ryan P. Vander Werff

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Parathyroid hormone-related protein (PTHrP) is expressed in many tissues and is often enriched in cancers. Tissue reaction to PTHrP varies as it is expressed as multiple isoforms, undergoes peptide processing, and has selective trafficking. PTHrP is secreted via a pre-pro -36 to -1 amino acid portion and interacts with extracellular receptors, including parathyroid hormone receptor 1 (PTH1R). PTHrP is also present within the cell and shuttles into and out of the nucleus via a nuclear localization signal (NLS) and a nuclear export signal (NES). Patients diagnosed with non-small cell lung carcinomas that express PTHrP, experience higher survival rates. PTHrP's benefits in non-small cell lung cancer may result from its previously demonstrated anti-proliferative effects. Because of PTHrP's highly intricate actions, its site of inhibitory action on proliferation is not known.

This project experimentally isolated the site of action and likely receptor responsible for PTHrP's inhibition on H1944 cells, an adenocarcinoma cell line. Early experimental results suggested that exogenous PTHrP has at least a partial role in growth inhibition. We successfully demonstrated that the nuclear trafficking of PTHrP is not necessary for inhibition of proliferation. We showed that PTHrP peptide introduced into the cell has no major effects on proliferation. Finally, we demonstrated that exogenous PTHrP treatments have the potential to inhibit proliferation at levels near stably transfected lines and that PTHrP does not inhibit cells with PTH1R knocked down. Based on these results we conclude that PTHrP inhibits proliferation of H1944 cells in an extracellular fashion dependent on the PTH1R.

INTRODUCTION

Parathyroid hormone related protein (PTHrP) is named so because its 1-34 amino acid residues share primary and secondary homology with Parathyroid hormone PTH 1-34, allowing both peptides to bind to a shared cell surface receptor PTH1R[1]. PTH1R can be both a Gs as well as a Gq protein-coupled receptor[2-4]. PTH1R has also been shown to act through a G-protein independent mechanism. In bone PTH(1–34) stimulation of PTH1R promotes the translocation of β-arrestin1 and 2 to the plasma membrane and receptor complex formation. The complexes are then internalized , leading to the activation of ERK1/2[5-7].

PTHrP is expressed in humans in any of three isoforms, PTHrP 1-139, 1-141, and 1-173, all of which are have the same initial 139 amino acids[8]. Previous experiments demonstrated that the PTHrP 1-34 portion and its associations with PTH1R is a major part of many of PTHrP's actions. PTHrP is also expressed as a multitude of derivative peptides. The entire protein undergoes extensive processing events, possibly creating up to 92 separate peptides[9]. These peptides can affect tissues by means of multiple receptors. PTHrP has a growth and calcium regulation domain in residues 38-94 and an anti-osteoclast portion from PTHrP 107-139[10]. Also, PTHrP 67-86, and PTHrP 107-138 inhibit proliferation of various cell lines[11].

PTHrP has the potential to localize to different aspects of the cell. Although PTHrP is considered a secretory protein, it is also found in the cytoplasm, nucleus and

nucleoli. Consequently, PTHrP can affect biological responses through paracrine and/or autocrine actions at the cell surface or intracrine effects in the nucleus or cytoplasm. A secretory function has been mapped to a Signal Recognition Particle (SRP), the 36 amino acid pre-pro segment[12]. The capacity to enter the nucleus or follow a secretory path requires a mechanism to switch off the signal for secretion. Several have been proposed, but discussion of these hypotheses is beyond the scope of this introduction. While PTHrP is in the nucleus it may regulate transcription or translation by binding to DNA, RNA, or protein factors. The nucleolus is also known to affect the cell cycle (including susceptibility to cancers as well as tumor suppression). It is not known how nucleolar localization precipitates these effects, but PTHrP that is localized in this area may activate or deactivate certain factors or bind to ribosomal RNA[13-14].

Residence in the nucleus results from a nuclear localizing Sequence (NLS) in residues 87-106. This site is a SV40 large T antigen-like NLS composed of two stretches of multi-basic amino acids, and amino-terminal flanking regions. It is recognized by importin ß1, a Ran dependent nuclear transport factor, which then binds and shepherds the PTHrP through the nuclear pore complex[15]. PTHrP localizes conditionally to the nucleus/nucleolus at G_1 and has been shown to stimulate proliferation and anti-apoptotic effects in vascular smooth muscle cells[16,17]. Cell cycle-specific nuclear transport appears to depend on phosphorylation of $p33^{cdk2}$ and $p34^{cdc2}$ at PTHrPs T85[16].

There is also a Nuclear Export Sequence (NES), PTHrP 126-136, which allows the protein to leave the nucleus. The NES is a leucine rich 109-139 region[18] that

mediates the export of PTHrP through specific binding to the nuclear export receptor CRM1(chromosome region maintenance protein 1/exportin-1/XPO1)[19]. Possessing both NLS and NES allows PTHrP to shuttle in and out of the nucleus, possibly in a regulated fashion.

The situation is complicated because GFP conjugated to PTHrP1-87, which is missing the NLS, has been observed in the nucleus and has demonstrated intracrine effects (dependent on amino acids 65-87) in prostate cancer. Specifically, this PTHrP form stimulates tumor growth through a five-fold increase in the angiogenic growth factor Interleukin-8[18]. Therefore PTHrP must have another means of nuclear transport residing upstream from the known NLS. Contrary to previous assertions, many of the effects on proliferation observed in cells expressing PTHrP 1-87 may still be mediated through nuclear effects.

PTHrP is expressed in a wide range of tissue including lung, bone, prostate, heart, breast, kidney, and brain. PTHrP is often enriched in tumors and has been connected to hyper-calcemia, multiple growth effects, regulation of apoptosis, angiogenesis, cell mobility, adhesion, and metastasis. PTHrP is known to regulate proliferation in many different ways. PTHrP 1-34 inhibits proliferation of vascular smooth muscle cells through activation of the PTH1R receptor. However, ectopic production of PTHrP stimulates proliferation of the same cells and the phenomenon depends on the presence of an active NLS[20]. Thus, the intracrine and paracrine effects of PTHrP oppose each other in smooth muscle cells. Also, after lung injury, PTHrP 1-34

and PTHrP 67-86 inhibit type II cell proliferation in a paracrine or autocrine manner[21]. PTHrP 67-86 also has growth inhibitory effects in breast cancer cells[11]. Thus, paracrine PTHrP appears to be growth inhibitory and the intracrine actions stimulatory. However PTHrP has also been found to stimulate proliferation through a paracrine route in different tissues and cell lines. For example, PTHrP 1-34 can augment mitosis in breast cancer cells and some osteoblast lines, but inhibit growth in others. With its multifunctional nature and versatility in engaging many signaling pathways, the PTHrP protein can induce a wide range of differing phenotypes.

PTHrP is expressed in two thirds of human non-small cell lung cancer. It is best known for causing the syndrome of hyper-calcemia of malignancy. However, effects on proliferation, apoptosis, motility and other properties make PTHrP likely to regulate other aspects of the disease process. In fact, women with PTHrP-expressing lung carcinomas have a longer life expectancy than women whose tumors lack the protein, while men see no significant benefit from the protein[22,23]. The sex-dependence is an interesting topic but is not the focus of this project. Instead, we are interested in PTHrP's inhibitory effects on tumor growth, which could explain the pro-survival effect in patients with lung cancer.

Previous studies have provided evidence that PTHrP has anti-mitogenic effects in several lung cancer lines and reduces growth in tumors. Exogenous PTHrP 1-34 treatment inhibited the growth of cultured BEN cells by approximately 40%. Further, in mice with orthotopic lung tumors neutralizing antibodies against 1-34 stimulated ben cell

tumor growth[23]. Recent studies have evaluated PTHrP-negative lung cancer cell lines that have been stably transfected with PTHrP 1-87. H1944 and MV522 human lung adenocarcinoma cell lines both express PTH1R, but neither line makes PTHrP in the wild type state. Clones expressing ectopic PTHrP show inhibited growth[24]. It is in this cancer model that we hope to find the site of action of PTHrP's inhibitory effects on cell proliferation.

PTHrP has tissue-specific processing and secretion systems that exhibit very different physiological responses to each derivative peptide, dependent on the properties of the expressing tissue as well as the many different PTHrP receptors that are believed to exist [25]. Partially due to the complexity of physiological effects and partially, the pathway in which PTHrP causes inhibition of non-small cell lung cancer the sites of action are still unknown. The PTHrP could manage lung cancer cell growth by signaling through surface receptors, or it could also have a direct role inside the cell, exerting intracrine effects in cytoplasm or nucleus. If PTHrP 1-87 mediates growth inhibition at the cell surface, we expect that the pathway would involve the interaction of the PTHrP 1-34 region with PTH1R or PTHrP 67-86 with a mid-molecule PTHrP receptor. Ostensibly, cell surface effects seems to be the most likely means for ectopic PTHrP 1-87 to inhibit lung cancer cell proliferation, since this particular form of PTHrP lacks the NLS. However, PTHrP 1-87 can enter nucleus through NLS-independent pathways in prostate cancer cells and we have observed immuno-reactivity for PTHrP 1-87 in a subset of cells transfected with that molecule. Thus, we cannot rule out intracrine effects.

Consequently, the aim of these experiments will be to find the site of action at which PTHrP is responsible for the inhibition of lung cancer growth.

EXPERIMENTAL DESIGN

In order to try and determine PTHrP's site of action steps, will be taken to manipulate the cell and/or protein with the intention of directing the peptide to certain areas and blocking it from others. Since PTHrP inhibits breast cancer and VSMC tissues growth through autocrine effects, we will begin by testing the effects of cell surface PTHrP on H1944 cells. We will add exogenous PTHrP to cell media of wild type H1944 adenocarcinoma cells, emulating a completely extracellular form of the protein's effects and paracrine stimulation. If growth inhibition results from an extracellular pathway, this should be sufficient to replicate the inhibition observed in PTHrP positive cells. We will also investigate autocrine effects in PTHrP-expressing H1944 cells by neutralizing the 1-34 peptide region of the PTHrP 1-87 molecule with blocking antibodies. If the ectopic PTHrP 1-87 induced by stable transfection inhibits H1944 cell division in an autocrine manner, then treating the peptide with the antibodies should cause the cells to approach a more normal proliferation rate. If Antibody Neutralization against the 1-34 region successfully rescues the cells from the inhibitory effect of PTHrP's proliferation, we would suspect the involvement of the PTH1R receptor, which binds PTHrP 1-34. We will then make stable H1944 cells with shRNA knockdowns of PTH1R. If PTH1R is the receptor responsible for PTHrP's proliferation inhibition signaling, then a knockdown should restore proliferation to normal levels even in the presence of PTHrP.

Another approach to the problem will be to determine whether secretion of PTHrP is required for the molecule's observed effects. Transient transfection of a PTHrP

construct that lacks SRP into H1944 human lung adenocarcinoma cells will allow the production of cytoplasmic PTHrP without secretion. This will be verified by radioimmunoassay (RIA) for PTHrP in the media, which should not contain PTHrP, and the cell lysate, which should. If secretion and/or extracellular receptors for PTHrP are necessary for lung cancer cell growth inhibition, the effect should not occur in cells expressing non-secretory PTHrP.

De Miguel et. al. have shown that this NLS is necessary for intracrine stimulation of proliferation in smooth muscle cells (17), and PTHrP has been observed in the nucleus and nucleoli during PTHrP induced arrests of invitro non-small cell adinocarcinoma cell cycles during the G2/M phase() suggesting that the nuclear transport may play a role in inhibition. To determine if the nucleus is PTHrP's site of action, selectively mutated PTHrP constructs lacking the NLS, residues 87-106, to reduce PTHrP transport into the nucleus. If PTHrP transport to the nucleus is necessary for cell cycle inhibition, then the NLS-deficient mutant should fail at inhibiting proliferation in comparison to wild type PTHrP. In addition, H1944 cells will be transfected with a PTHrP plasmid that is NES deficient. If successful nuclear levels of PTHrP should rise: the protein will pool in the nucleus and will not be able to exit. If our hypothesis is correct, forcing PTHrP to remain in the nucleus should increase the inhibitory effects on H1944 cell growth in comparison to wild type PTHrP and PTHrP deficient cells. This should show a profound decrease in proliferation inhibition.

As another approach to examine Intracrine effects, cells will have PTHrP 1-87 protein introduced directly into them using Chariot protein transfection reagents. We will verify the protein transfection using an Alexa fluor488-conjugated PTHrP peptide that will be visible inside the cell. If PTHrP is an intracellular effector, then we should be able to inhibit proliferation by directly inserting the peptide into the cell.

METHODS AND MATERIALS

Cell culture. H1944 cells have been obtained from the American Type Culture Collection (ATCC, Manassas, VA). All lines are grown at 37°C in 95% air/5% CO2 in RPMI 1640 medium plus 5% fetal bovine serum and L-glutamine.

Antibody protein G columns: Protein G agarose resin spin column kits (Thermo-Scientific, Rockford, IL) were equilibrated to room temperature, centrifuged at 5,000g for 1 min to clear storage buffer. 400ul of binding buffer was added followed by 50ug. 10ug/ml Antibodies + Protein added in separate tube. This mixture was then added to the column and incubated at RT for 10min with end over end mixing. Columns spun at 5000g for 2 min. Flow through was collected for RIA submission and columns were washed with Salt solution.

Antibody neutralization. Cells plated at 50,000 cells per well in 12well plate. Incubated for 24hrs at 37C. 5ug/ml of Antibody (generously provided by Dr. Deftos' Lab) was added to the cells and incubated for 24 more hours at 37C. Media was removed from the wells and lysates collected for thymidine incorporation assay.

Thymidine Incorporation Assay. For [^{3}H]-thymidine incorporation, cells will be plated at 4 x 10^5 cells/well in 24-well plates and studied at 60-70% confluency. then exposed for 24 h to growth media + 0..5 µCi [^{3}H]-thymidine/well with or without PTHrP treatments. Finally, cells will be washed, precipitated in trichloroacetic acid, lysed in

NaOH and counted. For cell cycle status determination, unsynchronized cells will be treated for 24 hours.

Stable and transient transfection. Expression plasmids will be complexed with a DNA binding agent (PLUS reagent - Life Technologies, Inc.) in serum-free DMEM media for 15 minutes, followed by addition of polycationic liposomes (DDAB:DOPE lipids or LipofectAMINE - Life Technologies, Inc.) in a minimal volume and for 4-6 hours. The DNA:liposome complexes will be removed and replaced with RPMI containing 10% FCS. Individual G418 resistant colonies will be isolated 7-14 days later by pipette tip picking and re-plated into 96-well plates. Appropriate mRNA and protein expression will be assessed by immunoassay and protein sizing studies, where appropriate.

Lentiviral shRNA gene knockdown. MISSION˙ TRC shRNA transduction-ready lentiviral particles (Sigma-Aldrich Chemical, St. Louis, MO) will be used for long-term inhibition of PTH1R expression. The shRNA kit provides lentiviral particles to 5 different PTH1R mRNA targets, each with a puromycin resistance element. The most effective lentiviral particle for our lung cancer cells line will be selected based on immunoblots and second messenger assays. Stable clones will be generated by treating with puromycin.

Chariot protein transfection. Protein will be transferred inside intact cells with the Chariot protein reagent (Active Motif, Carlsbad, CA) following the manufacterer's

instructions. Cells will be plated at 40-60% confluency in a twelve well plate. Protein will be mixed with the Chariot reagent in proportions of 0.5 µg PTHrP, 2 µl Chariot, 100 µl PBS and 100 µl sterile H_2O at room temperature for 30min. The cells will be washed once with PBS before adding 200ul/well of Chariot-macromolecule complex and 150ul/well serum free medium. After 3 hr incubation at 37C, the transfection mixture will be removed, cell will be washed once with PBS and replaced in normal media.

Alexa-fluor488 conjugation. .5mL of 2mg/ml PTHrP 1-87 was added to 50uL of 1M bicarbonate. Alexa fluor 488 protein (Molecular Probes, Eugene, OR) was equilibrated to RT. Protein mixture was added to the vile of reactive dye and stirred for 1hr using the vials internal stirbar. Size exclusion column was then poured. PTHrP-Alexa was observed as first band to run through while unbound Alexa came out the slowest.

Cell Titer 96 MTS growth assay. Cells will be plated at 1,000-7,000 cells/well in 96-well plates in growth media for proper cell adherence. Assays will be stopped in separate plates at 0, 1, 2,3, 5 and 7 d and cell quantity assessed with the proprietary MTS tetrazolium compound (Promega, Madison, WI) for 1h at 37 C. Reaction occurs when MTS is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium with an absorbance at 490nm.

Fluorescent microscopy. Fluorescent cell imaging was performed with an Olympus BX60 upright microscope with fluorescent attachments (Olympus America, Melville, NY). Light from a 100-watt mercury lamp was passed through an Olympus

ultraviolet-2E/C filter set for Hoescht 33,342 (H33342) or a G-E2E/C filter set for ethidium bromide excitation and emission wavelengths.

Cell death measurements. Conditioned media were collected seperately and adherent cells were washed once with PBS. Adherent cells were collected by washing with trypsin. 0.12 µM ethidium bromide and 4 µM Hoescht 33342 was added to each collection of tubes directly before counting. Ethidium bromide is excluded from viable cells, whereas H33342 is a cell permeant bis-benzimide dye that labels all nuclei. The fraction of dead cells was determined by counting ethidium bromide–positive cells and dividing by the total number of nuclei and total cells were determined by use of a hemocytometer.

Western blots. Equal quantities of cell lysate protein will be applied to Criterion 4-12% Bis-Tris gels (Bio-Rad, Hercules, CA), separated by electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes will be blocked with 5% non-fat dry milk for 1 hr at room temperature, probed with primary antibodies overnight at 4°C and exposed to goat anti-mouse or goat anti-rabbit IgG-HRP secondary antibodies for 1hr at room temperature. Chemiluminescence will be elicited by treatment with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and recorded with the UVP BioSpectrum 410 imaging system (Upland, CA). Band densities will be analyzed with ImageJ software (NIH).

PTHrP immunoassay. PTHrP was assayed in conditioned media and lysates of lung cancer cells by our own radioimmunoassays, using antibodies directed against PTHrP 1-34, PTHrP 38-64, and PTHrP 109-141, as described previously[26,27].

RESULTS

Antibody binding and neutralization of PTHrP 1-34:

Previous evidence suggested that PTHrP 1-34 may be a likely candidate for extracellular effects on proliferation. Our goal was to block these effects using neutralizing antibodies against the 1-34 amino acid domain. If the 1-34 portion of the peptide is necessary for extracellular inhibition of proliferation then we expect to see this inhibition rescued in cells treated with neutralizing antibodies. We first did a pilot study to determine how well our antibodies bound PTHrP to make sure that sufficient neutralization would take place.

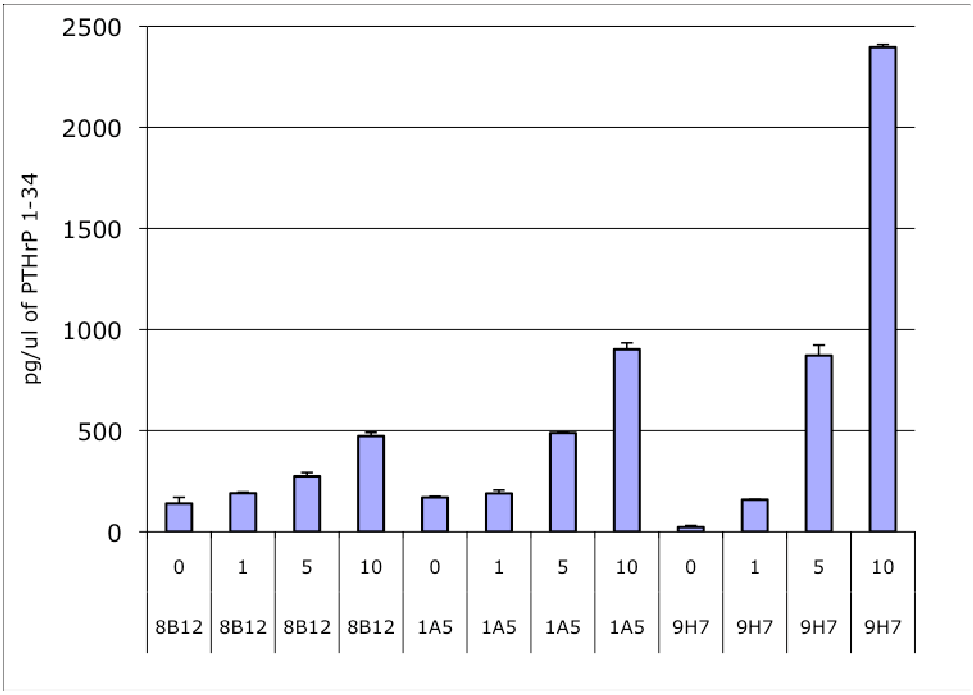


Figure 1: Protein G AB binding PTHrP Pilot Study: Antibody columns were used to bind PTHrP to determine the binding efficiency of 8B12 and 1A5. Antibody name and concentration (0,1,5,10 ng/mL) of PTHrP are found on the x axis. pg/mL of PTHrP in the elution is found on the y axis. 8B12 and 1A5 specific for the 1-34 portion of PTHrP reduced PTHrP compared to the 9H7 non-specific control. Columns with antibodies against PTHrP bind more PTHrP than the non-specific control.

Columns with the non-specific binding control 9H7 had about a 25% recovery rate of PTHrP with losses probably resulting from non-specific adhesion to column plastic or matrix. 1A5 and 8B12 columns had approximately 1/2 and 1/3 PTHrP recovery, suggesting they bound to 1/2 and 2/3^{rds} of all available PTHrP respectively. Stable clones of PTHrP 1-87 expressing H1944 cells express PTHrP at or below the levels used in this experiment. The PTHrP 1-87-expressing H1944 cells in a 24-well plate at 50% confluency produce 5000pg/ml per 24 hours or les. Thus using AB to PTHrP at a concentration of 10ug/ml will be sufficient to neutralize most secreted PTHrP.

After demonstrating effective antibody neutralization of PTHrP, we next tested the effect of these antibodies in PTHrP thymidine uptake assays. Our hypothesis was that neutralization of the 1-34 amino acids of PTHrP will rescue cells from the anti-proliferative effects of PTHrP and thus increase thymidine uptake.

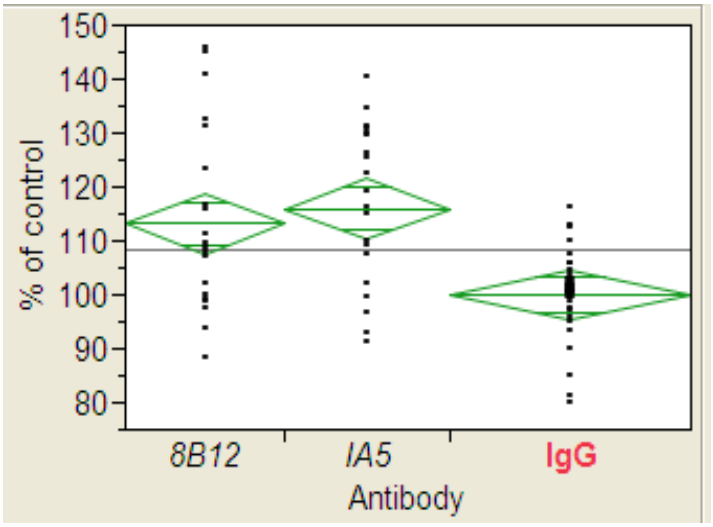


Figure2: Antibody neutralization of PTHrP: The percent change in insoluble thyumidine counts after 24 hr incubation, as adjusted to IgG control, is found on the y axis and treatment is found on the x axis. Dots are indivitual data points. 95% confidence interval represented by the green diamond and mean is the disecting line. Neutralizing treatments caused a significant increase in thymidine uptake

PTHrP 1-87 expressing cells were treated with neutralizing antibodies 1A5 and 8B12 or a non-specific control IgG while assaying uptake of H^3-thymidine over 24 hrs. Analysis of Variance displays a p-value of less than 0.0001 statistically demonstrating that PTHrP neutralization treatments affect levels of thymidine uptake. Dunnett's post-hoc test 8B12 and 1A5 treatment normalized to the IgG control had significantly different thymidine uptake with p-values of 0.001 and less than 0.0001and averages approximately 13% and 16% more uptake respectively.

These results indicate that extracellular PTHrP exerts an inhibitory effect on cell growth, but the neutralization only rescued a small portion of the 40% to 60% inhibitions displayed by PTHrP expressing clones. Our net goal was to determine whether PTHrP inhibition occurs through other mechanism or sites, making use of mutated PTHrP constructs that produced PTHrP in specific sites.

Pilot Work.

Pci-neo PTHrP constructs included wild type PTHrP 1-141, PTHrP 1-141 delta SRP (non secreted), PTHrP 1-141 delta 87-106(no NLS, reduced nuclear localization), PTHrP 1-173 delta 108-141 (no NES, increased nuclear localization), and PTHrP 1-87. DNA sequencing verified the correct gene structure. Pilot studies were conducted to ensure correct expression of the correct PTHrP protein and to optimize the timing of transfection efficiency, and expression. These constructs were then utilized to direct PTHrP towards or away from certain areas of the cell while assaying for changes in proliferation. Cells expressing PTHrP directed away from the site of inhibition should

have proliferation rates that resemble PTHrP free cells. PTHrP constructs designed to localize at inhibitory sites should inhibit cell growth or even increase inhibition.

A pGFP vector expressing GFP through the same promoter used for our PTHrP constructs was used to measure transfection efficiency and to find the optimal expression time. GFP fluorescence was expressed in 43% of cells directly after treatment and 74% 24 hours later indicating that 24 hours is the optimal experiment length to express PTHrP cloned into Pci-Neo clones in high cell numbers, but that significant protein expression can be achieved at earlier points.

A

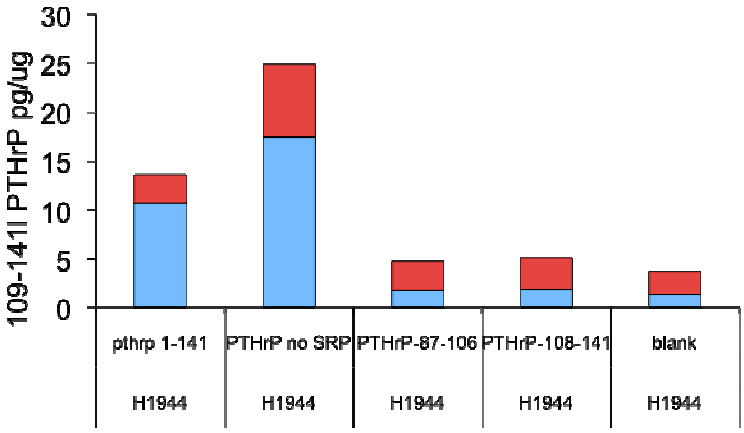
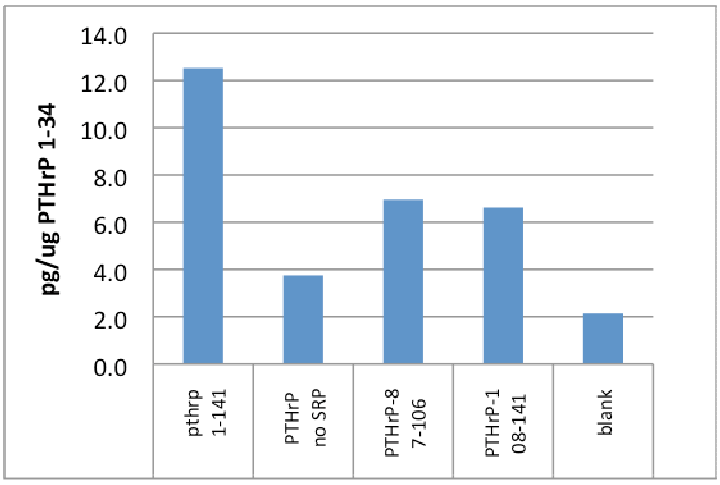


Figure 3: PTHrP expression in transiently transfected H1944 cells. PTHrP constructs transiently transfected into wild type H1944 cells. 48 hours after treatment Lysates and Conditioned media submitted for RIA to find total pg of PTHrP which was divided by total ug of cellular protein.(y axis) A) RIA targeting 109-141 portion of the PTHrP peptide. Blue at the bottom segment of each column represents PTHrP in conditioned media. PTHrP levels in lysates shown by red on top of the columns. B) Dual antibody RIA targeting 1-34 portion of PTHrP in conditioned media. All columns represent the Conditioned media PTHrP levels (this assay did not work for lysate samples). C) Expression of PTHrP 1-141 delta SRP showed unexpected amounts of PTHrP in conditioned media. A second experiment was preformed to investigate this phenomenon. WT H1944 cells were transfected with WT PTHrP 1-141 and PTHrP 1-141 delta SRP. One group had conditioned media and lysates collected 24 hours after treatment another group was collected at 48 hours and a third group was washed, and media replaced at 24 hours and then collected at 48 hours (labeled 2nd 24hr). PTHrP concentration were determined using RIA against amino acids 109-141. Conditioned media is represented the blue part of the column on the bottom and lysates are the red at the top of the column.

Figure 3 continued

B



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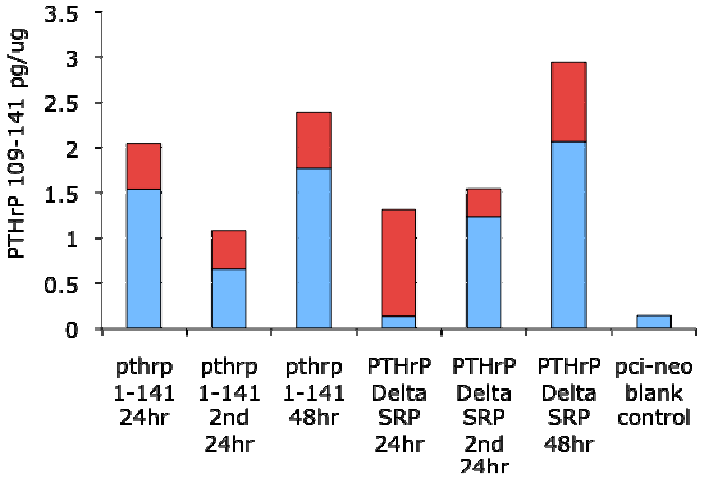


Next, we assessed PTHrP protein production in H1944 cells transiently transfected with the PTHrP constructs using RIA's to PTHrP 1-34 and PTHrP 109-141. **Figures 3A-B** show that cells transfected with PTHrP 1-141 and PTHrP 1-141 DeltaSRP expressed at least 4pg PTHrP 109-141/ug cellular protein, while the Delta108-141 constructs did not produce levels above background. The Delta108-141 result is understandable, since this construct lacks the epitope recognized by the assay, but the Delta87-106 result is somewhat surprising. However, this construct did produce

measureable PTHrP 1-34 protein (**Figure 3B**). Another surprise was the appearance of significant quantities of PTHrP DeltaSRP in conditioned media in the 48 hr experiments (red bars in 3A). We considered the possibility that PTHrP might have escaped from the cell with time, not necessarily because of secretion. Therefore, we conducted a time course study to evaluate what period was necessary for PTHrP to appear in the media after the PTHrP DeltaSRP transfection (**figure 3C**). The delta SRP construct's highest intracellular PTHrP (blue bars) and lowest extracellular levels (red bars) was at 24 hours after transfection. At later time points PTHrP appeared in the cell media. As a result of this finding, the time course for further transfection assay experiments was limited to 24 hours after treatment. Further cells washed at the 24hour time point seemed to reduce PTHrP in the lysates as compared to unwashed cells.

Cell lines that express PTHrP naturally have previously been tested in our lab showing no more than 1pg/ug in conditioned media and .5pg/ug in lysates. Stable expressing H1944 lines have between 1 and 4pg/ug in conditioned media and at most .2pg/ug in lysates. PTHrP constructs transiently transfected into H1944 cells show higher levels of peptide expression than cancer lines that normally produce PTHrP. The levels are comparable to stable PTHrP-expressing H1944 cell clones that we have already studied.

Thus these pilot studies demonstrate that our constructs are suitable for transfection experiments because they drive PTHrP expression at a high level in a large percentage of cells, possess the correct protein structure and localize as expected within

the cell. A PTHrP 1-87 vector will also be used in experiments. Its function has been characterized previously[24].

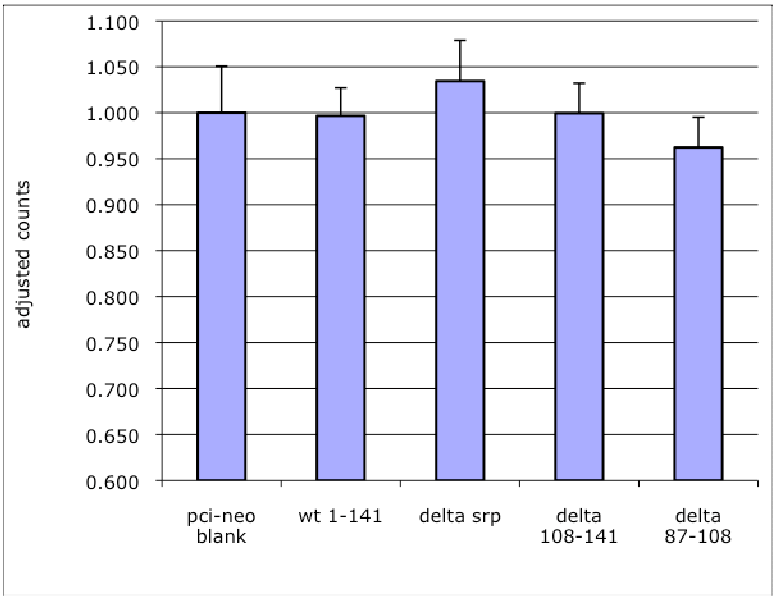


Figure 4: PTHrP selectively mutated for different transport in confluent H1944 cells: Cells at 90% confluence were transfected with vector or PTHrP mutant expression plasmids. Thymidine exposure began after 24hours transfection and continued for another 24 hours. Thymidine incorporation was assayed and data are presented as in figure 2. PTHrP variants had no significant impact on uptake of thymidine in confluent cells.

Effects of mutation-driven changes in PTHrP distribution on cell growth inhibition. **Figure 4** demonstrates the effect of the mutant PTHrP constructs on proliferation in confluent H1944 cells. Anova analysis showed no significant differences in thymidine uptake between confluent H1944 cells transfected with PTHrP constructs or the Pci-neo control. Confluence can have multiple effects on factors affecting in-vitro cell growth. Since PTHrP construct treatments did not have inhibitory effects on confluent cells, the experiments were repeated with sub-confluent cells.

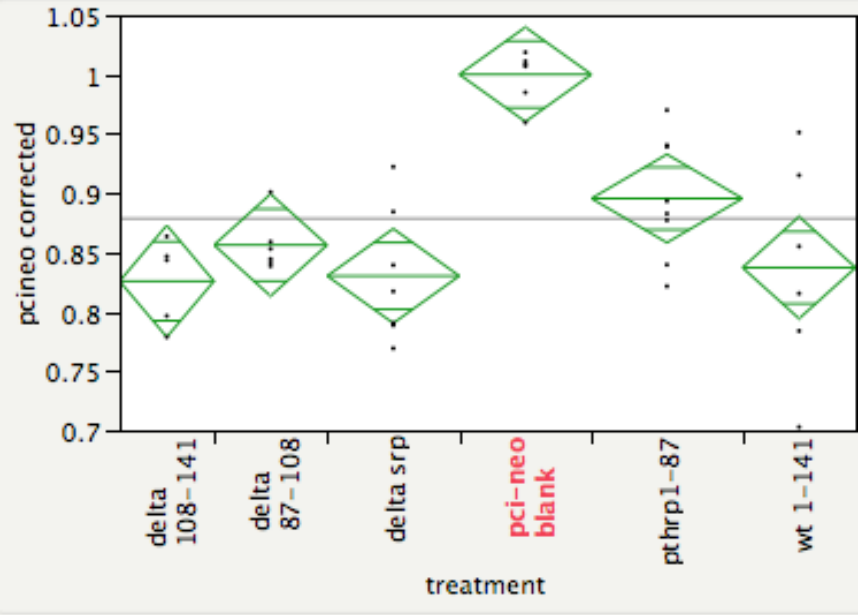


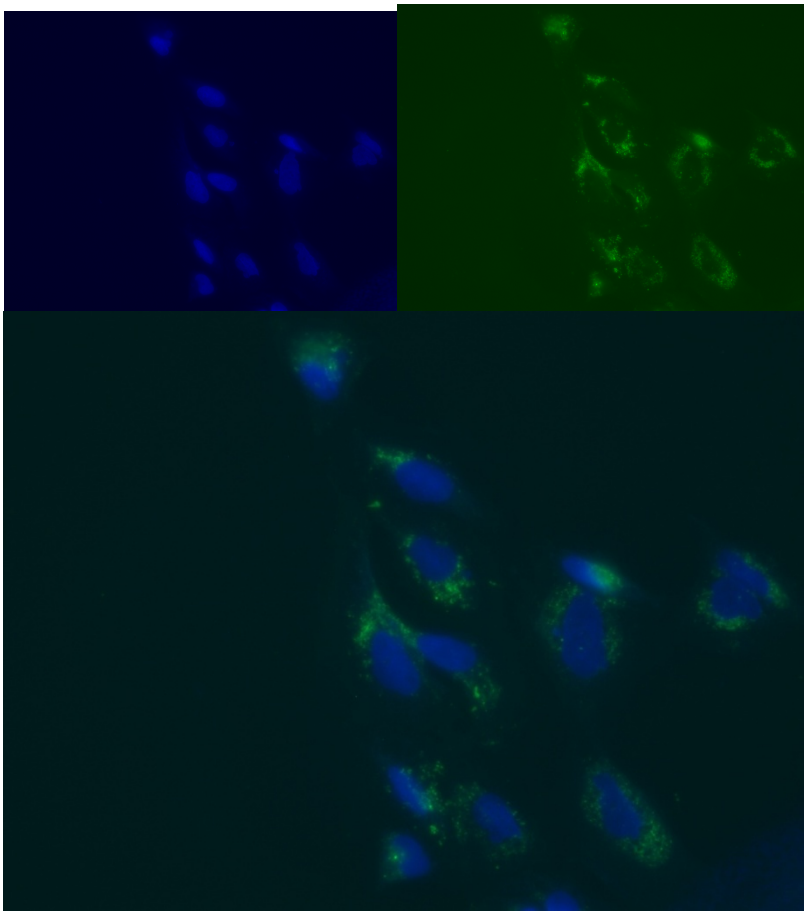
Figure 5: PTHrP selectively mutated to change transport: PTHrP constructs (shown on the x axis) were transfected into wild type H1944 cells, incubated and assayed for radioactive thymidine uptake. Counts were adjusted to counts for the Pci-neo vector control (y axis), with the normalized value Pci-ne value equal to 1. All PTHrP variants had significant less uptake of thymidine yet none were significantly different from one another. Dots are individual data points with the diamond representing 95% confidence intervals; the disecting line is the mean for each group. The results for a given group are generally significantly different if the 95% confidence interval does not overlap.

Sub confluent H1944 cells transfected with PTHrP constructs at 40-60% confluence had significantly slower proliferation rates than control cells as statistically demonstrated by Analysis of Variance (figure 5, <0.0001). The effects of PTHrP construct transfection were compared individually against Pci-Neo vector control by Dunnett's test and showed a P-value of less than 0.0001 except for PTHrP 1-87 which had a P-value of 0.002. None of the PTHrP constructs were significantly different from one another by Tukey's or Scheffe's post hoc testing. The positive result with PTHrP 1-141 D**elta**SRP suggested that intracellular PTHrP could have anti-proliferative effects. Thus, we tested the effects of intracellular PTHrP on cell growth with a second method involving direct delivery of PTHrP inside the cell.

Intracellular vs. Extracellular effects on proliferation

Pilot studies: Cells were treated with Chariot transfection reagent (Active Motiff, Carlsbad CA) and PTHrP peptide to introduce exogenous PTHrP peptide into the cell. Effects on proliferation were then assayed and compared against the effects of exogenous PTHrP treatments without internalization. Proliferation should only be inhibited by the protein transfection treatment if the site of the inhibitory action of PTHrP action is intracellular. If it is not intracellular then exogenous PTHrP should be the only treatment that inhibits proliferation suggesting a purely extracellular site of action for PTHrP. First, we performed pilot studies to show that PTHrP1-87 can be transfected into the H1944 cells in this manner, and to show that intracellular transfer was in a quantity sufficient to cause physiological effects.

A



B

C

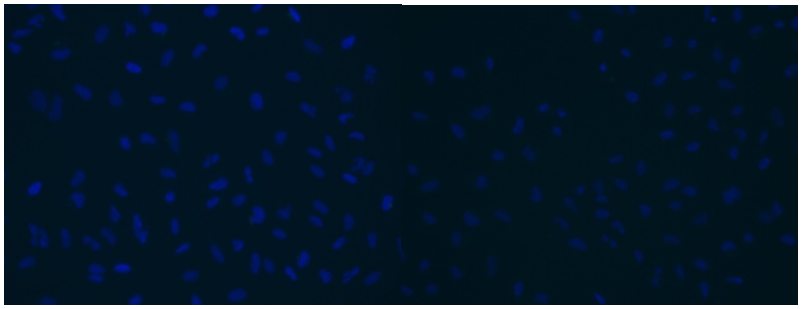


Figure 6: Imaging chariot protein transfection of Alexa conjugated PTHrP: PTHrP 1-87-Alexa fluor 488 conjugate was transfected into H1944 wild type cells and imaged with a fluorescent microscope A) 48hrs PTHrP-alexa transfection: green fluorescence represents PTHrP-Alexa, while blue is Hoechst 33342 staining cell nuclei B) 48 hr merged fluorescent image of cells treated with just Alexa and chariot (no PTHrP) C) 48hr merged image of cells treated with PTHrP-alexa without chariot.

PTHrP 1-87 was conjugated to Alexa 488 fluorescent dye and delivered into H1944 cells by Chariot transfection. **Figure 6A** demonstrates that cells that undergo chariot transfection of PTHrP conjugated to Alexa successfully incorporate the fluorescent conjugate and show little to no background. Fluorescence appeared in 60 out of 63 counted cells and appeared to localize to perinuclear organelles resembling the Golgi or the endoplasmic reticulum. Furthermore, cells treated with PTHrP-Alexa 488 in the absense of chariot and cells treated with Alexa488 alone plus chariot did not show fluorescence (**figure 6B-C**). Thus, the fluorescence in Figure 6A does not represent uptake of free Alex dye nor does it result from non-specific internalization independent of the Chariot reagent.

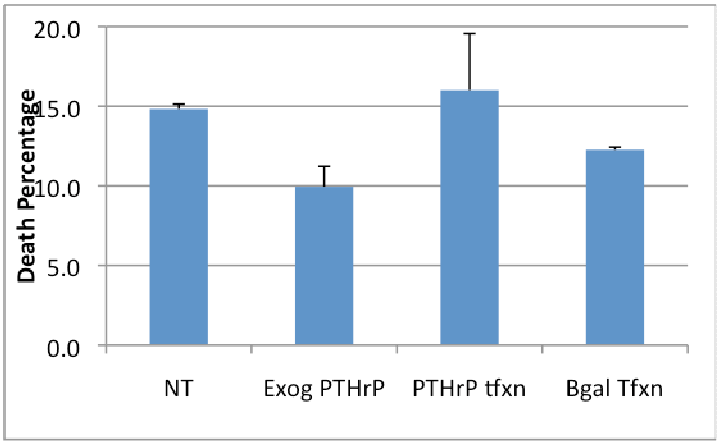


Figure 7: Death assay for chariot transfected H1944 cells. 48hours after treatments(found on x axis)Ethidium Bromide was used to count Condensed nuclei rates among cells on the plate as well as floating in conditioned media. This was divided by total cells counted and multiplied by 100 to get a percentage of cell death (found on Y axis). There was no significant difference between any of the treatments.

We also tested whether Chariot reagen or Chariot transfection with PTHrP caused H1944 cell death. There was no significant differences between viability percentages of controls vs. PTHrP treatments. Treatment of cells with exogenous PTHrP had a near significant decrease in the percentage of non-viable cells at a t-test p-value of 0.069. If

there is truly a difference here, it may result from the known effect of PTHrP in reducing lung cancer cell apoptosis (Hastings 2004), but should not play any role in the planned experiments.

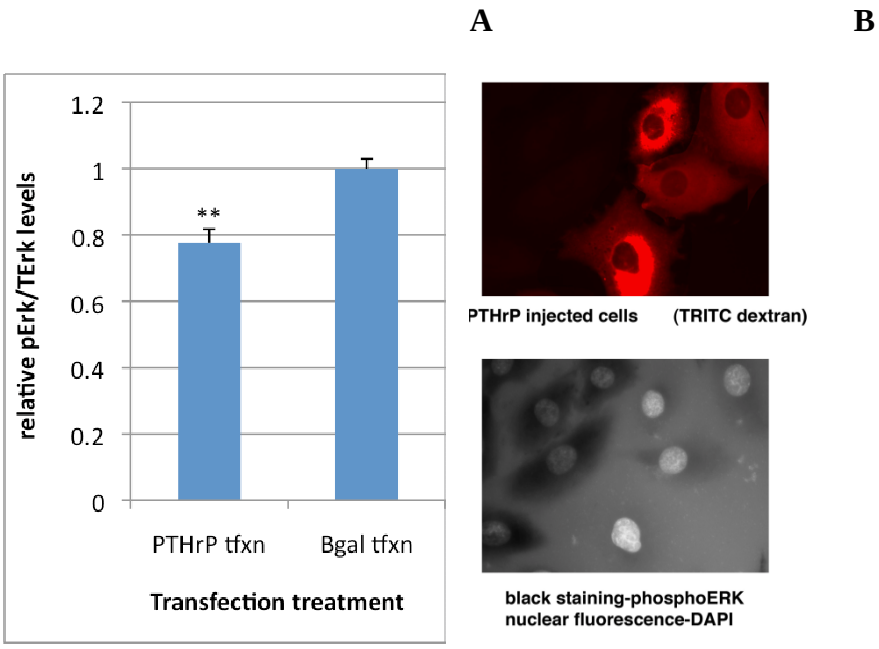


Figure 8: Changes in pErk/tErk levels in H1944 cells transfected or micro-injected with PTHrP: Treatments found on x axis show PTHrP peptide or control. Y axis shows relative Erk activation. A) Erk activation levels by chariot transfection of PTHrP 1-87 and assayed by western blot. B) Micro injection Erk activation. Top picture shows TRITC dextran (in red) a marker for micro-injected PTHrP 1-87 and bottom picture shows Erk activation black as a marker for phosphorylated Erk and DAPI a nuclear fluorescent marker. Both treatments of intracellular PTHrP decrease levels of Activated Erk

Finally, we tested whether the Chariot procedure delivered biologically significant quantities of PTHrP into cells by evaluating effects on Erk phosphorylation. **Figure 8A** results show a significant change in pErk/totalERK ratio with a T-test P-value of 0.0003. PTHrP caused an average 23% decrease in pERK/TERK. These results match those of our collaborator, Dr. David Rose, who assisted by demonstrating that microinjection of PTHrP 1-87 into H1944 cells inhibited serum-activated Erk phosphorylation **Figure 8B**.

This result suggests that the quantity of Chariot transfected PTHrP that gets into the cell is sufficient to show similar biological effects as microinjection of a known quantity of PTHrP.

Our pilot studies allow us to conclude that transfection with Chariot causes entry of PTHrP into H1944 cells at biologically active levels without injury to the cells. We then set out to test the effects of intracellular PTHrP on proliferation.

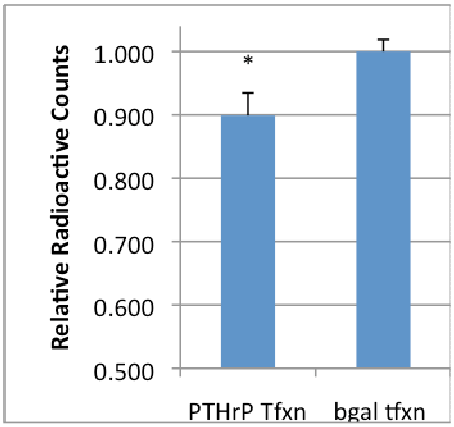


Figure 9: Thymidine uptake assay to measure proliferation changes in PTHrP transfected H1944 cells. Chariot protein transfection protecol was used to transfect PTHrP or control protein, Beta galactosidase, into H1944 cells. Thymidine was then administered within the cell media and after 48 hours of incubation radioactive thymidine uptake into the cell was assayed. Cells transfected with PTHrP 1-87 saw a slight decrease in thymidine uptake.

Effects of PTHrP 1-87 protein transfection on proliferation: Figure 9 demonstrates the difference in thymidine uptake between cells transfected with PTHrP protein or beta-gal protein control. PTHrP transfected cells had an average of 10%

inhibition on proliferation compared to control cells ($P<0.05$). This suggests that intracellular PTHrP might be partially responsible for inhibiting DNA synthesis.

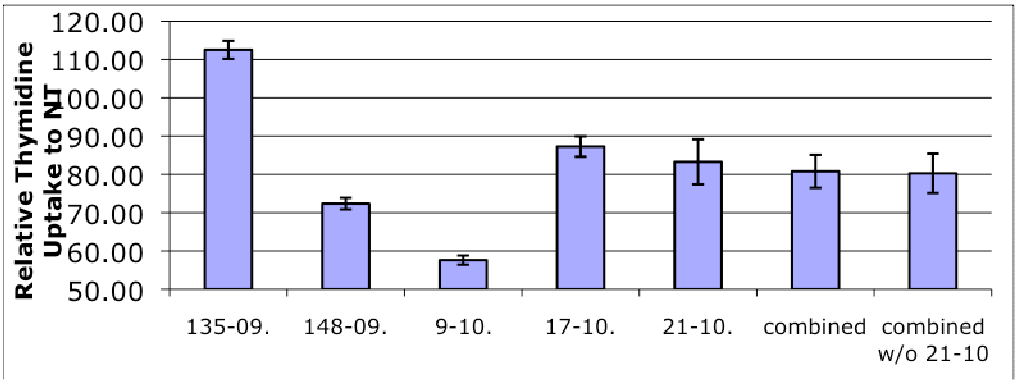


Figure 10: Thymidine uptake in extracellular PTHrP treated cells: Cells were treated exactly as though undergoing protein transfection but without Chariot treatment. After this treatment cells were incubated in media with and without PTHrP. Percent growth of PTHrP treated compared to non-treatment is found on the y-axis. Different experiments are found on the x-axis. Most experiments despite low variability within the experiment showed large variability between experiments.

Figure 10 shows the results of experiments on cells treated with PTHrP 1-87 or vehicle. The exogenous PTHrP decreased proliferation by 20% on average but results were variable among experiments ranging from slight stimulation to 40% inhibition in seven separate experiments. Variability within experiments was low compared to variability between experiments. The between experiments variability suggests that one or more experimental conditions might have varied across experiments and had effects on proliferation. The factors that might have varied include duration of PTHrP exposure, because the experimental period differed by ± 6-8 hrs, or confluence, which was not rigidly controlled. We next designed experiments to control for duration and confluence. We switched our growth measurement method to MTS assay because it easily accommodates growth assessments at multiple time points and confluences.

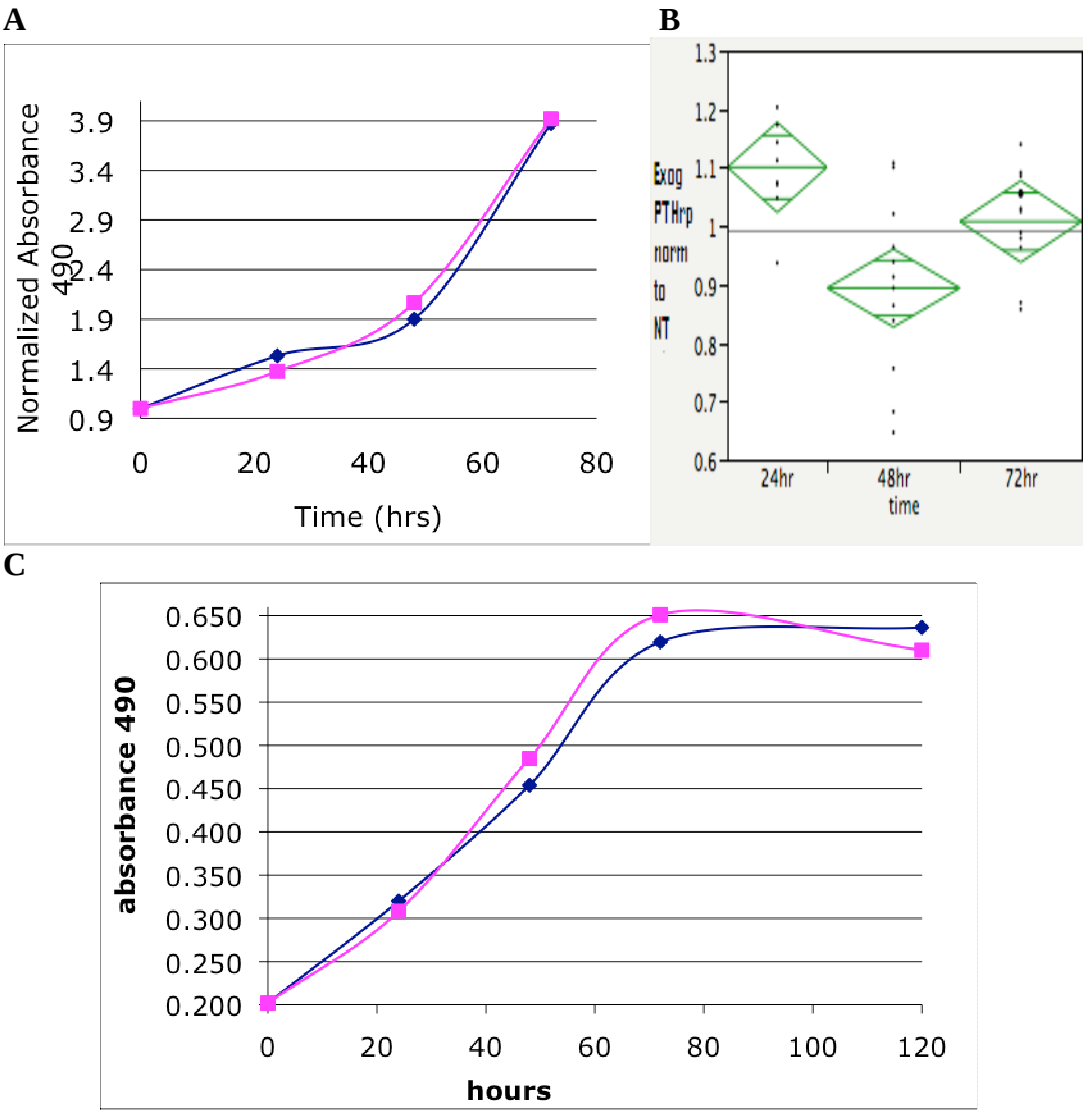


Figure 11: Exogenous PTHrP treatment of H1944 cells and MTS multiple time point proliferation assay. Cells underwent similar protocol as Figure 10 but with MTS as proliferation assay. They were treated with 500ug/ml PTHrP 1-87 (blue diamonds) or vehicle (pink squares) for 0, 24, 48, and 72 hours, then assessed for cell number with a commercial MTS kit. Absorbance at 490 adjusted to a media background was proportional to the quantity of cells for each time point. A) The first time course shows the results for confluent cells, with no consistent difference among PTHrP and vehicle groups over the experimental periods B) The same data from panel A, with cells adjusted to non treatment control levels at each time point. C) Experiment reproduced with half the cells plated per well and an extra time point at 120 hours of PTHrP (blue diamonds) or no treatment (pink squares). PTHrP appears to inhibition proliferation at intermediate time points, 48 and 72 hrs. Finally cell numbers return as cells begin to reach confluence at 120 hrs.

Figure 11 shows effects of exogenous, extracellular PTHrP 1-87 treatment on proliferation measured by the MTS assay. At the higher confluence (7000 cells plated per well), PTHrP had a significant effect on proliferation ($P<0.0001$), but effects were not consistent at each time point. A significant increase in proliferation in PTHrP treated cells was observed after the first 24hours(ttest p-value < 0.05) followed by an increase between the 24 and 48 hour with 48 hours having a 20% decrease in proliferation compared to the 24 hour time point, and these were the only time points statistically different with a p-value of 0.0008 by Tukey's post hoc test. Cells then returned to near no treatment levels at 72 hours but only gave a p-value of .056. In cells plated at a lower confluence (3,500 cells/well), PTHrP also affected growth but the action was consistent over 72 hrs. PTHrP inhibited the proliferation rate over that period, but the effect disappeared near the 100hour mark when cells were nearing confluence and growth rates had slowed.

These results show similarity to other proliferation tests done on exogenous treated H1944 cells. However, stable clones still exhibit more inhibition on cell growth than exogenous PTHrP alone. MTS on PTHrP chariot transfected cells was also performed to check for time dependent growth.

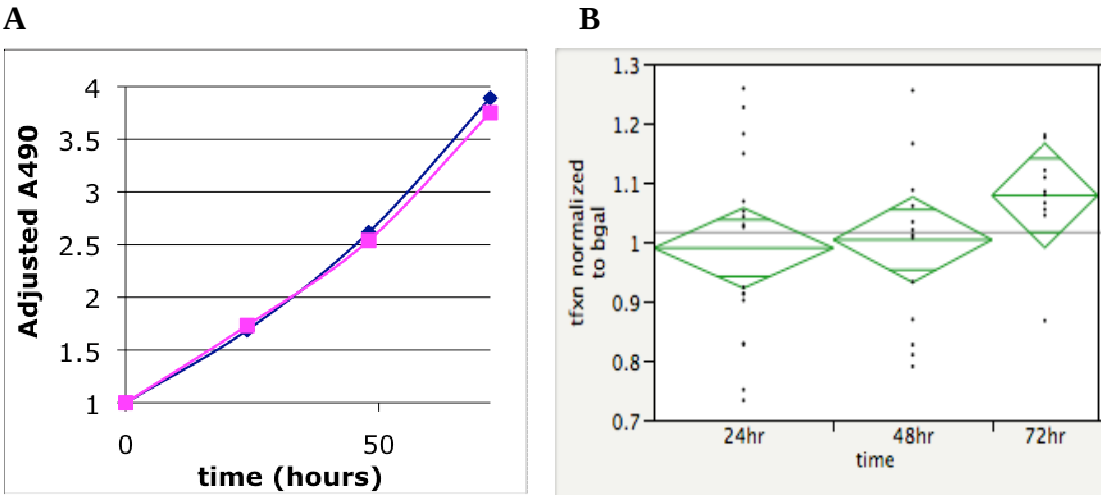


Figure 12: MTS of H1944 cells with PTHrP protein transfection: Cells underwent either a Chariot transfection of PTHrP 1-87(blue diamonds) or Beta galactosidase (pink squares) Cells were then incubated until MTS time point at 0, 24, 48, or 72 hours. A) Three experiments adjusted to media background to show relative cell counts per time point. B) PTHrP 1-87 treatment adjusted to B-Gal levels to demonstrate differences between PTHrP effects at each time point.

PTHrP and Beta Gal chariot transfections showed approximately equal levels of absorbance at each time point. ANOVA analysis determined that differences seen in absorbance were not significant and gave a p-value of .260, suggesting that intracellular PTHrP transfection has no significant effects on H1944 proliferation measured by the MTS method in near confluent cells.

shRNA knockdown of PTH1R

These prior experiments suggested that intracellular PTHrP had little if any responsibility for inhibitory effects on cell growth. By exclusion, this suggests that the inhibition must come from extracellular PTHrP, which has documented inhibitory effects supported by results from **Figure 2 and 10**. Our next plan was to evaluate the extracellular activities of PTHrP further by looking at PTHrP's interaction with PTH1R, a known receptor for PTHrP 1-34. ShRNA targeting of PTH1R allowed experimental

analysis of its role in PTHrP's growth inhibition. If PTH1R is the responsible receptor, then knockdown of PTH1R in clones that express PTHrP should return to normal levels of proliferation. H1944 cells that do not express PTHrP will also have PTH1R knocked down. If PTH1R is necessary for PTHrP to inhibit then these cells should lose sensitivity to exogenous PTHrP.

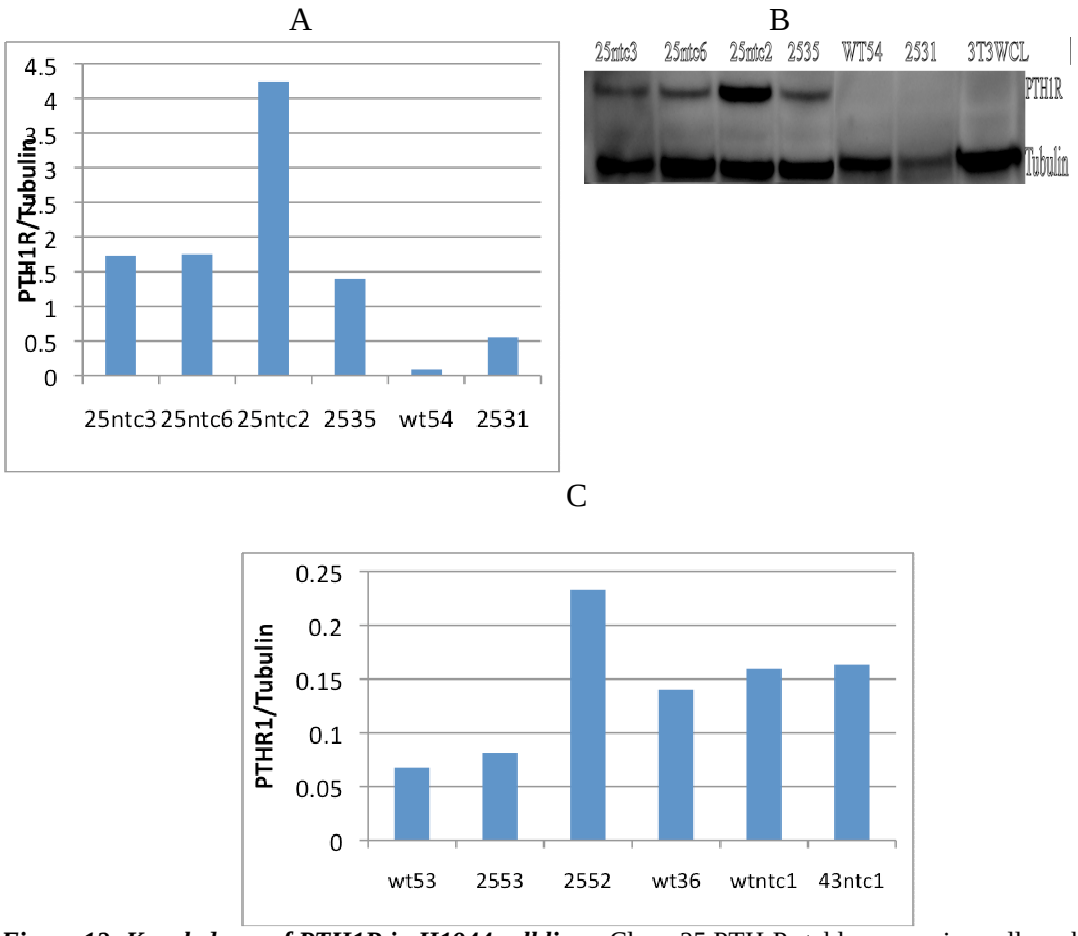


Figure 13: Knock down of PTH1R in H1944 cell lines: Clone 25 PTHrP stable expression cells and wild type H1944 cells were stably transfected with shRNA construct against PTH1R. Cells were then collected and lysed for western blot analysis of PTH1R vs. alpha tubulin as protein loaded control. A) Densitometry analysis of western blots. Relative PTH1R density/tubulin density in arbitrary units is plotted for different H1944 cell clones as noted on the x axis; the first two digits define the clone identity and the next digits indicate the shRNA treatment number (either a number of knock down or a non-target control (ntc)) B) picture of western blot for PTH1R (top band) and Tubulin (lower band) for some of the clones. C) Densitometry analysis of a 2nd western blots run the same way as the first but with different samples. Succesfull KD noted in 4Densitometrye 2 clones failed.

Pilot studies. PTHrP-negative and positive H1944 cells were stably transfected with non-target control (NTC) or shRNA lentiviral vector specific for PTH1R (Santa Cruz Biotech, Santa Cruz, CA). In **Figure 13** all non-target control shRNA cells exhibited approximately the same amount of PTH1R except for clone 25ntc2, which had almost three times the amount of PTH1R. In a relative comparison of non-target control cells (excluding 25ntc2) against all shRNA targeting PTH1R knocked down PTH1R protein expression by between 40 and 95% in all cell lines except 2535 which only had a 20% knockdown and 2552 which had an 50% increase in expression.

Knowing the PTH1R expression levels allowed selection for the clones with the best receptor knockdowns and also allowed insight into experimental results for analysis of proliferation through comparisons to expression levels.

A

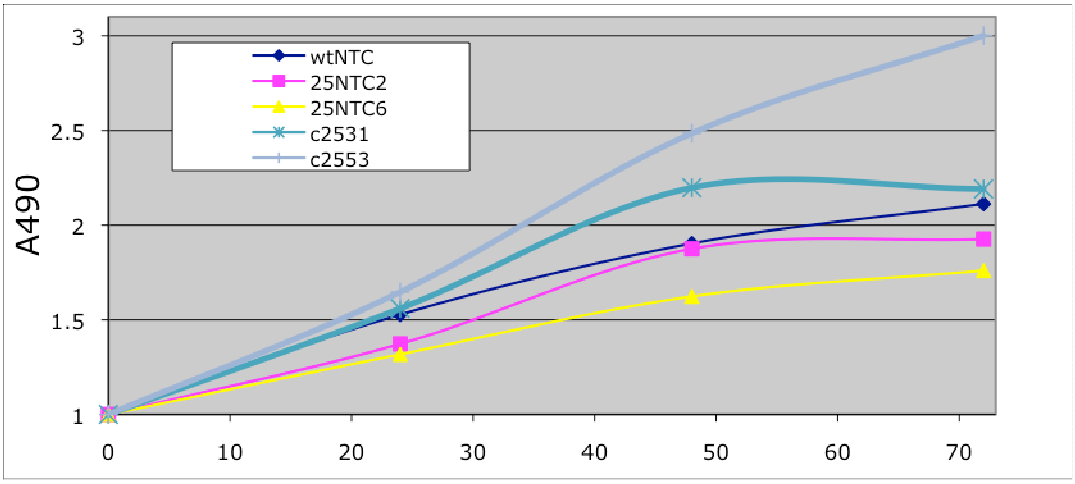
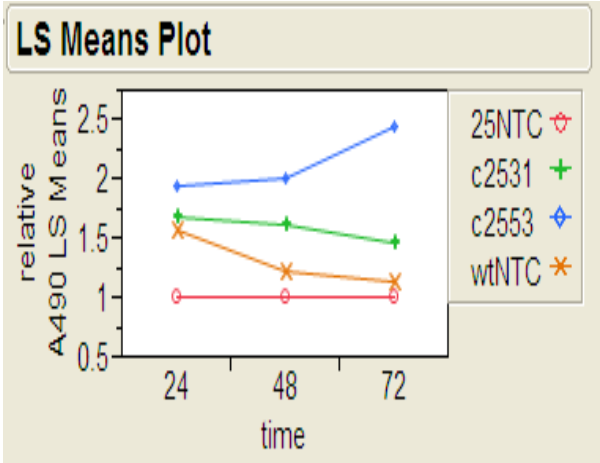


Figure 14: MTS on stable expressing PTHrP and stable shRNA against PTH1R H1944 cells: Cells that are stable for both expression of PTHrP and shRNA against PTH1R were plated in replicates of 6 in 96 well plates. At each time point 0, 24, 48, or 72 hours (x axis) MTS assay was preformed. A) is a graphic representation of A490 at each time-point. B) Is an LS Means chart for the difference in A490 as adjusted to the average clone 25ntc at each time-point with a table of connecting letters in which treatments not connected with the same letter are significantly different.

Figure 14 continued
B

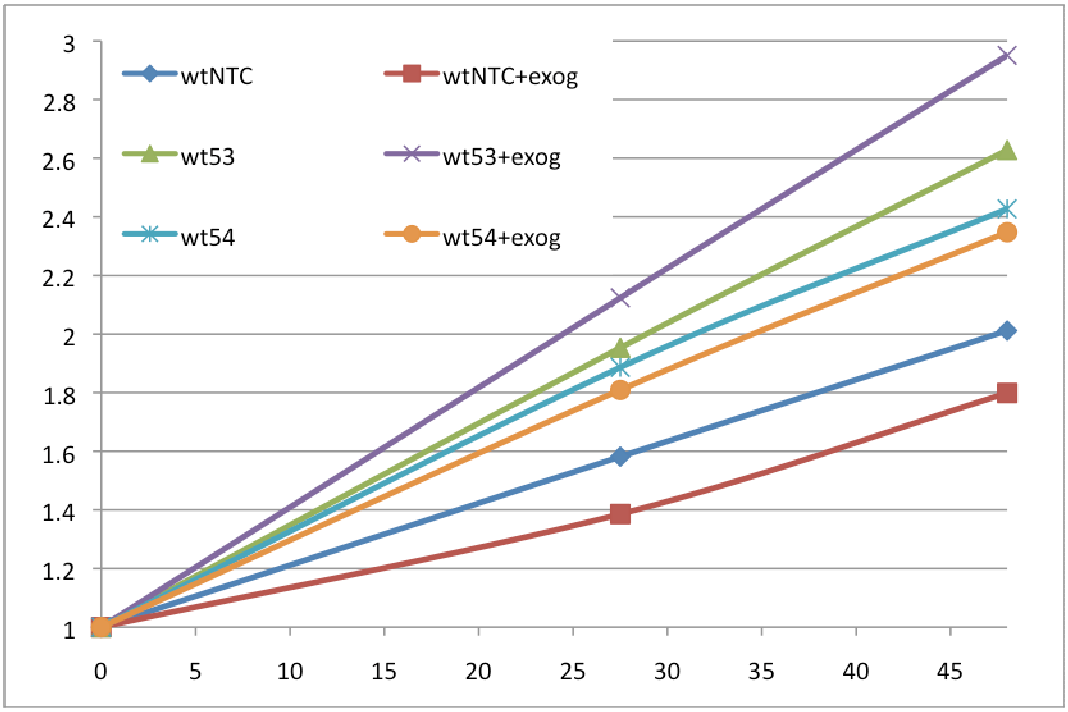


Level		Least Sq Mean
c2553,72	A	2.4384620
c2553,48	A B	2.0124996
c2553,24	B	1.9373563
c2531,24	B C	1.6762695
c2531,48	B C D	1.6228182
wtNTC,24	B C D E	1.5808024
c2531,72	C D E	1.4510518
wtNTC,48	D E F	1.2240292
wtNTC,72	E F	1.1264937
25NTC,48	F	1.0000000
25NTC,24	F	1.0000000
25NTC,72	F	1.0000000

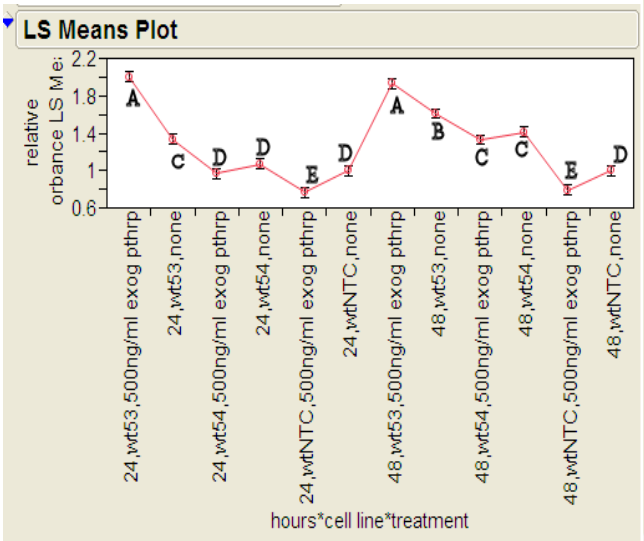
Effects of PTH1R knockdown on lung cancer cell proliferation. Cell growth was assayed by MTS assay. Clone 25 ntc (pink and yellow lines) showed significant decreases in absorbance in comparison to WTntc at 24 and 48 hrs of about 40 and 20%, respectively. The decrease is expected since Clone 25 expresses PTHrP. Clones 2553 and 2531 showed increased absorbance by 90-140% and 45-67% respectively compared to cell lines with intact PTH1R, but neither had significant changes in stimulation based on time.

shRNA targeting of PTH1R seemed to have an overall stimulatory effect on PTHrP expressing H1944 cells increasing proliferation to levels higher than even WTntc suggesting that PTH1R may be constitutively active and/or play a bigger role than expected. This suggests that PTH1R has effects on cell growth but it is unknown if they are still sensitive to PTHrP effects. Wild type H1944 cells with PTH1R knocked down by shRNA were then assayed for proliferation in the presence or absence of exogenous PTHrP 1-87 to determine if the receptor was necessary for sensitivity to PTHrP.

A



B



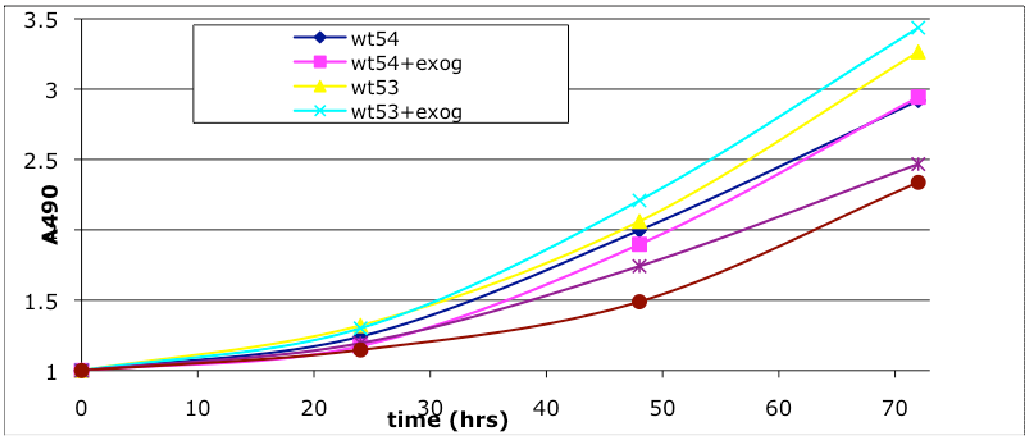
Level		Least Sq Mean
500ng/ml exog pthrp,wt53,24	A	2.0014813
500ng/ml exog pthrp,wt53,48	A	1.9283630
none,wt53,48	B	1.6091139
none,wt54,48	C	1.4105464
none,wt53,24	C	1.3374226
500ng/ml exog pthrp,wt54,48	C	1.3317856
none,wt54,24	D	1.0736117
none,wtNTC,48	D	1.0000000
none,wtNTC,24	D	1.0000000
500ng/ml exog pthrp,wt54,24	D	0.9696154
500ng/ml exog pthrp,wtNTC,48	E	0.7894865
500ng/ml exog pthrp,wtNTC,24	E	0.7681336

Levels not connected by same letter are significantly different.

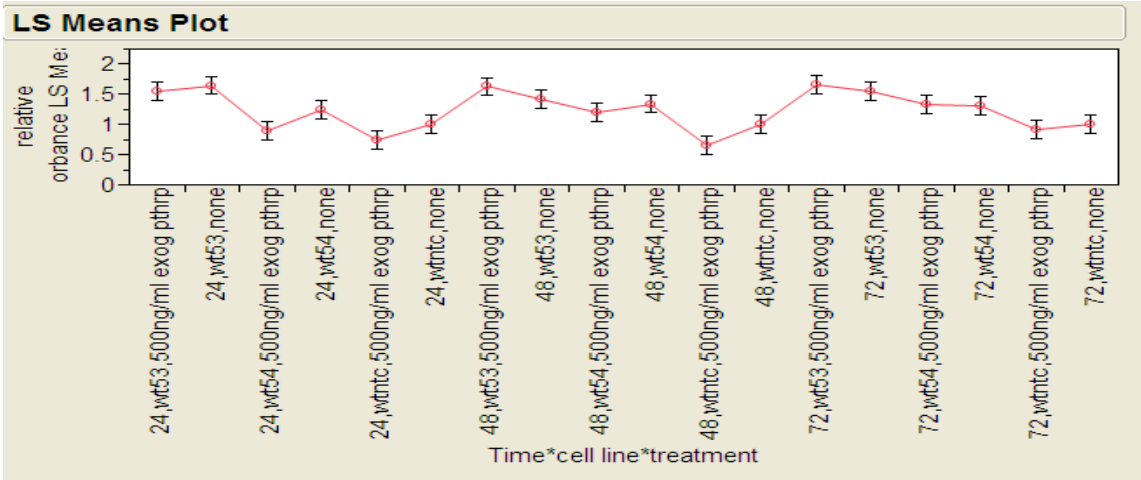
Figure 15: MTS for wild type cells stably transfected with shRNA against PTH1R and treated with PTHrP: Wild type H1944 cells were transfected with shRNA against PTH1R or a non-target control. Cells were then split into two groups one got regular media and the other received media with 500ug/ml of PTHrP and incubated for 24hours at 37celcius. Y axis is background adjusted absorbance at 490nm for each time point and treatments can be found in the legend. A) Cells were plated at aproximately 2,000 cells per well and an MTS assay was run on them at time points 0, 24, and 48 hours found on X axis. B) Cells adjusted to WTntc with no PTHrP treatment for each time point, and LS means diagramed. Table has treatments without the same letters are significantly different C) Cells plated at aproximately 1,000 cells per well and assayed at 0, 24, 48, and 72 hours. D) Cells adjusted to WTntc with no PTHrP treatment for each time point, and LS means diagramed. Table has treatments without the same letters are significantly different.

Figure 15 continued

C



D



Level		Least Sq Mean
72,wt53,500ng/ml exog pthrp	A	1.6602309
24,wt53,none	A	1.6480833
48,wt53,500ng/ml exog pthrp	A B	1.6288267
24,wt53,500ng/ml exog pthrp	A B C	1.5449674
72,wt53,none	A B C	1.5430332
48,wt53,none	A B C	1.4286953
48,wt54,none	A B C D	1.3434107
72,wt54,500ng/ml exog pthrp	A B C D	1.3243041
72,wt54,none	A B C D	1.3076905
24,wt54,none	B C D E	1.2433511
48,wt54,500ng/ml exog pthrp	C D E	1.2084342
72,wtntc,none	D E F	1.0000000
24,wtntc,none	D E F	1.0000000
48,wtntc,none	D E F	1.0000000
72,wtntc,500ng/ml exog pthrp	E F	0.9115532
24,wt54,500ng/ml exog pthrp	E F	0.8938697
24,wtntc,500ng/ml exog pthrp	F	0.7506100
48,wtntc,500ng/ml exog pthrp	F	0.6593048

Levels not connected by same letter are significantly different.

Exogenous treatment of PTHrP on WTntc cells induced significant inhibition on proliferation slowing cell growth by 25 to 35% for the first 48 hours **Figure 15**). PTH1R knockdowns grew significantly faster than WTntc cells and showed no inhibition of proliferation when treated with exogenous PTHrP. WT53 showed a significant increase in proliferation when treated with exogenous PTHrP. Suggesting that PTH1R is necessary for PTHrP's inhibitive actions and that PTHrP 1-87 could also have stimulatory effects with a mechanism that does not include PTH1R.

DISCUSSION

Neutralization of PTHrP 1-34

The objective of our study was to determine the site where PTHrP acted to inhibit proliferation of lung cancer cells. Previous studies in other cell lines suggested that PTHrP had inhibitory effects at an extracellular site on the cell membrane and that antibody neutralization of PTHrP 1-34 could eliminate most to all inhibitory effects of PTHrP (27). We began similarly by treating stable PTHrP 1-87-expressing H1944 cells with blocking antibodies to PTHrP 1-34. Figure 1 demonstrated that antibodies against PTHrP 1-34, 8B12 and 1A5, were effective in precipitating PTHrP immunoreactivity out of the media, reducing levels compared to treatment with a non-specific binding control 9H7, and that 1,000-fold excess of antibody to PTHrP treatments should be sufficient to bind nearly all exogenous PTHrP.

We assayed effects of the antibodies on growth measured by cell incorporation of tritiated thymidine, a marker for DNA synthesis. Figure 2 diagrams results showing that the antibodies stimulated growth by about 13%-16% compared to non-specific antibodies, suggesting that ectopic PTHrP produced by the cells inhibited their growth at an extracellular site. Neutralization at the 1-34 site suggests that this portion of the peptide is responsible for part of the inhibition, but it is possible that the antibody reduced PTHrP activity by blocking other active areas of the peptide or by precipitating the whole molecule. The antibody neutralization rescued approximately 1/3rd of the inhibition

observed between PTHrP expressing H1944 cells and wild type H1944 cells. Explanations for the failure of the antibody neutralization to block all growth inhibitory activity include incomplete neutralization of the peptide or the presence of an independent inhibitory mechanism that would not be blocked by reducing extracellular PTHrP levels.

Constructs designed for directed localization of PTHrP

PTHrP is transported to different areas of the cell and this transportation may allow PTHrP access to inhibit growth at sites other than the cell surface. To test for PTHrP's effects in different areas of the cell, PTHrP vectors were selectively mutated in order to drive PTHrP transport towards or away from different cellular loci. These mutants were sequenced at the CFAR molecular Biology Core and were analyzed to ensure the integrity of the sequence for WT PTHrP 1-141, PTHrP 1-141 delta SRP, PTHrP 1-141 delta 87-106, PTHrP 1-173 delta 108-141, and PTHrP 1-87. Pilot studies demonstrated that H1944 cells transfected with a GFP construct using the same promoter at high efficiency and that the PTHrP constructs drove expression of PTHrP protein at biologically relevant levels. RIA results for PTHrP expression are seen in figure 3. PTHrP 1-141 delta SRP produced a very high density of PTHrP within the cell but media conditioned by transfected cells for 48 hrs still contained PTHrP at high levels. This suggested that the delta SRP protein was secreted or that it leaked out of cells through another method. This prompted us to take a closer look at the role and time-course behind PTHrP 1-141 delta SRP secretion.

Figure 3c shows multiple RIA time-points after transfection. At 24 hours PTHrP 1-141 delta SRP transfected cells had 90% of its PTHrP within the cell. However, after the next 24hours only 20% remained similar to the levels in cells transfected with wild type PTHrP 1-. Washing cells seemed to lower the amount of PTHrP 1-141 and PTHrP 1-141 delta SRP in lysates compared to cells that were not washed. This was especially evident in the Delta SRP transfected cells, which had more than two fold the PTHrP within the cell when not washed. This may be from mechanical effects of washing the cell or may also suggest that PTHrP is passively secreted. There is no way to ensure that no PTHrP is flowing to the extracellular space but by performing and completing the experiment within the first 24 hrs, we knew that the PTHrP would be confined largely within the cells.

Figures 4 and 5 show the effect of the constructs on thymidine uptake in cells cultured under confluent conditions compared to sub confluent conditions. Interestingly, the PTHrP constructs produce no inhibition on confluent H1944 cells (Figure 4) but do reduce growth when the cells are not confluent. Dependence of the actions of PTHrP on confluency or differentiation state has been demonstrated before in other cell lines. In early osteoblastic cells, PTHrP up-regulated cyclin D1, a signal for proliferation, only in sub-confluent cells. PTHrP protects mesenchymal cells from apoptosis only when the cells are confluent.[28, 29]

As seen in figure 5, all variations of peptide significantly inhibit thymidine uptake when transfected into H1944 cells compared to transfection of the Pci-neo control vector,

indicating successful inhibition of proliferation. However, there are no significant differences between any of the variants themselves or any grouping of variants suggesting that inhibition is independent of intracellular trafficking. PTHrP 1-141 and PTHrP 1-141 DeltaSRP caused the same degree of inhibition, suggesting secretion was not absolutely necessary. NES and NLS deletions had little impact on inhibition as well, suggesting a minimal role if any for nuclear localization.

The NES allows PTHrP to exit the nucleus through CRM1 dependent transportation[18, 19]. PTHrP Delta 108-141 is missing the NES portion of the peptide and thus can traffic into the nucleus but should not be able to leave the nucleus. This should increase levels of PTHrP within the nucleus and if the site of inhibition is within the nucleus then this construct should display greater inhibitory effects than the constructs that do have the NES. This is not observed suggesting that either the nucleus is not the site of action for growth inhibition.

PTHrP's NLS has been shown necessary for proliferative regulation in certain tissues and allows for importin-beta dependent shuttling into the nucleus[15-17]. PTHrP 1-141 delta 87-108 maintains the NES but has a deleted NLS. This peptide will not transport to the nucleus and any PTHrP that may transport independent of the NLS should be exported through actions mediated by the NES, resulting in nuclear levels below physiological levels of wild type PTHrP. If PTHrP's site of action is dependent on nuclear localization then this construct should display very little to no inhibition on thymidine uptake. This is not the case as the NLS deletion mutant displays nearly as

much inhibition as its delta NES counterpart. The data from the NES and NLS deficient constructs strongly support that PTHrP's site of action is independent of nuclear localization.

The Delta SRP results can be interpreted in a number of ways. PTHrP that lacks the SRP displayed far more PTHrP within the cell at 24 hours than outside as seen in figure 3. This intracellular PTHrP was about 4-fold greater than the quantity of any of the other constructs. If the site of growth inhibition is intracellular we would expect greater inhibition from this construct than those with low intracellular PTHrP expression. On the other hand, if the site of action is extracellular then the Delta SRP PTHrP construct should cause less inhibition of cell growth. In fact, constructs that maintain their SRP display just as much inhibition as the other cells. This suggests that PTHrP could have dual sites of action inside and outside the cell. Given the lack of evidence for a nuclear inhibitory effect, one would postulate that the intracrine growth effect would occur in the cytoplasm. Such a mechanism is plausible because PTHrP has a number of cytoplasmic binding partners but completely speculative at this time. As an alternative to simultaneous extra- and intracellular effects, it is still possible that cells transfected with PTHrP delta SRP may leak enough PTHrP out of the cell (Figure 3) to have extracellular effects. Thus, further tests of PTHrP's intracellular vs. extracellular dependent growth inhibition were designed.

Endogenous vs. Exogenous PTHrP treatments

Our pilot studies in Figures 6,7 and 8 demonstrated that Active Motif's Chariot protein delivery reagent is a successful and safe means for delivering intracellular PTHrP into H1944 cells. Figure 6 demonstrates that PTHrP-Alexa488 could be successfully shuttled into H1944 cells. Figure 7 showed that the Chariot peptide transfection do not cause cells to lose viability. Figure 8 demonstrates that H1944 cells transfected or microinjected with PTHrP peptide exhibit show decreased Erk activation. These results suggest that PTHrP levels of peptide transfection are similar to those of microinjection and that quantities of peptide within the cell are sufficient to effect biologic changes.

The initial proliferation assay in Figure 9 showed that PTHrP protein transfection had some effects on thymidine uptake compared to transfection of beta-gal control protein. This 10% inhibition represents a fraction of the 40 to 60% inhibition found in stable PTHrP expressing H1944 cells. This suggests that PTHrP may engage an intracellular growth inhibition processes. However, the results of control manipulations complicated the issue.

Figure 10 shows a no treatment control and exogenous PTHrP treatment without Chariot that were also performed for this experiment. Exogenous PTHrP inhibited thymidine uptake by about 19% consistent with our prior results However, different experiments gave variable degrees of inhibition, ranging from some stimulation to 42% that seemed to depend on small variations in experimental protocol such as duration (which varied by 3-6 hrs in total time length), confluency, or cell handling. To control these variables better, we switched to MTS as a method of assaying proliferation. MTS

experiments incorporate a time course, allowing duration and confluence to be considered in the analysis. Also, In MTS assay cells need no washing or handling steps once treatment begins.

In figure 11A and B, exogenous PTHrP treated cells significantly increased proliferation in first 24 hours by 10%, as assayed by MTS, then dropped by 20% between the 24 to 48 hour marks compared to vehicle-treated cells, and then increased proliferation for the final 24 hr period up to the 72 hr mark. . The initial increase in proliferation may have to do with PTHrP's effects on other cell processes, such as protection against cell death. PTHrP may also stimulate cells at short time points while chronic PTHrP exposure decreases growth. In figure 11 C cells plated at half the confluence were treated with exogenous PTHrP and assayed for growth by MTS. At the lower cell density, exogenous PTHrP inhibited proliferation until 72 hours of treatment. The narrowing of the difference between PTHrP-treated and vehicle control groups after 72 hrs may be a matter of confluence These experiments maintain the impression that at least part of the inhibition of proliferation ascribed to PTHrP must come from extracellular actions. The results also reinforce the concept that experimental conditions, particularly confluency (see Figure 4) modify the effect of PTHrP and may explain the variability shown in Figure 10.

We repeated the test of PTHrP protein transfection on growth using the MTS assay to provide a comparison with the effects of extracellular PTHrP. Figure 12 surprisingly shows that MTS assay of chariot transfected PTHrP peptide did not inhibit

growth measured by a cell mass assay, MTS. This observation conflicts with results from thymidine experiments (figure 9). The disparate results may occur because of methodological differences; MTS is a measure of metabolic break down rates and intracellular PTHrP may increase this metabolic rate, while thymidine uptake is a measure of DNA synthesis, which may be slowed down by intracellular PTHrP. Thymidine also entails extensive wash steps while MTS protocol has no wash steps. PTHrP has been shown to increase invasion of cancerous cells and this may be do to lowered cell adhesion[30]. If this is the case wash steps in thymidine experiments may wash away cells. In any case, if intracellular PTHrP has effects on cell growth, they are small and do not explain the entire growth inhibition induced by stable PTHrP gene transfer. Thus, we turned our attention at this point to extracellular effects.

ShRNA knockdown of PTH1R

Since exogenous PTHrP 1-87 inhibits growth and neutralization of PTHrP 1-34 augments growth, PTH1R, the receptor for PTHrP 1-34, may be involved in proliferative inhibition of H1944 cells. Of note, the findings with protein and antibody treatment in H1944 cells are consistent with similar studies in Ben squamous lung carcinoma cells in-vitro and in-vivo[23]. To study the possible role of PTH1R, stable clones of shRNA targeting PTH1R were created in wild type H1944 cells as well as clone number 25 and 43 which are stable PTHrP expressing H1944 clones. Figure 13 shows that the shRNA reagents successfully knocked down PTH1R by up to 95%. We picked the stable clones with the greatest PTH1R knock down for subsequent growth studies.

Figure 14 shows the MTS assay for proliferation of these knockdowns. figure 14 A-C, two knockdown clones for a PTHrP expressing H1944 line showed an increase in proliferation compared to the same clone treated with a non-silencing shRNA (NTC). Proliferation increased to a level greater than even a wild type, PTHrP-negative clone transfected with the ntc clone vector (WTntc). . 25ntc clones demonstrated 40% growth inhibition compared to WTntc cells over a 24 hr period, consistent with previous findings of growth inhibition due to PTHrP. These results indicate that PTH1R is involved and very important for inhibition of proliferation .

To delve deeper into this, the effects of shRNA targeting PTH1R were assayed on growth of PTHrP-negative WT cells. At this time a new method was used for looking at effects of exogenous PTHrP. Cells were pre-incubated in media containing PTHrP for 24hours and then were plated with PTHrP treatment continuing through the experiment. This, we believed, should better mimic the conditions in stable expressing PTHrP clones, which experience constant exposure to ectopic PTHrP and may be arrested in a quiescent state as a result, even before they are plated. As seen in Figure 15, exogenous PTHrP treatment with this new protocol inhibited proliferation of WTntc cells significantly by around 25 to 35%, similar to the reduction of inhibition seen in 25ntc clones compared to Pci-Neo transfected clones. This degree of inhibition with exogenous PTHrP is much larger than achieved previously and its magnitude is great enough to suggest that exogenous PTHrP may be responsible for nearly all inhibition of proliferation on H1944 cells. Furthermore, shRNA-mediated knock down of PTH1R eliminated sensitivity of PTHrP-negative H1944 cells to exogenous PTHrP treatment. The rates of proliferation

of wt54, a PTHrP-negative PTH1R knockdown clone and wt54 treated with exogenous PTHrP were not significantly different. This suggests that the PTH1R/PTHrP interaction contributes to the inhibitory effects of PTHrP on cell growth. Inhibition of proliferation with exogenous PTHrP acting through PTH1R inhibition has also been observed in vascular smooth muscle cells.[31] Interestingly, PTH1R knockdown appeared to stimulate proliferation in PTHrP-negative cells, even in the absence of exogenous PTHrP suggesting that PTH1R is constitutively active and mediates partial inhibition of cell growth independent of the presence PTHrP or other ligands,

CONCLUSION

Through stepwise experimentation, the site of action of PTHrP in inhibiting lung cancer cell proliferation has been identified as an extracellular location, presumably involving PTH1R ligation. The studies involved intentional direction of PTHrP towards or away from certain areas of the cell, followed by growth assays. Neutralization of the 1-34 amino acid segment of PTHrP partially blocked PTHrP from inhibiting cell growth. Transfection of PTHrP constructs directed toward or away from the nucleus certain areas of the cell made it apparent that PTHrP's inhibitory effects could not have been taking place within the nucleus. Growth inhibition by intracellular PTHrP has not been ruled out completely. However, the PTHrP protein transfection experiments showed little to no decrease in proliferation suggesting most to all effects come from outside the cell. PTH1R knockdowns in clone 25 PTHrP expressing H1944 cells demonstrated PTH1R's importance to inhibit growth in H1944 cells and suggested that the receptor was constitutively active and further stimulation may increase inhibition. A protocol for exogenous PTHrP treatments showed inhibition on WTntc cells more comparable to stable PTHrP lines suggesting possible complete PTHrP inhibition from extracellular sources, a greater exogenous effect than previously thought. Finally, PTH1R knockdowns in wild type H1944 cells that do not express PTHrP again showed an increase in proliferation but also lost all sensitivity to the inhibitory effects of exogenous PTHrP, which even stimulated some growth. This suggested that PTHrP's inhibition is solely from its interaction with PTH1R at the cell surface.

Future experiments should include tests for other PTH1R activating ligands including PTH and different segment lengths of PTHrP, such as PTHrP 37-86 and PTHrP 67-86. We will also second messenger signaling by PTH1R after exogenous treatments and see if PTH1R remains active or is down regulated as seen in VSMC[31]. We will attempt to discover what PTH1R pathway is involved in growth inhibition. The possibilities include G protein-coupled receptor pathways involving Gs and/or Gq or internalization of receptor and PTHrP complexed to β-arrestin1 or 2 leading to activation of ERK[2-4]. Second messenger signals will be assayed upon treatment of exogenous PTHrP looking for activation. G-protein coupled inhibitors can also be used to see if one pathway or the other is necessary for PTHrP's inhibitory effects. Finally antagonists can be used, as done in a Song et al. experiment, to block β-arrestin1 and 2 stimulation[31]. Further, ligands will be screened for optimal inhibitory PTH1R interaction. Finally, experiments will be performed to investigate whether PTHrP interacts with other signaling pathways active in non-small cell cancer, such as EGFR or Akt pathways. Knowledge about how PTHrP regulates cancer cell growth could translate into experiments involving tumor growth in animal models. PTHrP and the inhibitory pathway it activates through PTH1R could become important in future therapeutic agents that could one day be used to treat human non-small cell lung carcinoma through inhibiting proliferation.

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