UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Effects of PTHrP-Mediated ERK Activation on Non-Small Cell Lung Carcinoma

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

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by

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The thesis of Jennifer Phun is approved, and it is acceptable

in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

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Dedication

For my loving mother.

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List of Abbreviations

cAMP	Cyclic adenosine monophosphate
ERK	Extracellular signal-regulated kinase
GPCR	G-protein coupled receptor
MEK	Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase
NES	Nuclear export signal
NSCLC	Non small cell lung carcinoma
pERK	Phosphorylated extracellular signal-regulated kinase
PKA	Protein kinase A
PKC	Protein kinase C
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
siRNA	Small interfering ribonucleic acid
tERK	Total extracellular signal-regulated kinase
UV	Ultraviolet

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Randolph H. Hastings, Chair

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Each year, more people die from lung cancer than any other cancer related death. Parathyroid hormone-related protein (PTHrP) is expressed in two-thirds of human non-small cell lung carcinoma (NSCLC), the most common form of lung cancer. Patients diagnosed with PTHrP expressing NSCLC are seen to have better prognosis. When further studied, PTHrP positive cells increased the levels of ERK1/2 phosphorylation.

Because ERK1/2 has implications on cell growth, we postulated that ERK activation in PTHrP positive cells may contribute to PTHrP's growth inhibition. To understand the role of PTHrP mediated ERK activation, an siRNA model was employed to knock down ERK1, ERK2 or

ERK1 and ERK2 combined, where decreasing total ERK protein will lower phosphorylated ERK as well. ERK knockdown in ectopic PTHrP expressing cells was then tested for effects in cell proliferation and localization.

ERK2 knockdown decreased proliferation in PTHrP positive cells. ERK1 knockdown increased ERK2 levels as well as increased proliferation at 72 hours, thereby suggesting a feedback mechanism between ERK1 and ERK2 in PTHrP positive cells. ERK1/ERK2 combination knockdown decreased proliferation at 72 hours, suggesting that ERK1 opposes ERK2 and inhibits proliferation. Examination of pERK cellular localization in cells ectopically expressing PTHrP demonstrates that pERK1/2 localizes to the nucleus, therefore pERK1 and pERK2 localization does not contribute to the differences in effect between isoforms. These results allude different effects between ERK isoforms where ERK2 increases proliferation and ERK1 inhibits proliferation. Therefore, PTHrP decreases proliferation through a mechanism that overpowers the pro-proliferative actions of ERK2, which may involve ERK1 activation.

1 INTRODUCTION

1.1 Lung cancer and Non-small Cell Lung Carcinoma

Every living thing is comprised of cells. Although a basic component of life, a cell has the potential of being dangerous and often times, lethal. Normal cells have programs and controls directing them on when to divide or die. Cancer arises when a cell becomes loses control over its lifespan, proliferative rate and death regulating. If not treated and removed, a carcinoma is capable of spreading to neighboring sites in the body. Typically resulting from tobacco smoke exposure, lung cancer is the leading cause of cancer-related deaths in both men and women. Each year, more deaths result from lung cancer alone than breast, colorectal and prostate cancer combined. The 5-year survival rate of those with lung cancer is a grim 15% or less due to the tendency for early metastasis and lack of early diagnosis. Lung cancer is divided into small cell lung carcinoma and non-small cell lung carcinoma (NSCLC), with 85% of lung cancers are categorized as NSCLCs (American Cancer Society, 2011).

1.2 Parathyroid Hormone-related Protein

Parathyroid hormone-related protein (PTHrP) was first discovered as a product of cancer from cancer patients displaying symptoms of hyperparathyroidism and hypercalcemia (Moseley et al., 1995). It is synthesized and expressed by various normal and malignant tissues such as skin, bone, blood vessels, smooth muscles neuronal tissues and the lungs (Schluter, 1999). In humans, the PTHrP gene codes for three isoforms derived from alternative mRNA splicing. They include PTHrP 1-139, PTHrP 1-141 and PTHrP 1-173, where the first 139 amino acids are identical in all three isoforms (Yang and Stewart, 1996). PTHrP is named for its amino terminal primary and secondary structure homology to parathyroid hormone (PTH), where 9 amino acid residues out of the 1-13 sequence are identical (Schluter, 1999). Because of this homology, PTHrP can bind and activate PTH's G-protein coupled receptor, the PTH/PTHrP

receptor PTH-1R (Juppner et al., 1991). Its affinity for the PTH-1R is how PTHrP mediates humoral hypercalcemia of malignancy (Suva et al., 1987). While PTH has global endocrine functions, PTHrP acts in a paracrine or autocrine/intracrine manner and functions in cell growth, apoptosis, calcium transport and smooth muscle relaxation (Goltzman et al., 1989).

PTHrP can exert effects through a number of pathways besides binding to PTH-1R. PTHrP has the potential to localize to the cytoplasm, nucleus and nucleoli (Farmer et al., 2008). It enters the nucleus with its nuclear localizing sequence in residues 87-106. One report found that PTHrP localizes to the nucleus or nucleolus during the G_1 phase and stimulates proliferation (de Miguel et al., 2001; Lam et al., 1999). On the other hand, others report that nucleolar PTHrP reduces rRNA synthesis in chondrocytes and could possibly prevent progression through the cell cycle (Aarts et al., 2001).

The site of PTHrP's action is important because of the potential for differential effects on cell growth. For instance, cells treated with PTHrP peptides acting through cell surface receptors inhibited cell proliferation although proliferation was stimulated with the same peptides when introduced with a nuclear targeting site. The cell surface receptors and nuclear targeting site trigger diverse effects through different cytokines and have implications on cancer (Goomer et al., 2000; Gujral et al., 2001; Massfelder et al., 1997).

1.3 PTHrP in Lung and Lung Cancer

During lung development, PTHrP is involved in alveolar type II cell maturation, where PTHrP or gene knockout results in underdeveloped lungs (Rubin et al., 1997). In normal adult lungs, PTHrP 1-34 negatively regulates type II cell proliferation (Hastings et al., 1997) and causes a differentiated phenotype (Hastings et al., 1996). This decrease in cell growth can possibly be attributed to PTHrP 1-34's capability to sensitize alveolar type II cells to apoptosis (Hastings et

al., 2003b). On the other hand, PTHrP is pro-apoptotic in BEN squamous lung carcinoma (Hastings et al., 2004b).

PTHrP is expressed in two-thirds of human non-small cell lung cancers (Brandt et al., 1991). Although some studies suggest that the presence of PTHrP in cancer signifies poor prognosis (Pecherstorfer et al., 1994), there is evidence that PTHrP is favorable for patients with lung cancer. Studies in immunocompromised mice treated with PTHrP antibodies had an increase in lung carcinoma growth (Hastings et al., 2001), suggesting that PTHrP inhibits lung tumor growth. Other studies found that women with PTHrP expressing lung carcinomas have a survival advantage compared to women without PTHrP, but the protein had no apparent implications in males (Hastings et al., 2006). Since context elements such as sex affects the cellular response to PTHrP, it is important to know which cellular pathways are involved in inhibiting proliferation.

Certain domains of PTHrP function to inhibit cell proliferation, such as PTHrP 38-94 in breast cancer tissues (Luparello et al., 2001), and PTHrP 107-111 and PTHrP 107-139 in rat osteosarcomas (Valin et al., 1997). Opposing effects of PTHrP can also be seen in the same tissue depending on whether the cell was stimulated in an intracrine or paracrine manner (Massfelder et al., 1997). Recently, PTHrP is found to reduce the expression of integrin α2 and α3 in an NSCLC cell line, which may be a mechanism to reduce lung carcinoma invasiveness (Hastings et al., 2010). PTHrP has effects on cell growth, apoptosis, matrix protein expression and invasiveness (Luparello et al., 1995).

1.4 Extracellular Signal-Regulated Kinase

Extracellular signal-regulated kinase (ERK) is a classical mitogen activated protein kinase (MAPK) involved in a wide range of cellular functions, commonly thought to stimulate growth. Under resting conditions, ERK proteins are found anchored in the cytoplasm. Upon

phosphorylation, ERKs translocate to the nucleus and can phosphorylate various substrates that include enzymes, transcription factors and cytoskeletal proteins (Nishida and Gotoh, 1993). These, in turn, regulate processes such as transcription, DNA replication and other various activities (Robinson and Cobb, 1997) that ultimately lead to effects on processes including cell-cycle progression, differentiation, migration, and cell death (Cagnol and Chambard, 2009; Huang et al., 2004; Marshall, 1995; Schlessinger and Ullrich, 1992). While many of ERK's effects occur when ERK is localized in the nucleus, ERK's extranuclear component is important as well. Approximately 180 ERK substrates have been identified, and nearly half of them do not reside in the nucleus (Yoon and Seger, 2006). After stimulation, half of ERK's content remains in the cytoplasm and regulates processes such as cell-matrix contact (Fincham et al., 2000), adhesion (Glading et al., 2001), and anti-apoptotic signaling (Ajenjo et al., 2004).

ERK is generally considered a pro-proliferative mediator, but it can also be anti-mitogenic depending on whether phosphorylation is sustained or shortlived (Arany et al., 2004; di Mari et al., 1999). Another factor that can modify the growth effects of ERK is its subcellular localization. For instance, it is possible that cytosolic retention of ERK1/2 prevents contact with transcription factors in the nucleus that allow mitogenesis (Torii et al., 2004), although these instances are not absolute. Factors altering growth response to ERK vary with several factors, including tissue type, differentiation state and cell density (Marshall, 1995).

1.5 PTHrP and ERK

PTHrP can activate ERK in various cell lines. While PTH-1R can act as a G_s or a G_q protein coupled receptor to stimulate the PKA and PKC pathways (Abou-Samra et al., 1992; Mahon and Shimada, 2005), it can also act through G-protein independent mechanisms. Evidence now support the involvement of the MAPK pathway (Chan et al., 2001; Miao et al., 2001), where

some cell types expressing PTHrP elevate the levels of phosphorylated ERK (Chen et al., 2004a; Hastings et al., 2009).

PTHrP-mediated ERK activation can be either pro- or anti-proliferative depending on factors such as cell differentiation, whether the stimulation were acute or sustained, and the spatial organization of ERK. For instance, PTH-1R stimulation by PTH increases cyclin D1 through a process dependent on MAPK (Datta et al., 2007) in proliferating cells. On the other hand, PTHrP signaling can induce growth arrest through a MAPK pathway involving the decrease in cyclin D1 (Datta et al., 2005). Differentiated cells show decreased ERK phosphorylation while undifferentiated cells increase ERK phosphorylation (Chen et al., 2004a). PTHrP activated ERK can be acute or sustained depending on the signaling pathway. PTH-1R stimulation can activate a G-protein/PKA pathway to stimulate acute ERK activation while a G-protein independent β-arrestin pathway allows sustained ERK activation (Gesty-Palmer et al., 2006; Sneddon and Friedman, 2007). Sustained ERK activation is typically reported to cease cell division (Clark et al., 2004; Greene and Tischler, 1976; Heasley and Johnson, 1992). Accordingly, intermittent exposure to PTHrP is seen to stimulate anabolism of osteoblastic cells through the ERK pathway (de Gortazar et al., 2006). These effects depend on cell type since sustained ERK activation in fibroblasts stimulates proliferation (Meloche et al., 1992). The effects mediated by the transient or sustained activation of ERK may result from ERK's ability to localize to the nucleus (Dikic et al., 1994; Nguyen et al., 1993; Traverse et al., 1992; Traverse et al., 1994). Nuclear ERK is associated with cell survival due to its access to pro-proliferative genes. On the other hand, PEA-15, an anti-apoptotic protein in the cytosol can tightly bind to ERK proteins (Callaway et al., 2007). Nonetheless, ERK can stimulate or inhibit growth.

1.6 PTHrP, ERK and Lung Cancer Proliferation

A 2009 study compared the proliferation two lung adenocarcinoma cell lines H1944 and MV522. These cell lines do not produce PTHrP, but express the PTH-1R. When PTHrP was stably transfected into these cell lines, both exhibited slower growth due to a slower transition through the G1 phase. These PTHrP expressing cells exhibited lower levels of cell cycle proteins cyclin D2 and cyclin A2 while increasing levels of the cyclin dependent kinase inhibitor $p27^{Kip1}$. When observing the signaling effects of PTHrP, both H1944 and MV522 cells had constitutive ERK phosphorylation, but no changes to other proliferative proteins such as Src (Hastings et al., 2009). ERK's anti or pro-proliferative properties make it a likely candidate for affecting cell proliferation in PTHrP positive cells. In either case, the activation of the ERK pathway has impacts on the proliferative effects of PTHrP.

1.7 Aims and Approach

The PTHrP mechanisms that could regulate lung cancer progression have not been established. We have therefore proposed that the ERK activation could have effects on cell proliferation in NSCLC. Since PTHrP is commonly expressed in NSCLC patients, it could be a reasonable target for lung cancer therapy. Further, as it is also unclear how ERK activation is related to cell proliferation, we have come up with three possible scenarios:

1. Although ERK is well known for increasing cell proliferation, the increase in ERK activation by PTHrP directly could decrease cell proliferation, thus contributing to the anti-proliferative effect of PTHrP
2. ERK activation has no effect on proliferation in the lung cancer cells. Thus, PTHrP decreases cell proliferation without an ERK influence
3. ERK activation stimulates lung cancer proliferation, opposing an independent effect of PTHrP to inhibit growth. If ERK were not activated, the PTHrP-mediated growth inhibition would be greater.

The experiments presented in this thesis investigate the role of ERK activation in PTHrP positive cells by determining which cellular functions are affected by ERK and PTHrP. To approach this project, lung cancer cells expressing the active PTHrP region will be treated with siRNA to knock down total ERK, thereby decreasing ERK activity. After ERK knockdown, cells will be subjected to a multitude of tests including tests for cell proliferation, integrin expression levels, apoptotic levels, and possible transcription factor up or downregulation.

Since PTHrP is expressed in two-thirds of NSCLC in humans, it could be a reasonable target for lung cancer therapy. Determining the function of this particular PTHrP mediated cellular inhibition pathway—ERK in this case—allows the pathway to be exploited in possible therapies for NSCLC.

2 MATERIALS AND METHODS

2.1 CELL CULTURE

NCI-H1944 human lung adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were grown in a humidified incubator with 5% CO_2 and 95% oxygen. The H1944 media used contained RPMI 1640 medium plus 8% fetal bovine serum supplemented with D-glucose, HEPES and sodium pyruvate. PTHrP expression in H1944 cells was induced by a cationic liposome-mediated transfection of a pCi-Neo-PTHrP 1-87 expression plasmid as described in Pache et al. 2006. Control cells received an empty pCi-Neo vector. Cells were incubated with a lethal dose of geneticin to remove non-transfected cells. Colonies were isolated, expanded, and screened for PTHrP secretion by immunoassaying the media for PTHrP 1-34. PTHrP 1-87 was transfected instead of the full length PTHrP 1-141 protein to focus on the effects of amino terminal region containing the PTH1R ligand. A shorter expression plasmid with PTHrP 1-34 peptide was not used because PTHrP 1-34 expression is much lower than plasmids containing PTHrP 1-87 (Ditmer et al., 1996). H1944 cells were used to study the effects of PTHrP because these cells express the PTH-1R receptor, but not PTHrP. This means that experimental manipulations can be compared to unaltered H1944 cells.

2.2 siRNA TRANSFECTIONS

H1944 PTHrP positive and negative cells were seeded 2.5×10^5 cells/well in 6-well plates to 40-50% confluency and transiently transfected with siRNA oligonucleotides targeted against ERK1, ERK2 or non-silencing control siRNA oligonucleotides with a scrambled sequence (sc-44205, sc-35335, and sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were transfected for 48 hours at 37^{o}C in 1.5 mL of growth medium. Each transfection used 20 pmol of siRNA oligomer in 500 µL serum-free RPMI mixed with 3 µL Lipofectamine 2000. Efficiency of siRNA transfection was assessed by Western blot.

2.3 WESTERN BLOTS

Cell pellets were extracted using a lysis buffer with phosphatase inhibitors (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 µM PMSF, 1x NaF and 1 µg/mL leupeptin), freeze-thaw, sonication and vortexing. Lysates were cleared by centrifugation. Total protein concentrations were measured using a BCA assay (Pierce, Rockford, IL, USA). Equal quantities of cell lysate protein were applied to Criterion 4-12% Bis-Tris gels (Bio-Rad, Hercules, CA), separated by electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 3% bovine serum albumin or 3% non-fat milk for 90 minutes at room temperature, probed with primary antibodies overnight at 4°C. Antibodies included α-Tubulin, p-ERK (E-4), ERK 1 (K-23), Histone H1 (AE-4) (sc-5286, sc-7383, sc-94, sc-8030, Santa Cruz Biotechnology, Santa Cruz, CA), Complex IV subunit Vb-OxPhos (459110, Invitrogen, Carlsbad, CA), and Cleaved Caspase-3 (Asp175) (#9661S, Cell Signaling, Beverly, MA). Blots were then exposed to goat anti-mouse (sc-2031) or goat anti-rabbit (sc-2030) IgG-HRP conjugated secondary antibodies for 1 hr at room temperature. Chemiluminescence was elicited by treatment with Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) and recorded with the UVP BioSpectrum 410 imaging system (Upland, CA). Band densities were analyzed with VisionWorksLS Analysis Software (UVP).

2.4 CELL FRACTIONATION

For experiments involving separation of the cytosol and nuclear fractions, H1944 cells were resuspended in lysis buffer (50mM $NaPO_4$, 20mM NaF, 2mM EDTA, 2mM EGTA, 1mM DTT, 1x Leupeptin, 300µM PMSF, 200µM Benzamide, .05mM Sucrose) and freeze thawed twice with liquid nitrogen. Cells were then passed ten times through a tuberculin syringe to completely lyse. Whole cell lysates were centrifuged at 1,000 x g for 5 min at 4° C, and the resulting

supernatant (Cytosol) was transferred to a new tube. The pellet was washed five times to remove any remaining cytosolic components, and resuspended in 150µL of lysis buffer containing 1% Triton X-100. Total proteins were measured and western blots were run on all three fractions. Blots were measured for pERK, Histone H1, a nuclear marker, and the cytoplasmic marker Oxphos.

The percent purity of the nuclear and cytoplasmic fractions were assesed based on the enrichment of the histone H1 (H1) and oxphos (ox), respectively, compared to the respective densities for those markers in the immunoblots of total cell protein. The formula for purity of the nuclear fraction was % = 100 * (nuc H1/cell H1)/(nuc H1/cell H1 + nuc ox/cell ox). Similarly, the formula for the purity of the cytoplasmic fraction was cyto % = 100* (cyto ox/cell ox)/(cyto ox/cell ox + cyto H1/cell H1).

2.5 CELL TITER 96 MTS CELL PROLIFERATION ASSAYS

H1944 PTHrP positive and negative cells were transfected with siRNA as directed. They were re-plated at 2,500 cells/well in 96-well plates in growth media for proper cell adherence. Assays were stopped in separate plates at 0, 1, 2, and 3 days. Viable cell quantities were assessed with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium MTS compound (Promega, Madison, WI) after 1 hour incubation at 37^{o}C. MTS is bioreduced by metabolically active cells into a colored formazan product soluble in tissue culture media, which can be read at 490 nm absorbance. The absorbance is directly proportional to the number of living cells, where a standard curve for each cell line was created.

2.6 FLOW CYTOMETRY

H1944 PTHrP positive and negative cells were treated with ERK 1 and control siRNA as stated. The cells were trypsinized, harvested, washed with PBS and resuspended in 1 mL of PBS. The following antibodies were used: Integrin α-2, Integrin α-3, Integrin α-5 and Integrin β-1 (555669,

556025, 555617, 555443, BD Pharmingen, San Diego, CA). Cells were incubated with the antibody for 20 minutes at room temperature, and washed with PBS. Cells were then fixed with 2% paraformaldehyde and analyzed for surface integrin expression via PE- and FITC-fluorescence intensities by a Coulter Elite flow cytometer (Beckman Coulter Electronics, Miami, FL).

2.7 APOPTOSIS ASSAY

To analyze whether the effects of ERK sensitized or desensitized PTHrP positive cells to apoptosis, H1944 PTHrP positive and negative cells were treated with ERK 1 and ERK 2 siRNA. To analyze whether the effects of ERK sensitized or desensitized PTHrP positive cells to apoptosis, cells must undergo apoptosis, but not completely. To induce apoptosis, cells were switched to fresh media containing .5mM staurosporine (9953S, Cell Signaling, Beverly, MA) and incubated for 24 hours. Cells were then collected, washed, and analyzed for Cleaved Caspase-3 (9661, Cell Signaling, Beverly, MA) via western blot. Apoptosis was not induced by UV irradiation because UV activates ERK.

2.8 TRANSCRIPTION FACTOR SCREENS

2.8.1 RNA isolation

RNA was isolated from subconfluent cells transfected with siRNA using the RNeasy mini kit (74104, Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA was digested with RNase free DNase (79254, Qiagen, Valencia, CA). The integrity and purity of the RNA was verified using the Nanodrop 2000, a micro-volume UV-Vis Spectrophotometer for nucleic acid quality (ThermoScientific, Wilmington, DE).

2.8.2 Transcription factor array

To evaluate gene expression in the individual ERK 1 siRNA treated cell lines, the RT^2qPCR profiler Human Signal Transduction Pathway array (PAHS-075, SABiosciences, Frederick, MD)

was used according to manufacturer's instructions. This represents 84 genes that control when, where and the extent genes are expressed, plus five housekeeping genes and three controls. cDNA was generated from 1 µg total RNA using the RT^2 First Strand Kit (330401, SABiosciences, Frederick, MD) in accordance with the manual. The template was combined with the RT^2 SYBR Green/Rox qPCR master mix. 25 µL of this mixture was added to each well of the RT^2 qPCR profiler plate containing the predispensed gene-specific primers. The cDNA was amplified using a two step cycling program for the ABI Prism 7000 real time PCR machine with SYBR green I dye detection. Data analysis was based on the $\Delta\Delta C_T$ method with the aid of an Excel (Microsoft Excel, Microsoft, Redmond, WA) spreadsheet containing algorithms provided by the manufacturer. To determine the expression levels of each gene, the C_T was normalized to the expression of the RPL13A, GAPDH and HPRT1 housekeeping genes. A positive value indicated that the gene was upregulated and a negative value indicated that the gene was downregulated.

2.9 STATISTICAL ANALYSIS

Data was transferred to Excel (Microsoft Excel, Microsoft, Redmond, WA) and GraphPad Prism (GraphPad, San Diego, CA) software (version 5.03). The mean measurement values for experimental and control groups were compared by t-test or ANOVA (GraphPad). Statistical significance was accepted at $p < 0.05$.

3 RESULTS

To study the effects associated with PTHrP induced ERK activation in lung cancer, ERK levels were knocked down using siRNA in search of differing behaviors between ERK knockdown and nonsense siRNA. A secondary level of comparisons was also made between PTHrP positive and negative cells.

3.1 PTHrP AND ERK

3.1.1 PTHrP increases ERK levels

The basis of these experiments lies in the findings in Hastings et al., 2009 that PTHrP increases phospho-ERK (pERK) levels in lung cancer cells. To verify these findings, whole cell lysates from PTHrP expressing H1944 lung adenocarcinomas were analyzed for total ERK (tERK) and pERK levels by immunoblot. All cells were grown subconfluently because ERK levels were greater in subconfluent cells than confluent cells (results not shown). PTHrP positive cells were compared to the PTHrP negative pCi-Neo empty vector clones of H1944. Similar to the 2009 findings, PTHrP positive cells contained higher levels of activated ERK1 and ERK2 than the PTHrP negative control (**Fig. 1A, 1B**). While the levels of pERK increases in PTHrP positive cells, the tERK levels do not change between PTHrP positive and negative cells. pERK2 seems to be the predominant active ERK form in H1944 cells (**Fig. 1C**). The presence of PTHrP increases the activated ERK forms by nearly 2 fold.

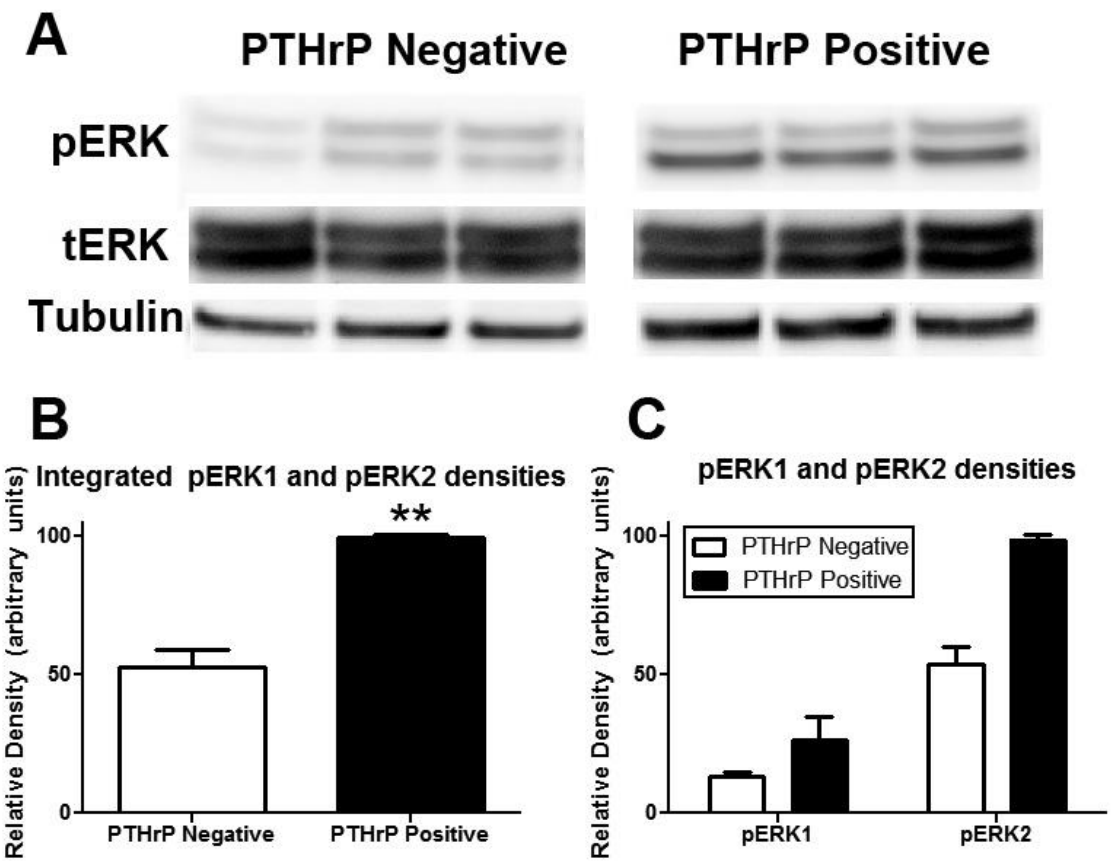

Figure 1. PTHrP increases activated ERK levels. (A) PTHrP-positive H1944 clones demonstrated increased levels of extracellular signal regulated kinase (ERK) phosphorylation (pERK) compared to the PTHrP-negative vector controls. **(B)** The bar graph shows average values of 3 independent clones of each group, representative of the pERK1 and pERK2 combined densities above in Figure 1A, where PTHrP positive pERK is taken as 100. **, $P < 0.01$ vs. pCi-Neo vector control. **(C)** This bar graph shows the relative densities of pERK1 and pERK2 in PTHrP positive and negative clones. Values were an average of 3 independent clones of each group. All relative densities were normalized to the housekeeping gene α-Tubulin. Blots here were performed multiple times with reproducible results.

3.1.2 ERK siRNA is able to decrease ERK levels

The means for testing the effects of activated ERK levels in the cells is to alter ERK levels. In this experimental setup, siRNA was used to knock down the ERK levels within the cells. The most crucial step in effectively knocking down gene expression is the successful delivery of the siRNA. In these experiments, ERK siRNA was administered using Lipofectamine 2000. This lipid was chosen over other available lipids because H1944 cells had the greatest amount of

green fluorescent protein transfected into the cell using this reagent compared to other lipids (data not shown).

ERK1 siRNA transfection is capable of knocking down ERK1 levels. **Figure 2** shows that there is significantly less (roughly ~ 70 - 80%) ERK1 levels in the ERK1 siRNA treated cells, in both PTHrP positive and PTHrP negative cells. The non-silencing siRNA used as a control contains a scrambled RNA sequence not known to bind to any genes within the cell. MCF-7 is a breast cancer cell known to express ERK. It was used as a positive control for ERK to indicate that the 44 and 42 kDa bands detected on the blot were ERK1 and ERK2 respectively. MCF-7 cells are known for low levels of phospho-ERK, with pERK1 levels less than pERK2 levels (Mcdaid and Horwitz, 2001). Similarly, pERK1 levels did not show for MCF-7 positive controls, but nonetheless indicated the positive detection of ERK on the blots.

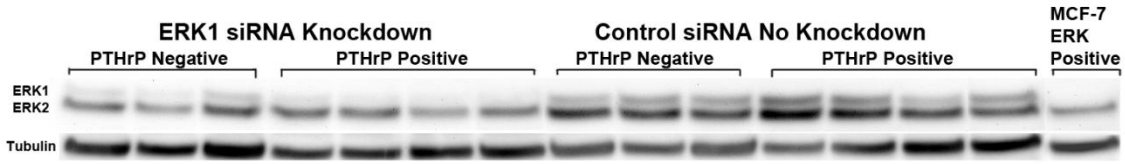

Figure 2. ERK1 siRNA is capable of knocking down ERK1 levels in all of the H1944 pCi-Neo and PTHrP positive clones. Each PTHrP negative band represents one of the three H1944 pCi-Neo vector clones and the PTHrP positive represents each of the PTHrP 1-87 positive clones. MCF-7 breast adenocarcinoma is a positive control to show the location of ERK.

3.1.3 ERK2 knockdown slows PTHrP positive cell proliferation

H1944 cell proliferation experiments was performed over a timecourse of 72 hours after ERK2 knockdown, itself a 48 hour procedure as described in the Materials and Methods. To ensure that the siRNA knockdown lasted the entire 120 hours, timecourse was done on both H1944 pCi-Neo and PTHrP clones treated with ERK2 siRNA (**Fig. 3A**). The control siRNA timecourse over the 72 additional hours remains steady, with little to no change over time in both pCi-Neo and PTHrP clones (**Fig 3B, 3C**). The ERK2 siRNA continuously knocks down pERK2

levels over the 72 hours in both pCi-Neo and PTHrP cells. Selectivity to ERK2 was high since the levels of ERK1 did not decrease upon transfection of ERK2 siRNA.

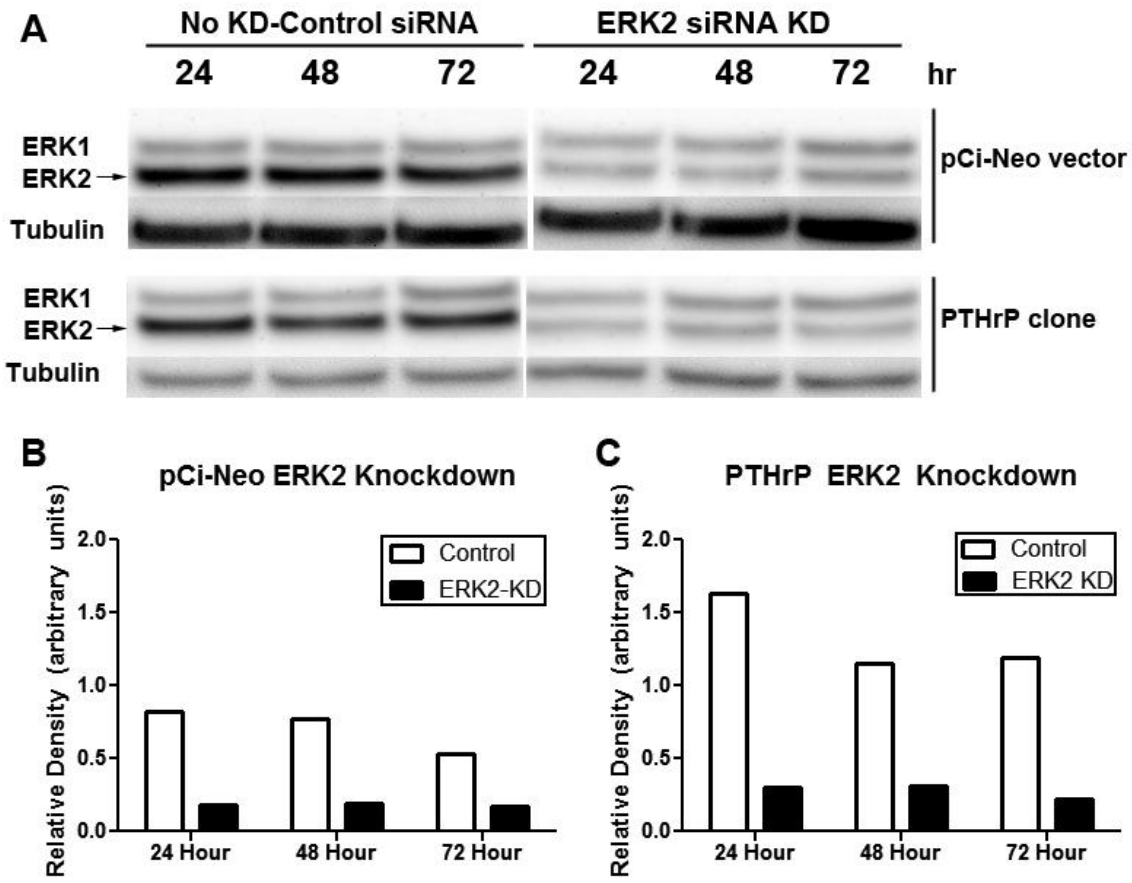

Figure 3. Time course of ERK2 siRNA knockdown. Western blot analysis of ERK2 siRNA knockdown efficiency. H1944 **(A)** pCi-Neo and PTHrP positive clones were transfected with 20 pmol ERK2 siRNA for 48 hours. Cells were then removed from siRNA and placed into fresh media for the indicated times in hours up to an additional 72 hours. A control siRNA was transfected to show the ERK levels without knockdown over 72 hours and to expose both sets of cells to similar conditions. Cell lysates for each time point were analyzed for pERK by Western Blotting. Relative density of pERK2 in **(B)** pCi-Neo PTHrP negative clones and **(C)** PTHrP positive clones from Figure (3A). Densities were normalized to α-Tubulin.

The level of activated ERK2 decreased approximately 70 - 80% upon siRNA transfection for the 72 hours in the H1944 PTHrP positive and negative cells. Reducing the level of phospho-ERK2 in growing cells reduced the rate of cell proliferation in PTHrP positive cells (**Fig. 4A, 4C**), but not in PTHrP negative cells (**Fig. 4B, 4D**), when measured by MTS as described in the Materials and Methods. The results presented in **Figure 4** are representative of two

independent experiments. ERK2 knockdown was compared against control siRNA at each time point measured for each PTHrP positive and negative cell line using a paired t-test.

Most PTHrP positive cell lines showed drastic decrease in cell proliferation with ERK2 knockdown, while one showed steady growth between the two conditions. Taken together, there was a drastic reduction in growth at 24 hours ($P < 0.01$), with significant reduction in cell proliferation at 48 and 72 hours ($P < 0.05$). On the other hand, ERK2 knockdown in PTHrP negative cells did not show any considerable changes in cell proliferation. One of the cell lines increased in cell proliferation, while others decreased slightly. There were no significant changes in PTHrP negative cell proliferation when ERK2 levels were decreased. Because the lack of pERK2 showed a decrease in PTHrP positive cell proliferation, it can be implied that ERK2 activation in PTHrP positive cells is necessary for cell proliferation to proceed normally, and may possibly stimulate proliferation.

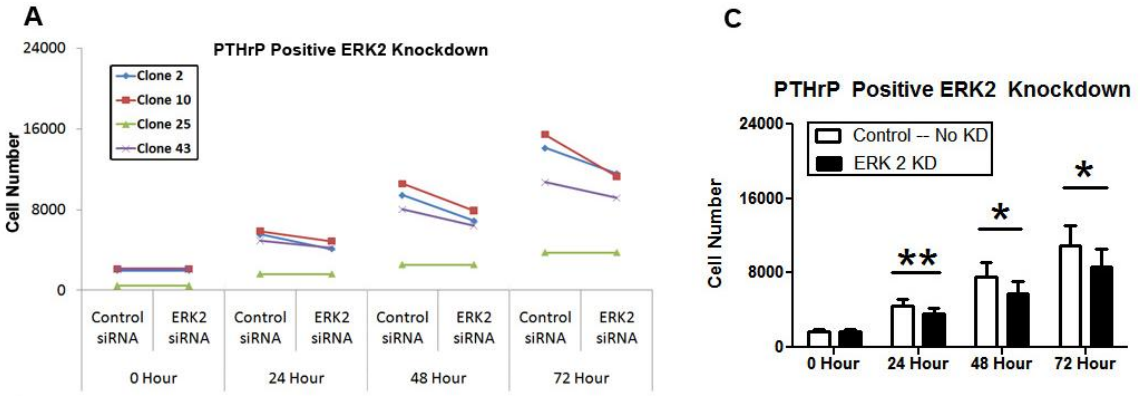

Figure 4. Effects of ERK2 siRNA on proliferation on PTHrP positive H1944 cells. H1944 cells **(A)** PTHrP positive clones 2, 10, 25 and 43 or **(B)** PTHrP negative pCi-Neo 1, 2 and 3 were transfected with ERK2 siRNA or control siRNA (20 pmol each) for 48 hours, seeded in 96-well plates, and incubated for up to an additional 72 hours. Cell proliferation was measured by the MTS assay every 24 hours up to 72 hours. Cell numbers were calculated using a standard curve created for each cell line by measuring known cell counts treated with the MTS protocol. Line segments connect results from the same clone with and without ERK2 knockdown. Proliferation decreased with knockdown in 3 out of the 4 cell lines. Results present the average cell counts across clones tested with control siRNA or ERK2 siRNA at each time point in two independent experiments. **(C)** Quantitative representation of Figure (4A), where the population (n=4) of both experiments were averaged together. *, $P < 0.05$ and **, $P < 0.01$ vs. control siRNA by paired t-test. (n=4) **(D)** Quantitative representation of Figure (4B) where the population (n=3) of each time point and condition of both experiments were averaged together.

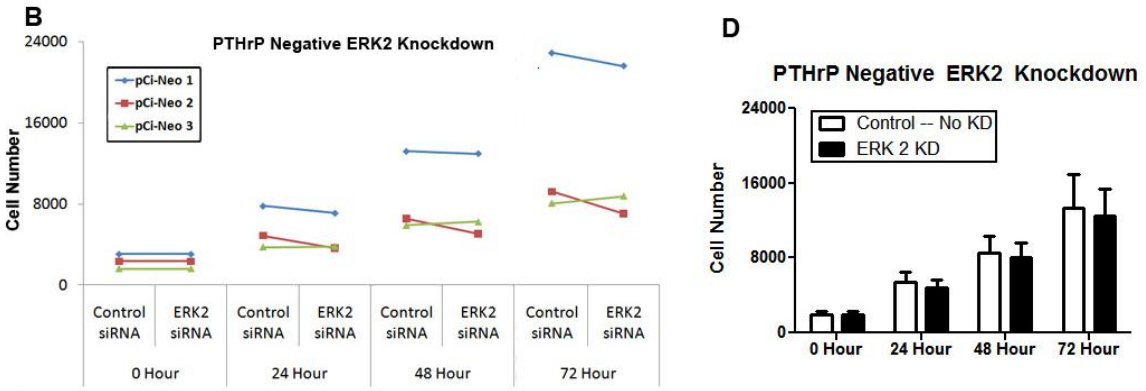

Figure 4 continued

3.1.4 Removal of ERK1 may increase PTHrP positive cell proliferation

The proliferation experiments were repeated with ERK1 knockdown for 72 hours. To verify that ERK1 siRNA knockdown is maintained throughout the 72 hours, an ERK1 timecourse on a H1944 PTHrP positive cell was performed (**Fig. 5**). This timecourse did not include a pCi-Neo since knockdown occurred in both pCi-Neo and PTHrP positive cells with ERK2 siRNA knockdown. The timecourse also did not include control siRNA over the 72 hours because ERK2 siRNA knockdown also showed no difference in control siRNA over the 72 hours (**Fig. 3**).

ERK1 siRNA knocked down the pERK1 siRNA over 72 hours (~70-80%), whereas the control siRNA did not decrease ERK1 levels. Interestingly, it appears that the constant pERK1 knockdown concurrently increases pERK2 levels over the 72 hours, where pERK2 levels increase by almost 50% 24 hours after the siRNA was removed from the media (**Fig. 5B**).

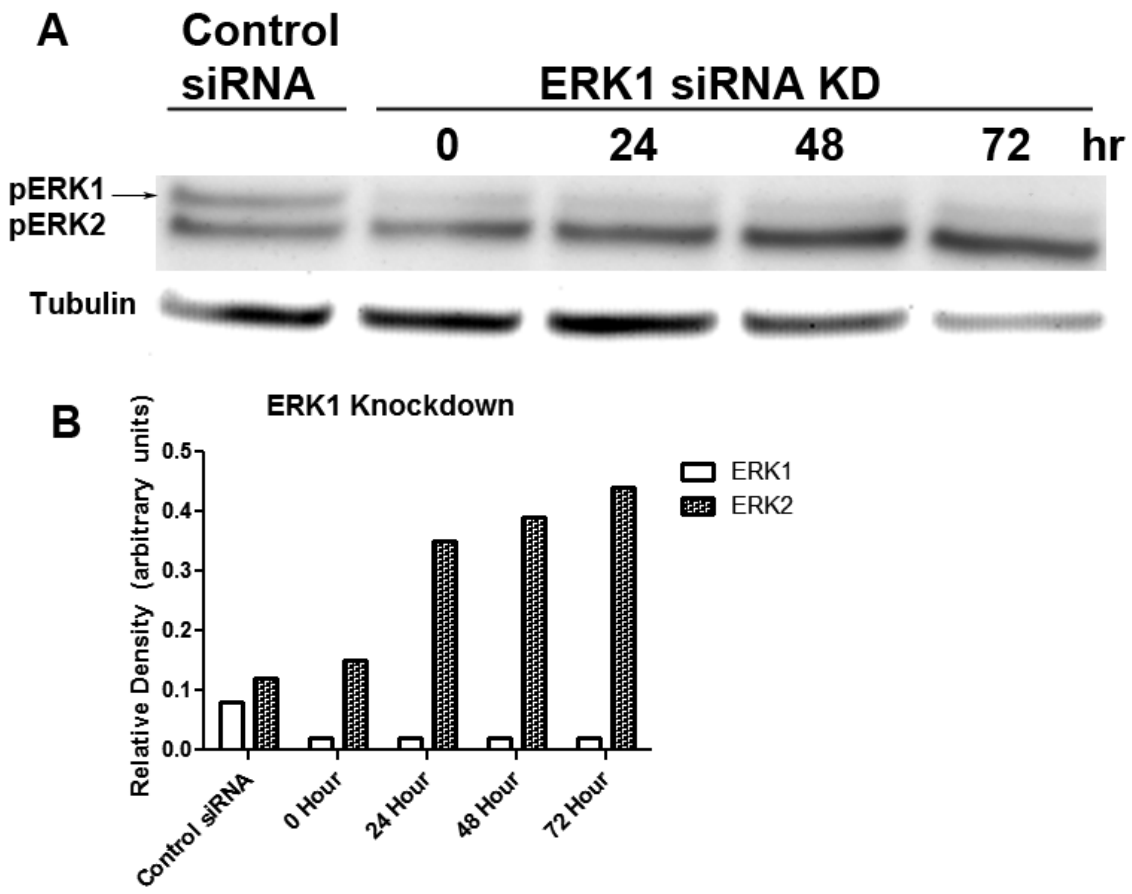

Figure 5. Time course of ERK1 siRNA knockdown. Western blot analysis of ERK1 siRNA knockdown efficiency. H1944 PTHrP positive clone was transfected with 20 pmol ERK1 siRNA for 48 hours, and placed into fresh media for the indicated times up to an additional 72 hours. A control siRNA was transfected at 20 pmol to show the levels of ERK1 without knockdown. Cell lysates at each time point were analyzed for pERK1 and pERK2 by Western blotting. **(B)** Relative density of pERK1 and pERK2 from Figure (5A). Densities were normalized to α-Tubulin. ERK1 knockdown decreased ERK1 by 70-80% but upregulated ERK2 by 4-fold.

PTHrP positive and negative H1944 cells were transfected with ERK1 siRNA and control siRNA as described in the Materials and Methods to decrease activated ERK1 levels. The results presented in **Figure 6** are representative of four independent experiments. In both PTHrP positive and PTHrP negative cells, the decrease in pERK1 did not have major effects on cell proliferation (**Fig. 6A, 6B**). PTHrP positive cells after knockdown lacking ERK1 grew at the same rate as the control siRNA transfected cells for the first 48 hours. There was no change for the first 48 hours. However, at 72 hours, PTHrP positive cells demonstrated increased proliferation

as a result of siRNA (**Fig. 6C**). The individual PTHrP negative cell lines were inconsistent with cell proliferation behavior, some decrease slightly and others increasing in cell number with the ERK1 siRNA treatment. Ultimately, there were no differences in cell proliferation for the first 48 hours with or without ERK1 knockdown.

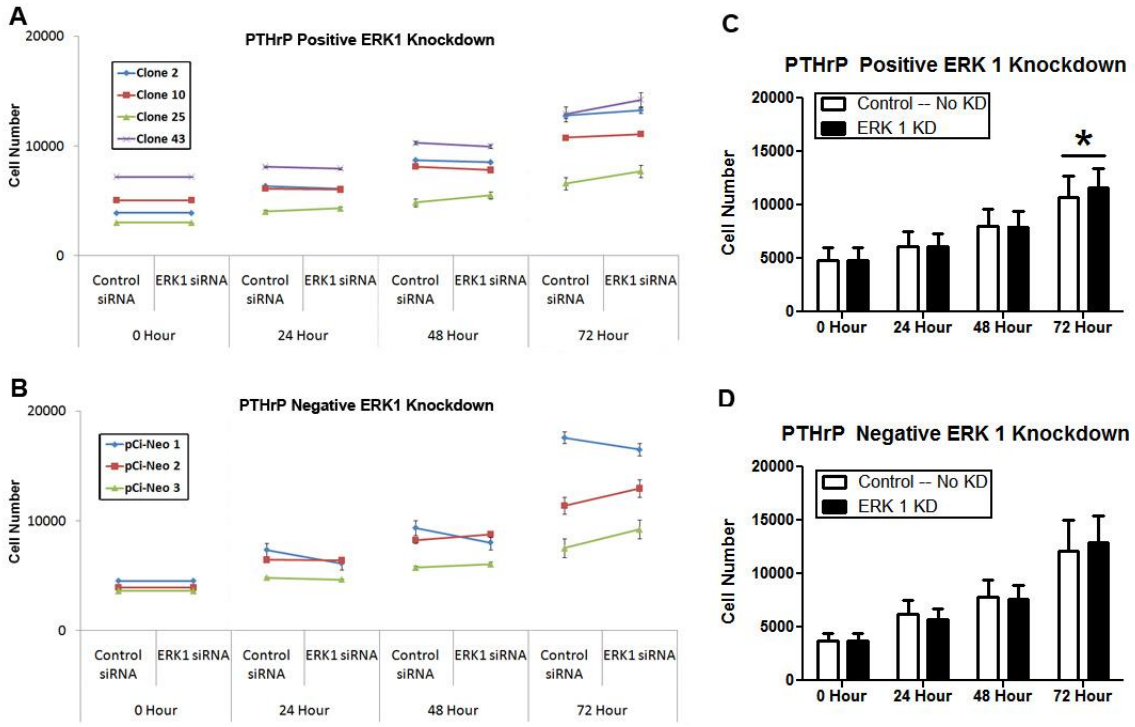

Figure 6. Effects of ERK1 siRNA on proliferation on PTHrP positive H1944 cells. H1944 cells **(A)** PTHrP positive clones 2, 10, 25 and 43 or **(B)** PTHrP negative pCi-Neo 1, 2 and 3 were transfected with ERK1 or control siRNA (20 pmol each) for 48 hours, seeded in 96-well plates, and incubated up to an additional 72 hours. Cell proliferation was measured by the MTS assay every 24 hours up to 72 hours. Cell numbers were calculated using a standard curve created for each cell line by measuring known cell counts treated with the MTS protocol. Line segments connect results from the same clone with and without ERK2 knockdown. Results present the average cell counts across clones tested with control siRNA or ERK1 siRNA at each time point in four independent experiments. **(C)** Quantitative representation of average cell number by clone in Figure (6A), where the population (n=4) of both experiments were averaged together. *, $P < 0.05$ vs. control siRNA by paired t-test. **(D)** Quantitative representation of Figure (6B) where the population (n=3) of each time point and condition of both experiments were averaged together.

3.1.5 Reducing both ERK1 and ERK2 levels in PTHrP positive cells may decrease PTHrP positive cell proliferation, similar to the removal of ERK2

The negligible effect in proliferation with ERK1 knockdown could be explained by the lower levels of ERK1 relative to ERK2 in H1944 cells. To examine the effects of ERK1 knockdown sans the confounding effect of ERK2 upregulation, combined ERK1 and ERK2 siRNA knockdown was analyzed. **Figure 7A** shows the 72 hour timecourse of ERK1 and ERK2 siRNA knockdown on pERK1 and pERK2 levels. pERK1 and pERK2 levels were knocked down, but the decrease for both isoforms of pERK was less than knocking down ERK1 or ERK2. The reduction in pERK1 and pERK2 ranged from 20-50% knockdown of pERK1 and 50-75% pERK knockdown depending on the hours passed with the siRNA oligonucleotides. Both ERK siRNAs gradually lost their knockdown efficiency over time, but at the end of the 72 hours, there was still some reduction compared to the control siRNA (**Fig. 7B**).

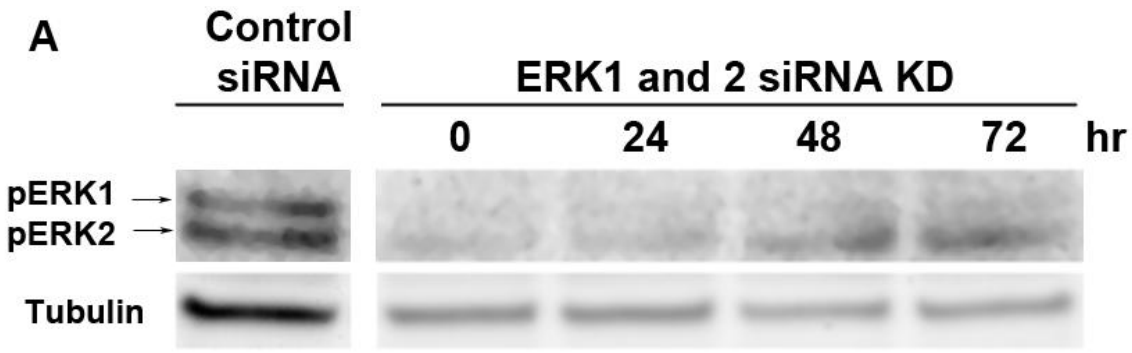

Figure 7. Western blot analysis of combined ERK1 and ERK2 siRNA knockdown efficiency. H1944 PTHrP positive clone was transfected with combined 20 pmol ERK1 siRNA and 20 pmol ERK2 siRNA for 48 hours, and placed into fresh media for the indicated times up to an additional 72 hours. A control siRNA at 40 pmol was used to show the levels of ERK1 and ERK2 without knockdown. Cell lysates at each time point were analyzed for pERK1 and pERK2 by Western blotting. **(B)** Relative density of pERK1 and pERK2 from Figure (7A). Densities were normalized to α-Tubulin.

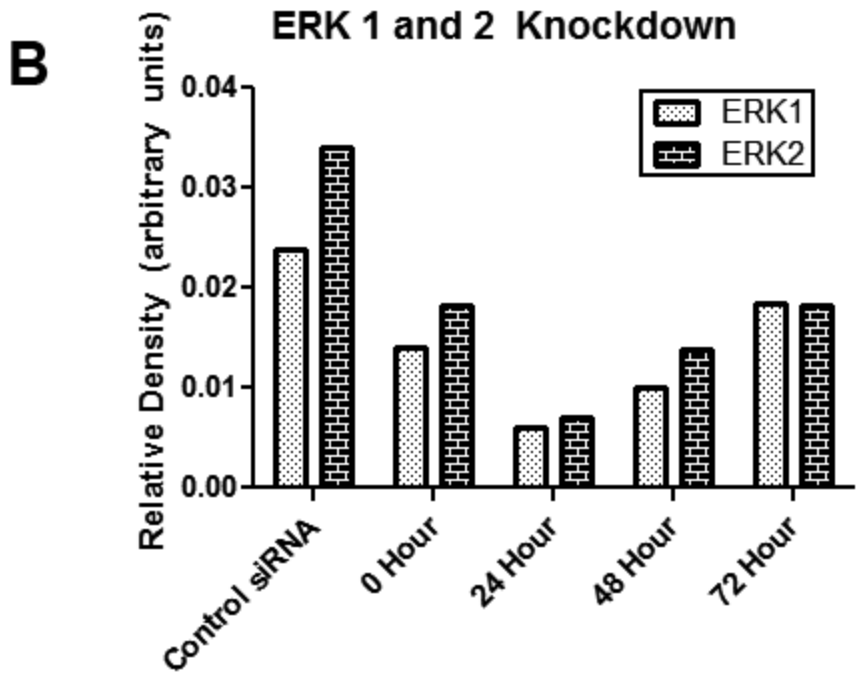

Figure 7 continued

Cell proliferation experiments on PTHrP positive and negative H1944 cells were then performed with simultaneous knockdown of ERK1 and ERK2 (**Fig. 8A, 8B**). The results are representative of two independent experiments. Similar to single ERK2 siRNA knockdown, ERK1 and ERK2 combination knockdown significantly decreased proliferation of PTHrP positive cells at 72 hours post transfection. Interestingly, the PTHrP positive cells with ERK1 and ERK2 knockdown grew at a similar rate to the control cells 24 and 48 hours after replating, although ERK2 knockdown was more efficient at the 24 and 48 hour time points (**Fig. 8C, 8D**). Only when pERK1 levels become close to the control pERK1 levels did the decrease in pERK2 levels show significant changes to cell proliferation. ERK1 and ERK2 siRNA knockdown in PTHrP negative cells did not cause any significant changes in proliferation over 72 hours. There was great variability in growth rates of the individual pCi-Neo vector clones.

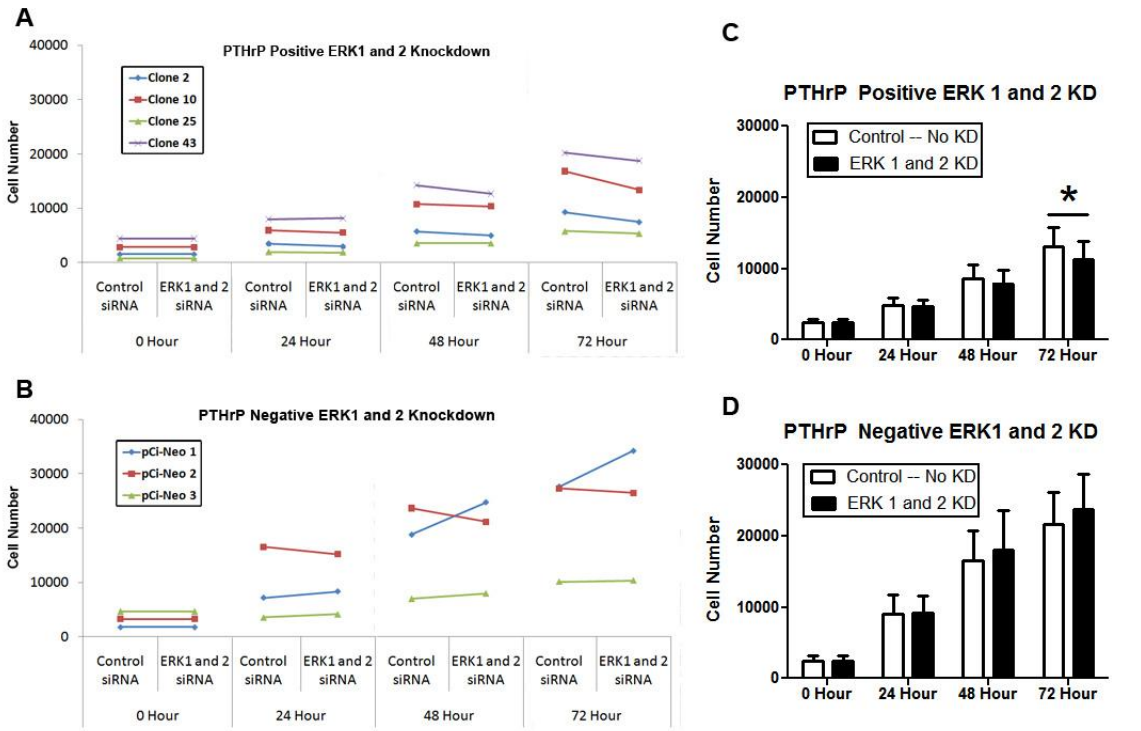

Figure 8. Effects of ERK1 and ERK2 siRNA on proliferation on PTHrP positive H1944 cells. H1944 cells **(A)** PTHrP positive clones 2, 10, 25 and 43 or **(B)** PTHrP negative pCi-Neo 1, 2 and 3 were transfected with ERK1 and ERK2 siRNA combined (20 pmol ERK1 and 20 pmol ERK2) or control siRNA (40 pmol) for 48 hours, seeded in 96-well plates, and incubated for up to an additional 72 hours. Cell proliferation was measured by the MTS assay every 24 hours up to 72 hours. Cell numbers were calculated using a standard curve created for each cell line by measuring known cell counts treated with the MTS protocol. Line segments connect results from the same clone with and without ERK1 and 2 knockdown. Results present the average cell counts across clones tested with control or ERK1 and ERK2 siRNA at each time point in two independent experiments. **(C)** Quantitative representation of average cell number by clone in Figure (8A), where the population (n=4) of both experiments were averaged together. *, $P < 0.05$ vs. control siRNA by paired t-test. **(D)** Quantitative representation of Figure (8B) where the population (n=3) of each time point and condition of both experiments were averaged together.

3.2 LOCALIZATION OF pERK IN PTHrP POSITIVE NSCLC

The changes in proliferation with ERK knockdown might depend on the localization of ERK when activated by PTHrP. To study the localization of pERK, PTHrP positive and negative H1944 cells were treated with ERK2 siRNA, separated into nuclear and cytoplasmic fractions and examined for pERK location.

3.2.1 Successful separation of pure nuclear and cytoplasmic fractions

The percent purity for H1944 cell nuclear and cytoplasmic fractions exceeded 90% on average (**Table 1**). Purities did not differ between the PTHrP-negative pCi-Neo clones and the PTHrP clones (data not shown), so values were averaged across those groups.

Table 1. Purities of Cytoplasmic and Nuclear Fractions (%)

Group	Fraction	n	Mean ± SE	Range
Control siRNA	Nuclear	3	92.5 ± 2.5	83.9 - 99.5
ERK siRNA	Nuclear	3	91.6 ± 3.3	79.7 - 98.6
Control siRNA	Cytoplasmic	3	93.7 ± 0.8	83.8 - 99
ERK siRNA	Cytoplasmic	3	95.5 ± 0.9	93.0 - 97.4

3.2.2 pERK in the PTHrP positive cell

When phosphorylated, ERK may translocate to the nucleus or remain in the cytoplasm. Nuclear ERK is commonly associated with mitogenesis. The goal of these studies was to determine whether the cellular distribution of activated ERK varied as a function of ectopic PTHrP expression. In addition, these experiments examined whether ERK knockdown affected nuclear pERK, cytoplasmic pERK, or both and tested whether the pro-mitogenic properties of activated ERK2 in PTHrP positive cells can be attributed to nuclear or cytoplasmic pERK. Since ERK2 knockdown decreases cell proliferation, it is hypothesized that ERK2 knockdown will be associated with greater levels of cytoplasmic pERK.

Figure 9A shows the densities of pERK2 in the nuclear and cytoplasmic compartments after ERK2 knockdown compared to the pERK treated with control siRNA in PTHrP negative and positive cells respectively. **Figure 9B** shows the levels of pERK1 in the nuclear and cytosolic fractions in H1944 cells. The effects of ERK2 knockdown were greater in PTHrP positive cells. PTHrP increases predominantly nuclear pERK. ERK knockdown mainly decreases nuclear pERK, therefore the difference between ERK1 and ERK2 is not due to compartmentalization.

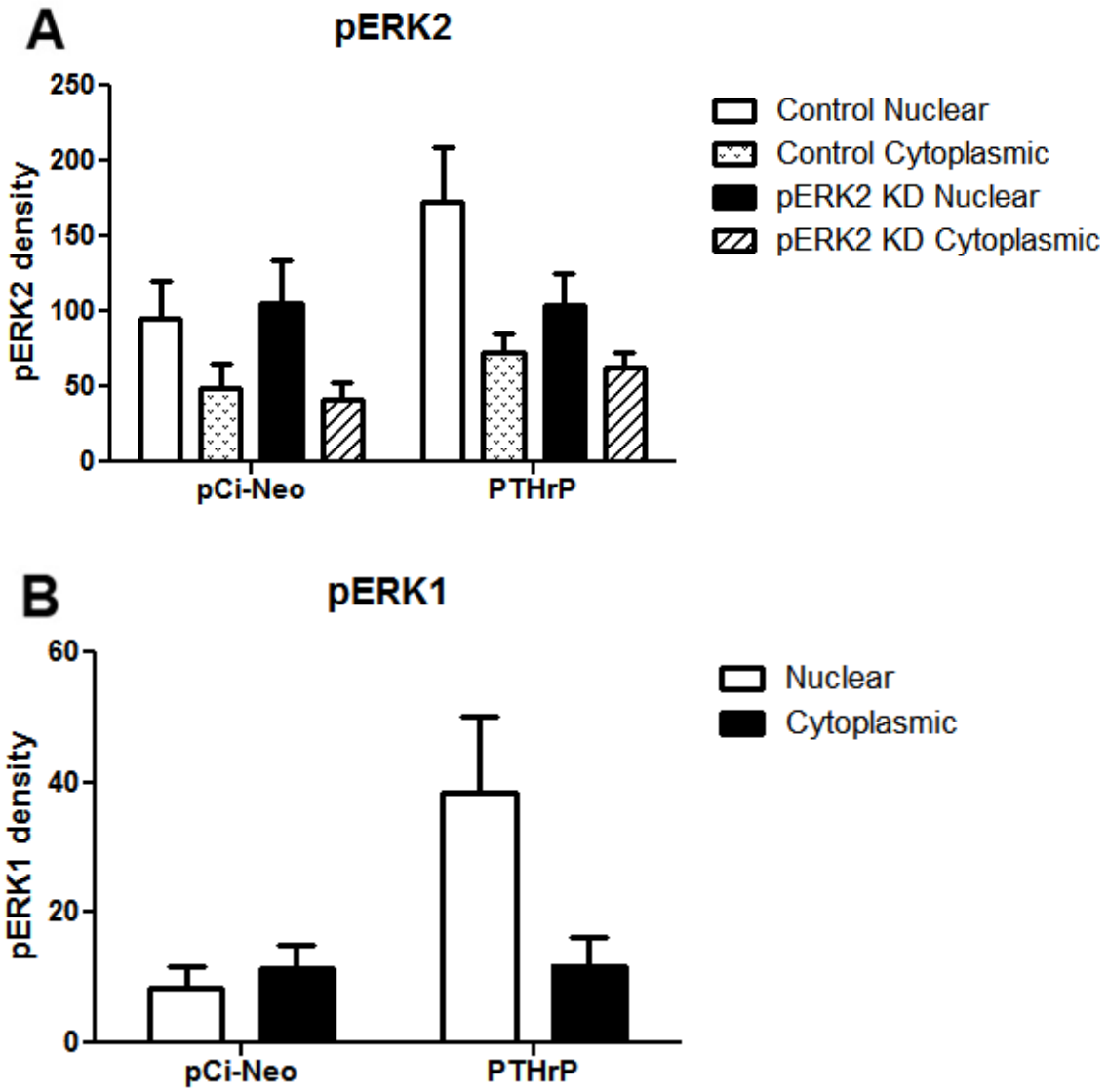

Figure 9. Fraction of pERK2 between nuclear and cytoplasmic compartments as a function of ectopic PTHrP expression and ERK2 KD. H1944 PTHrP negative (pCi-Neo) and positive (PTHrP) cells were treated with 20 pmol ERK2 siRNA for 48 hours, collected and separated into nuclear and cytoplasmic fractions by centrifugation. Western blot analysis was done for nuclear pERK2 and pERK1. Figures **(A)** and **(B)** show compartmental distributions for pERK2 and pERK1, respectively. Ectopic PTHrP primarily increased pERK1 and pERK2 in nuclear compartments rather than cytoplasmic. Similarly, ERK2 knockdown decreased pERK predominantly in the nuclear compartment, not the cytoplasmic compartment in PTHrP-positive cells. Effects were small in the PTHrP-negative cells.

3.3 OTHER FACTORS EXAMINED FOR RELATION TO PTHrP INDUCED ERK ACTIVATION

The following series of experiments attempts to understand the mechanisms that PTHrP induced ERK activation undergoes to manage cell proliferation. These experiments look at effects on integrin subunits, apoptosis and gene expression.

3.3.1 ERK1 removal does not affect cell surface integrins α2, α3, α5, β1

Integrins take part in cell signaling, which can affect the cell cycle and possibly lead to effects on cell proliferation. Our lab has found that PTHrP affects cell matrix adhesion by integrin α2, α3, and α5 subunits, therefore we examined the possibility that PTHrP induced ERK activation may affect these integrins. H1944 PTHrP positive and negative cells were treated with ERK1 siRNA and control siRNA and analyzed for changes in integrin subunit expression via flow cytometry. ERK1 siRNA was chosen because results showed that both pERK1 and pERK2 had roles in cell proliferation. Results in **Figure 9** are representative of one independent experiment. The 0 antibody control shows no integrin binding for any of the conditions. Similar to previous results, there were small changes in integrin α2, α3, α5 expression between PTHrP positive and negative cells, and no difference in integrin β1 expression. Compared to the control siRNA, ERK1 siRNA knockdown in both PTHrP positive and PTHrP negative cells showed no changes in the number of cells stained with any of the integrins.

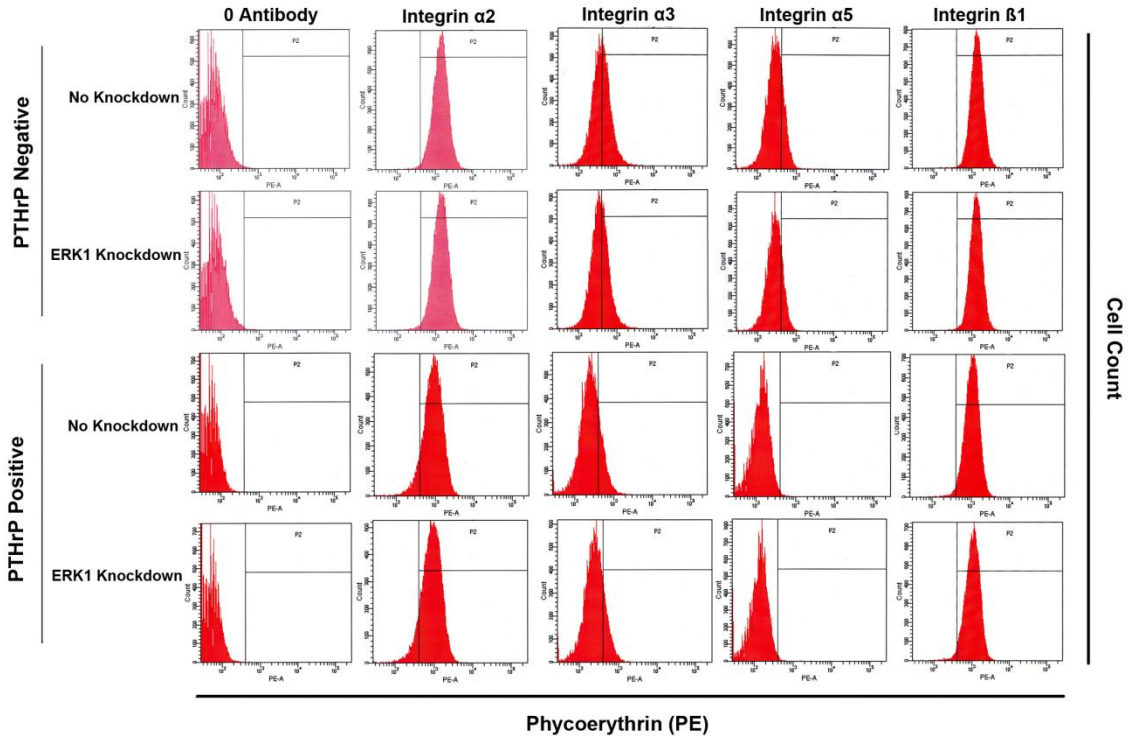

Figure 9. Flow cytometry analysis of H1944 cellular Integrin α2, α3, α5, β1 with ERK1 knockdown. Flow cytometry was performed on all four of the H1944 PTHrP positive and all three PTHrP negative cell lines for integrin subunits using antibodies coupled to phycoerythrin fluorochrome. Histograms are representative of the PTHrP positive and negative clones, and is representative of one experiment.

Table 2 is representative of all of the PTHrP positive and negative clones' mean phycoerythrin fluorescence emittance averaged together for each independent treatment of siRNA and integrin. It includes the surface expression of Integrin α2, Integrin α3, Integrin α5, and Integrin β1 when PTHrP positive and negative cells were treated with ERK1 siRNA. The values presented in Table # are representative of an average of the phycoerythrin fluorescence for each PTHrP negative (n=3) and PTHrP positive (n=4) cell line for each condition. Comparable to **Figure 9**, there are no differences between the mean fluorescence of ERK1 siRNA and control knockdowns.

Table 2. ERK1 knockdown on cell surface integrins mean phycoerythrin fluorescence

	PTHrP Negative		PTHrP Positive	
Antibody	*ERK1 Knockdown*	*Control siRNA*	*ERK1 Knockdown*	*Control siRNA*
No Stain	652 ± 175	623 ± 150	456 ± 156	461 ± 121
Integrin Alpha 2	1648 ± 481	1680 ± 469	1043 ± 657	1106 ± 745
Integrin Alpha 3	2834 ± 1928	2669 ± 1738	2435 ± 1881	2342 ± 1756
Integrin Alpha 5	1365 ± 737	1324 ± 691	783 ± 525	749 ± 508
Integrin Beta 1	3028 ± 1464	2959 ± 1215	1957 ± 1052	1887 ± 1052

Similar to **Table 2**, **Table 3** is representative of all of the PTHrP positive and negative clones' % parent P2 analysis of the population of cells from flow cytometry for each ERK1/control siRNA condition stained for the different integrins. It includes every PTHrP negative (n=3) and positive (n=4) clone for each individual ERK1 knockdown and control treatment in regard to the specific integrin α2, Integrin α3, Integrin α5, and Integrin β1 antibody stain. This table compares the population of cells past the positive stain threshold from. There are some population differences between PTHrP positive and negative cells for integrin α2, α3, and α5, but no changes with integrin β1. This signifies that PTHrP affects these integrin α2, α3, and α5 subunits, but not integrin β1. Again, there are no changes between ERK1 siRNA and control siRNA knockdown in either PTHrP negative or positive cells. These results suggest that the PTHrP induced ERK activation does not have a role in regulating cell proliferation by cell surface integrin expression.

Table 3. ERK1 knockdown on cell surface integrins % parent P2 analysis

	PTHrP Negative		PTHrP Positive	
Antibody	*ERK1 Knockdown*	*Control siRNA*	*ERK1 Knockdown*	*Control siRNA*
No Stain	0.27 ± 0.06	0.37 ± 0.15	0.07 ± .0.8	0.07 ± 0.08
Integrin Alpha 2	88.50 ± 17.67	88.03 ± 18.31	66.67 ± 34.22	68.10 ± 33.75
Integrin Alpha 3	80.33 ± 33.46	82.20 ± 30.31	96.27 ± 4.52	96.63 ± 4.06
Integrin Alpha 5	62.77 ± 39.12	65.10 ± 37.74	48.37 ± 39.02	45.77 ± 39.17
Integrin Beta 1	99.63 ± 0.25	99.77 ± 0.83	99.03 ± 0.83	99.27 ± 0.66

3.3.2 PTHrP is not affected by staurosporine induced apoptosis

Previous results suggest that PTHrP has pro-apoptotic effects and sensitizes cells to apoptosis (Hastings et al., 2003b). We chose a staurosporine induced apoptosis model because previous results determined that apoptosis induced by UV stimulated ERK activation (results not shown). While the mechanisms of staurosporine induced apoptosis are not entirely known, one possible mechanism is that staurosporine induces apoptosis by disrupting mitochondrial function (Wei et al., 2001). At 0.5 µM, staurosporine readily induced cleavage of caspase-3 into two fragments 19 kDa and 17 kDa in size, but did not cause all of the cells to die (data not shown). ERK1 and ERK2 combination knockdown on H1944 PTHrP positive and negative cells were treated with staurosporine to induce apoptosis (**Figure 10**). Analysis of the cleaved caspase 3 immunoblots revealed that there were no changes in apoptosis between PTHrP positive and negative cells. Densities between pCi-Neo and PTHrP positive cells do not show differences in levels of cleaved caspase-3. These results indicate that staurosporine does not have any effects on PTHrP-related apoptosis. The lack of differences between PTHrP negative and positive cells in this staurosporine induced apoptosis model suggest that the effects of ERK activation by PTHrP are not related, and further pursuing the effects of pERK1 and pERK2 was not worthwhile. To justify, **Figure 10** includes ERK1 and ERK2 siRNA knockdown and no differences are seen between cleaved caspase-3 levels in the knockdown and the control siRNA.

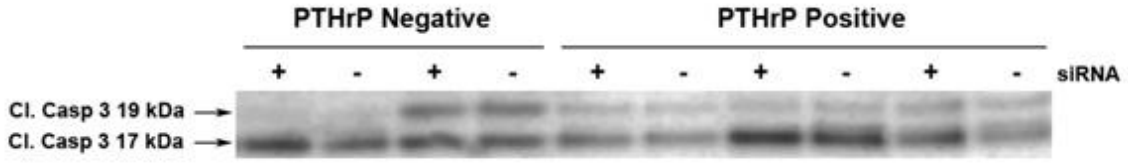

Figure 10. Staurosporine induced apoptosis of cultured H1944 PTHrP positive and Negative cells. H1944 PTHrP positive and negative cells were transfected with combined ERK1 and ERK2 siRNA for 48 hours. Cells were then exposed to 0.5 µM staurosporine for 24 hours to induce apoptosis. Caspase-3 cleavage was identified by immunoblot with an antibody specific for the cleaved caspase-3 19kDa and 17kDa fragments.

3.3.3 PTHrP induced ERK activation and gene expression

PTHrP increases ERK levels and results in changes in cell proliferation. To check the changes in transcription factor when ERK levels are altered, a transcription factor PCR array was used to screen 84 possible human genes in PTHrP positive and negative cells. The genes with the greatest fold changes with ERK 1 siRNA knockdown in either pCi-Neo PTHrP negative cells or PTHrP positive cells are seen in **Figure 11**. The transcription factors with the greatest fold changes were ATF1, STAT3, STAT4, STAT5B, ETS2, FOS, E2F1, and RB1. All of these transcription factors except STAT5B and E2F1 were upregulated by PTHrP negative cells, where all but FOS were upregulated by PTHrP positive cells. The greatest change in fold regulation was three-fold with FOS. Despite low changes in fold regulation from the highest hit transcription factors, confirmatory qPCR was done on the remaining cell lines with primers specific for FOS, ATF1, ETS2, STAT3, STAT4, and STAT5B. There was no change in transcription factor gene levels with or without ERK1 siRNA knockdown in either PTHrP positive or PTHrP negative cell lines (data not shown). These results indicate that these genes may have an effect on PTHrP positive cells and ERK activation, although this topic needs to be examined further. Other possible transcription factors that may be affected by PTHrP induced ERK activation have not been identified.

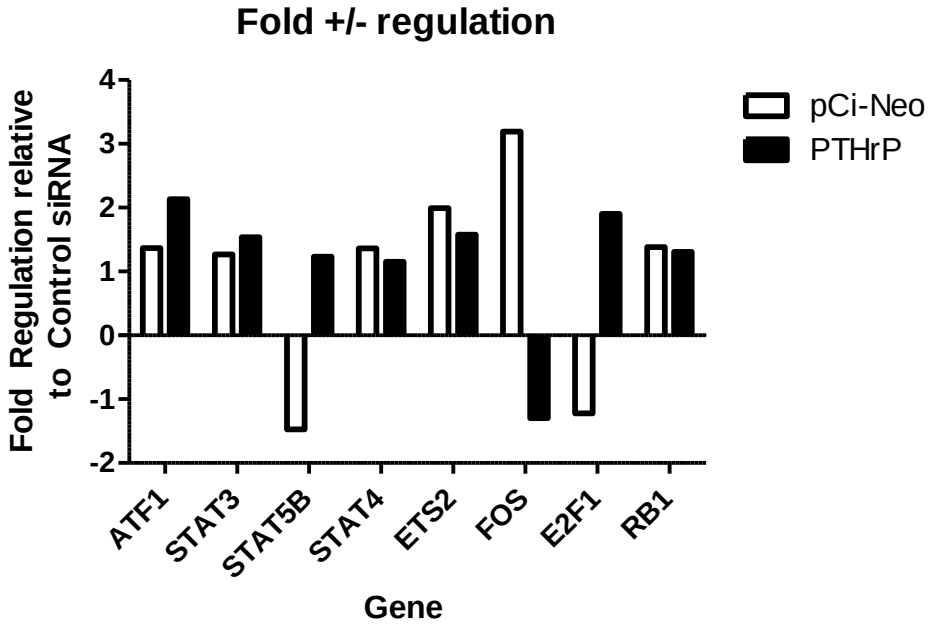

Figure 11. Quantitative real-time PCR analysis for differential gene expression with ERK knockdown in PTHrP positive cells. PTHrP negative and positive cells were treated with ERK1 or control siRNA and analyzed for up or downregulation of transcription factors using a human transcription factor array and qPCR. The eight transcription factors with the greatest changes in fold regulation are displayed. Downward-pointing bar indicates decreased expression and upward-pointing bar indicates increased expression. Results are representative of three independent experiments.

4 DISCUSSION

4.1 ERK1 and ERK2 knockdown

The objective of this study was to ascertain the role of PTHrP mediated ERK1/2 phosphorylation on cell growth. The specific question was whether activity of either ERK isoform contributed to or opposed the effect of PTHrP in inhibiting lung cancer cell proliferation, the observed effect of PTHrP. This research focuses specifically on the effects of ERK1 and ERK2 and does not include effects of upstream ERK mediators or other ERK proteins. For instance, ERK5 is irrelevant because although it is the closest related protein to ERK1/2, it is not an ERK1/2 isoform since they are activated by different kinase molecules (Zhou et al., 1995). In addition, utilization of Raf inhibitors induces changes to cell viability (Panka et al., 2006), an effect that is too problematic and not feasible for this study.

These experiments took advantage of the RNAi technology to study the effects of ERK activation. siRNA for ERK1 and ERK2 decreased levels of their specific target and also reduced perk in PTHrP transfected H1944 lung adenocarcinoma cells. Successful knockdown of up to 80% ERK1 and ERK2 siRNA are seen in **Figures 1-3**, and **Figure 5** indicating that 20pmol of ERK1 or ERK2 siRNA is sufficient in knocking down total-ERK and more importantly, phospho-ERK levels. Protein levels were measured through western blots with antibodies specific for total or phospho-ERK. A quick immunoblot for a known ERK positive cell line, MCF-7 was successfully performed to ensure that the p44 and p42 bands for ERK1 and ERK2 respectively were indeed ERK1/2.

4.2 ERK and proliferation

PTHrP's effects vary with cancer type and can be harmful or beneficial. In lung cancer, PTHrP was demonstrated to reduce lung carcinoma growth (Hastings et al., 2001) and extend

patients with NSCLC's survival time (Hastings et al., 2006). This decrease in lung cancer cell proliferation may or may not be attributed to the finding that found that PTHrP increases the level of ERK phosphorylation (Hastings et al., 2009), a result duplicated in these experiments (**Fig. 1**). Because ERK is known to have pro and anti-proliferative effects, ERK is a likely candidate for implicating effects in NSCLC proliferation. ERK phosphorylation in PTHrP positive cells is hypothesized to decrease NSCLC proliferation because sustained ERK activation is reported to be antimitogenic (Clark et al., 2004). If ERK activation is involved, in the anti-proliferative effect, then ERK knockdown would stimulate growth in PTHrP expressing cells. On the other hand, ERK would oppose the effect of PTHrP if activation increases proliferation.

ERK knockdown did in fact alter cellular proliferation. ERK2 knockdown decreased proliferation in PTHrP positive H1944 cells but not PTHrP negative cells (**Fig. 4**). Restriction to the PTHrP-posiitive cells could be due to the increased amount of pERK in cells expressing PTHrP since ERK's primarily cause effects when activated. Similar to other studies (Carcamo-Orive et al., 2008; D'Souza et al., 2008; Lefloch et al., 2008), decreasing the levels of activated ERK2 significantly decreased mitogenesis signifying that activated ERK2 levels increases proliferation.

Intriguingly, ERK1 knockdown did not decrease proliferation in PTHrP positive cells, contrary to the effects of ERK2 knockdown. Reduction in ERK1 caused minimal changes early, 48 hours after knockdown was complete, but increased proliferation after 72 hours (**Fig. 6**). One interpretation for this result could be that activated ERK1 is a negative modulator of cellular proliferation. On the other hand, the increased proliferation could follow from the increase in pERK2 levels that occur over time with ERK1 knockdown (**Fig. 5**). A clear delineation of cause and effect is impossible because ERK1 knockdown affected both pERK1 and pERK2.

To gain more information about the specific role of ERK1 in cancer cell proliferation, we treated the cells with siRNA for both ERK isoforms together. The expectation was that dual

knockdown would eliminate the proliferative response to treatment with ERK1 siRNA alone if that response were due to the upregulation of ERK2. However, the dual ERK1/ERK2 knockdown had a similar effect as knocking down ERK1 alone, even though the latter maneuver increased ERK2. Proliferation of the PTHrP-positive was unchanged in the first 48 hours after siRNA treatment and then increased at 72 hours (**Fig. 8**). These findings uphold a likely interaction between ERK1 and proliferation in PTHrP-positive H1944 cells, since knockdown of ERK2 alone slows cell division at 24-72 hours (**Fig. 3**) and these changes did not occur with dual knockdown. ERK1 could have two possible roles in this regard. One possibility is that growth inhibition with ERK2 knockdown requires activated ERK1. This would account for the differences in proliferative responses at 24-28 hours in **Figure 3** and **Figure 8**, but it would not explain the increase in proliferation at 72 hours in **Figure 8**. Thus, we favor another hypothesis that activated ERK1 has anti-proliferative effects in H1944 cells, opposing the effect of ERK2 but parallel with the growth inhibitory effects of PTHrP.

Similar proliferation effects to ERK1 knockdown were seen when ERK1 and ERK2 were knocked down together. No significant changes occurred for the first 48 hours but the combined ERK knockdown caused growth at 72 hours in PTHrP positive cells (**Fig. 8**). Presumable, the changes were restricted to PTHrP positive cells because of their greater level of ERK activation. The change in pERK2 levels with ERK1 knockdown suggest a possible feedback mechanism between the two isoforms that might allow ERK2 to compensate for the loss of ERK1. An explanation could be that ERK1 and ERK2 compete for binding to the upstream MEK (Vantaggiato et al., 2006), where ERK2 is more successful since ERK2 levels are higher than ERK1 levels by at least 2 fold (**Fig. 1**). One study that found that only 60% of ERKs were activated when stimulated (Fujioka et al., 2006), indicating that there is a reserve of ERKs that can still be activated. ERK1 might show effects only if there is a great reduction of overall ERK1 levels. Since

H1944 cells have lower amount of ERK1, little effects were seen with ERK1 knockdown and changes were only observed when ERK2 increased. Among all of the responses of ERK activation, only those that directly require ERK activity will be affected by the loss of ERK1 activation.

ERK1 and ERK2 are very similar isoforms. They both have the same subcellular localization patterns prior and after activation (Lenormand et al., 1993) have the same substrate specificities (Yoon and Seger, 2006) and are activated by the same upstream kinase molecule. While similar, ERK1 and ERK2 have differences in function as seen with E*rk1* and *Erk2* gene invalidations in mice. *Erk2* deficiency is embryonic lethal in mice (Hatano et al., 2003), while mice defective in *Erk1* live and reproduce normally (Pages et al., 1999). In these cases, it is thought that ERK2 can compensate for loss of ERK1, similar to what is seen in **Figure 5** where ERK2 levels increase over time with ERK1 knockdown.

The proliferation results suggest a third possibility, an additive result in lung cancer cells, where the increase in ERK activation by PTHrP does not lead to either an increase or a decrease in lung carcinoma proliferation. Rather, the increase in activation of ERK isoforms by PTHrP seems to have minimal effects on growth in lung cancer. PTHrP must work in an ERK independent, alternative route to decrease proliferation. A possible mechanism could be that these PTHrP positive lung adenocarcinoma cells decrease cyclin A2 levels, resulting in decreased cyclin A2 and CDK2 association (Hastings et al., 2009). Decreasing the levels of a protein known to facilitate G_1/S and G_1/M transitions in the cell cycle could explain how PTHrP slows cellular proliferation. Another possibility is that PTHrP targets cyclin D1 to induce growth arrest (Datta et al., 2005).

How PTHrP alters ERK activation is unclear. PTHrP transduction involves receptors coupled to guanosine triphosphate binding proteins (GPCRs). Its effects depend on which G

protein is involved. It can stimulate cyclic AMP (cAMP) formation by adenylate cyclase (AC) to then activate protein kinase A (PKA). Alternatively, PTHrP can also activate phospholipase Cβ (PLC) and lead to the formation of protein kinase C (PKC) (Abou-Samra et al., 1992). Both cAMP and PKC cascades can affect MAP kinases, including ERK (Liebmann, 2001; Sugden and Clerk, 1997). Inhibitory growth effects by PTHrP are mediated by the cAMP/PKA second-messenger pathway where the PKC and PLC pathways can increase proliferation (Fortino et al., 2002). On the other hand, the PKA acute stimulation of ERK can cause it to be pro-proliferative. It is possible that the effects of ERK depend on whether PTHrP is regulated by a PKC or PKA pathway.

4.3 pERK subcellular localization

ERKs are activated by a wide variety of stimuli and can regulate many processes. One of the mechanisms that confer content specificity on the regions ERK activate is the localization of the kinase. The experimental goals for pERK subcellular localization were twofold: 1) to determine whether cellular distribution of activated ERK1 and ERK2 varied from ectopic PTHrP expression to explain their differences in proliferation and 2) to identify which subcellular pool of activated ERK was affected by knockdown. Differences in affects could be attributed to pERK localization.

MAP kinases such as ERK change their subcellular localization when stimulated. In quiescent cells, ERK1/2 is found in the cytoplasm anchored to MEK1/2. MEK1 and MEK2 are localized in the cytoplasm due to their nuclear export sequences (NES) in the amino terminal domain (Fukuda et al., 1996). MEKs are not the only ERK retaining molecules because there are significantly less MEK molecules than ERK molecules. Other known scaffold interactions include those with Sef1, MP1, and the death-domain containing phosphoprotein, PEA-15, also known to contain a NES (Formstecher et al., 2001). When ERK is activated, it dissociates from MEK and translocates to the nucleus along with MEK (Jaaro et al., 1997). In the nucleus, ERK can

phosphorylate and activate many transcription factors that are the prerequisites for many cellular responses such as proliferation. Some activated ERKs may be retained in the cytoplasm due to the cytosolic retention residues 312-320 in ERK (Rubinfeld et al., 1999).

The localization of pERK1 and pERK2 was postulated to allow them to behave differently. Because pERK2 was seen to increase proliferation, we hypothesized that it would be localized to the nucleus. Accordingly, ectopic PTHrP increased localization of pERK1 and pERK2 to the nucleus (**Fig. 9**). This is similar to other studies finding that 60-70% of activated ERK molecules translocate to the nucleus (Lenormand et al., 1993). An independent test for ERK1 knockdown and localization was not done, but because ERK2 knockdown decreased primarily nuclear pERK2, ERK1 knockdown was presumed to affect nuclear pERK1. Therefore, the difference in activity seen between pERK1 and pERK2 was not a function of their subcellular localization. In no way does this that pERK2 localization in the nucleus does not allow for its pro-proliferative actions.

The subcellular localization of ERK2 is likely to be important for access to transcription factors in the nucleus that stimulate cell cycle entry and mitogensis. These results only signify that pERK1 is also found in the nucleus, although it may have different effects when there. The pERK1/2 in the cytosol could regulate the balance between proapoptotic and prosurvival proteins to contribute to PTHrP's inhibitory effects (Strasser et al., 2008). Activated ERK is sustained in cells with ectopic PTHrP expression, supporting the idea that sustained ERK activation is required for certain cells to proliferate (Balmanno and Cook, 1999). Sustained ERK activation allows greater opportunity for pERK to translocate to the nucleus. Sustained ERK activation augments cell motility and invasion (Silletti et al., 2004), although other studies report that sustained ERK activation can lead to differentiation as well (Marshall, 1995).

The temporal pattern of ERK activation may affect the localization of ERK (Chen et al., 2004b), where ERK can transiently translocate to the nucleus for several minutes or sustain its activation for up to 3 hours (Yoon and Seger, 2006) . While this is so, the constant production of PTHrP in the H1944 stably transfected cells should persistently stimulate and activate ERK. Its subcellular localization at the time the cells were separated into nuclear and cytoplasmic compartments would be the same at any time and this factor would be irrelevant.

4.4 ERKs and Integrin subunits

Cancerous cells are malignant when they over-proliferate and metastasize. One process that has effects on carcinoma progression is the regulation of integrin subunit expression. This experiment aimed to link PTHrP's activation of ERK to changes in integrin subunits levels. PTHrP influences integrin expression and related processes including, extracellular matrix adhesion in some cancers (Shen et al., 2004). There are 18 integrin α subunits and 8 β subunits that form heterodimers to one another. Studying the changes in all of the subunits was not feasible. We focused on a specific group that included integrin α2, α3 and α5 which are known to decrease in PTHrP positive lung adenocarcinoma cells (Hastings et al., 2010). These integrin subunits have known implications on metastasis and malignant phenotypes (Yoshimasu et al., 2004).

The effect of PTHrP on integrin expression is thought to be mediated by an intracrine pathway (Shen et al., 2004), yet the mechanism by which PTHrP decreases integrin expression in lung adenocarcinomas is unknown. ERKcan regulate integrin expression in other cell types (Lai et al., 2001), as well as be affected by integrin expression (Aplin et al., 2001). Thus we hypothesized that ERK activation by PTHrP contributed to changes in integrin subunit levels.

The results described in **Figure 9** confirm that PTHrP expression decreases integrin α2 and integrin α3 expression, while also decreasing integrin α5 expression. There was no shift in cells with integrin β1 expression. ERK1 was then knocked down to examine the alteration in

integrin levels by PTHrP, because only activity that is fully dependent on ERK activation will be affected by ERK1 knockdown. The little change in integrin β1 demonstrates that this subunit is continuously binding to other subunit, while α2, α3, and α5 expression decrease. The integrin levels remained the same in cells with ERK1 knockdown compared to treatment with a scrambled siRNA sequence was used in both PTHrP positive and PTHrP negative cells.

The lack of change in integrin expression with ERK1 knockdown indicates that PTHrP alters certain integrin levels in lung adenocarcinoma through mechanisms other than ERK activation. In this case, while ERK activation by PTHrP can increase proliferation, it does not singlehandedly contribute to increased integrin subunit expression. We did not test ERK2 knockdown in this regard and cannot infer that the effects would be the same as for ERK1.

4.5 PTHrP and apoptosis

PTHrP is reported to be both pro and anti-apoptotic depending on the cell type and context such as confluency and the specific amino acid residues. In type II alveolar cells, PTHrP was found to increase the sensitivity of the cells for undergoing apoptosis (Hastings et al., 2003b). To study whether the increase in ERK activation had a role in the sensitivity to apoptosis, H1944 PTHrP positive and negative cells were treated with ERK1 and ERK2 siRNA. Cells were then exposed to an apoptotic stimulus and analyzed for apoptosis by caspase 3 cleavage.

4.5.1 UV induced apoptosis

There are multiple methods for inducing apoptosis in experimental systems. One such method is through ultraviolet (UV) radiation. UV can cause apoptosis through an extrinsic pathway that includes the activation of death receptors such as Fas and the tumor necrosis factor receptor (Micheau and Tschopp, 2003; Wajant, 2002). Alternatively, UV can activate an intrinsic mitochondrial pathway by inducing events such as DNA damage (Rich et al., 1999). UV

was initially chosen as the stimulus for apoptosis because it was effectual in inducing apoptosis with reproducibility in preliminary studies with untreated cells. These experiments were unsuccessful in that while apoptosis was induced with 15 minutes of UV exposure, analysis of ERK levels demonstrated that ERK activity was increased by UV radiation (results not shown). The ERK activation by UV may be a survival signal against apoptosis, possibly mediated through a Src-dependent epidermal growth factor receptor phosphorylation (Kitagawa et al., 2002). Results could not be extrapolated from these results since the stimulus also altered the variable being tested.

4.5.2 Staurosporine induced apoptosis

Staurosporine is an antibiotic also known for inducing apoptosis. While the mechanism of its actions is not entirely known, studies have found that staurosporine induces apoptosis through caspase dependent and independent mechanisms (Belmokhtar et al., 2001). Staurosporine was then chosen to induce apoptosis in this system since we chose to measure caspase-3 cleavage for the amount of apoptosis. **Figure 10** is representative of the caspase-3 cleavage in PTHrP negative and positive H1944 cells with and without ERK knockdown. While staurosporine was successful in inducing apoptosis, PTHrP producing cells did not show any changes in apoptosis sensitivity compared to PTHrP negative cells. Both PTHrP negative and positive cells had variable amount of caspase-3 cleavage depending on cell line, where there were no significant changes between the cells. The minimal change between PTHrP negative and positive cells when treated with staurosporine renders this apoptotic model unusable for examining the effect of ERK. The increase in ERK phosphorylation in PTHrP positive cells would not be involved in the lack of an effect by PTHrP.

Consistent with these findings, other studies noted that PTHrP 1-34 and PTHrP 140-173 had no effect on caspase-3 activity after staurosporine treatment (Hastings et al., 2003a).

Investigations have shown that staurosporine is a potent protein kinase C (PKC) inhibitor (Tamaoki et al., 1986). PTHrP signals through PKC or PKA dependent mechanisms after stimulation of its GPCR (Orloff et al., 1994). Little changes in apoptosis were seen by inhibiting one of PTHrP's pathways in the PTHrP positive cells, though PTHrP can still regulate apoptosis in lung cancer through its PKA pathway (Hastings et al., 2004a).

Protein kinase C is known to activate the ERK pathway independent of Ras and Raf (Ueda et al., 1996). Staurosporine can also act as a non-specific kinase inhibitor (Ruegg and Burgess, 1989). In this case, staurosporine can directly inhibit ERK since ERK is its own kinase. Recent studies from Antonsson and Persson showed that staurosporine induced apoptosis and was supplemented with changes to ERK activity (Antonsson and Persson, 2009). This indicates that staurosporine induced apoptosis is also an unusable protocol for determining the role of ERK in PTHrP's sensitivity to apoptosis. Future studies would entail inducing apoptosis through other mechanisms such as the protein synthesis inhibitor anisomycin, not known to activate ERK (Cano et al., 1994).

4.6 Transcription factor analysis with ERK knockdown

To gain insight to which genes are being altered when ERK is activated in PTHrP positive cells, a human transcription factor array was used. To examine whether the changes are fully dependent on ERK activation, PTHrP positive and negative H1944 cells were treated with ERK1 siRNA and control siRNA. A disadvantage to this method is the mathematical model and stringency used to limit the results, where on occasion, excluded genes may have provided important data. To compensate for this, the genes were sorted and ranked by greatest change in fold regulation. One-third of the genes with the greatest changes were further sorted and compared to the PTHrP negative cells' ERK knockdown results, which included genes not deemed significant by the analysis software. The common genes were also cross-referenced for

possible involvement with PTHrP. The analysis could also be done between PTHrP positive and negative cells to delve into the complexities between cells expressing PTHrP and those without, but the purpose of this experiment was to study the effects of ERK activation in these cells. The top hits for fold up or downregulation for both PTHrP positive and negative cells were ATF1, STAT3, STAT4, STAT5B, ETS2, FOS, E2F1, and RB1 (**Fig. 11**). While these genes had the greatest changes in regulation, the largest fold change was only 3-fold from FOS.

Followup was done on ATF1, STAT3, STAT4, STAT5B, FOS, and ETS2 by confirmatory quantitative PCR. *ATF1*, which encodes cyclic AMP dependent transcription factor ATF1, was chosen because PTHrP can stimulate the formation of cAMP to activate the PKA pathway and produce growth inhibitory effects (Fortino et al., 2002). In addition, ATF1 is closely related to CREB, where PTHrP is seen to regulate cell differentiation through CREB (Ionescu et al., 2001). STAT3 and STAT5B were further studied because previous results found that PTHrP regulates these genes (Hoogendam et al., 2006) through the AC/PKA pathway . Since PTHrP is seen to regulate some members of the STAT family, STAT4 was selected as well. FOS was selected for its association with PTHrP induced differentiation. Finally, ETS2 is seen to regulate PTHrP in conjunction with PKCε (Lindemann et al., 2003).

The low hits from the transcription factor superarray do not indicate that activated ERK levels do not alter gene expression. The 84 genes in the superarray consist of a vast array of transcription factors downstream of signaling from cytokines, chemokines, androgens, toll like receptors and transcription factors target of signal transduction pathways such as MAP kinase. The variety of target genes on the array could just not have been the main target genes of PTHrP activated ERK. A possible method to pursue to possible genes up or downregulated by PTHrP mediated ERK phosphorylation is to incorporate the use of microarrays with thousands of possible gene targets. The negative result from pursuing the changes in fos levels are consistent

with other studies where the PTHrP mediated upregulation of c-fos were independent of MAP kinases. (Chen et al., 2004a). The identification of *fos* from the superarray could just from its involvement in PTHrP induced cell differentiation. Notably, the confirmatory qPCR did not show the involvement of the *STAT* genes when ERK was knocked down since STAT3 is known to be phosphorylated in the ERK1/2 signaling pathway (Chung et al., 1997). One study found MAP kinases to downregulate STAT signaling where the targets affected can sensitize melanoma cells to apoptosis (Krasilnikov et al., 2003). ERK is also known to phosphorylate ATF1(Gupta and Pyrywes, 2002) and ETS2 (Svensson et al., 2005).

4.7 Clinical Implications

While PTHrP decreased cell proliferation in lung adenocarcinoma, it was increasing the activation of a protein that increases cell growth in these studies. Mechanisms to retain ERK in the cytoplasm may decrease the proliferative effects of ERK in PTHrP cells, such as a MEK induced cytosolic retention (Rubinfeld et al., 1999) or injection of anti-ERK antibodies. Inhibiting the ERK2 activation in PTHrP positive cells may contribute to the antiproliferative properties in lung cancer, with greater emphasis on targeting the ERK2 pathways. The increase in ERK1 activity may also counter the effects of ERK2. Because ERK is involved in many signaling aspects, targeting the substrates involved in ERK mediated proliferation may be less disruptive than inhibiting ERK itself. In this sense, PTHrP will be able to decrease cell proliferation in lung cancer without the interference of ERK for better prognosis. The overall goal is to inhibiting cancer cell growth and slow carcinoma progression to improve patient survival.

4.8 Limitations and future experiments

4.8.1 Other mechanisms of ERK knockdown

These experiments took advantage of RNAi to study the effects of activated ERK in PTHrP positive cells. RNAi consists of siRNA nucleotides or shRNA, where shRNA can be

introduced to the cell by a lentiviral vector. Stably transfected ERK shRNA into the cells would constantly knock down ERK. For the purposes of these studies, siRNA was chosen over shRNA because siRNA was fairly effective in knocking down ERK and last throughout the timespan of the experiment. The internal cell system is not tampered with a transient transfection. The amount of knockdown via shRNA varies and is not 100% (Boedeker et al., 2006), a result similar to siRNA where a varied amount of protein still remains within the cell. Being that *erk2* deficiency is embryonic lethal, a complete *ERK* gene knockout did not seem worthwhile. Because there is a reserve of ERK from using siRNA, the effects seen in these experiments may not be as large or may be masked by the ERK activity from the 20-30% ERK remaining in the cell. Nonetheless, an effect was seen. Future experiments encompassing longterm ERK knockdown will include a stable ERK knockdown using shRNA.

Highly selective MEK inhibitors such as U0126 can be used as an alternative mechanism for decreasing ERK activation in PTHrP positive cells. Proliferation could be measured to see if the results are reproducible. Another possibility is to overstimulate ERK and examine proliferation rates.

4.8.2 Difficulties in experimental analysis

4.8.2.1 MTS assay

Cellular proliferation was measured with the MTS assay, which requires conversion of 490nm absorbance to cell number with a standard curve. Standard curves were made for each cell line due to differences in cell size. In addition, standard curves were made for each siRNA treatment for each cell line in the event that the siRNA changed the cell shape or size and affects the absorbance, although it was found that there were no changes with siRNA transfection. Difficulties in this experiment lie in the seeding of cells into 96 well plates, where cell counts are more difficult to manage. Alternatively, cells could be sorted, counted and plated

with a flow cytometer, but such procedures require a large quantity of cells and risk contamination. In addition, sorting through a flow cytometer introduces a new variable that may affect cell proliferation.

4.8.2.2 Subcellular localization

Difficulties in with the subcellular localization experiment lie in the data analysis, where it is difficult to establish baseline pERK levels and protein load accuracy due to the lack of an appropriate marker found at equal levels in both the nucleus and cytoplasm. This experiment shows the difference in activated pERK levels within PTHrP positive and negative cells. To followup on this experiment, ERK localization can be studied through immunofluorescence at different time points of the ERK knockdown. The western blots done in this experiment was a mere snapshot of the occurrences in the cell when ERK2 levels were altered. In addition to these experience, fluorescently tagged pERK molecules can be added to the cell to visually monitor the ERK localization with and without the presence of PTHrP. Other improvements to this experiment will also incorporate ERK1 siRNA knockdown.

4.8.3 Expand integrin subunit examination

It is possible that PTHrP affects other integrins besides the subunits we tested. It may or may not affect other integrins dependent or independent of ERK. Further studies could be done on the other subunits. In addition, ERK2 siRNA can be used to monitor changes to integrin subunit levels.

4.8.4 Rescue effects from siRNA knockdown

While a scrambled sequence nonsilencing siRNA was used as a control for the ERK siRNA, another way to control for the siRNA is to perform rescue effects. To do this, ERK protein will be introduced into cells that have been treated with siRNA to see if the reintroduction of ERK will restore the typical growth rate of the cells.

4.8.5 Extracellular effects of PTHrP on ERK

ERK knockdown was done in cells already expressing PTHrP, therefore the effects seen may or may not be completely attributed to the presence of PTHrP. Because the decrease in proliferation from ERK2 knockdown was only seen in PTHrP positive cells, we speculated that the presence of PTHrP was a contributing to the effect. Another way to verify this is to use knock down ERK in H1944 wildtype cells and stimulate them with exogenous PTHrP. This method may provide greater insight to whether the effects seen are a result of PTHrP and ERK since the H1944 cell lines used in the experiments were overexpressing PTHrP from the stable transfection and could possibly overwhelm the extracellular signal regulated kinases. This method would mimic extracellular effects of PTHrP, where one study noted that PTHrP acts on an extracellular site for to inhibit proliferation (Vander Werff, 2010).

4.8.6 Cell cycle effects from ERK activation

PTHrP was found to decrease the levels of cyclin A2(Hastings et al., 2009), a protein needed for cell cycle progression. Further confirmation that ERK activation by PTHrP has the potential for proliferative effects could be to measure the levels of cyclin A2 after ERK knockdown. If ERK activation counters PTHrP's actions, then ERK would increase cyclin A2 levels. If this is the case, then ERK2 knockdown in PTHrP positive cells should further decrease cyclin A2 levels.

4.8.7 Expansion of cell lines and cell types

The results presented in this report only included studies on one lung carcinoma cell line. A more expansive study would include other cell lines and cell types to ensure that the results seen are not merely a characteristic of the specific cell line. This would also prevent problems arising from the clonal heterogeneity of the H1944 cell line. For instance, possible cells

available for use are MV522 lung adenocarcinoma with stably transfected PTHrP and BEN human squamous lung carcinoma cells that already express PTHrP.

5 CONCLUSION

Previous studies found that ERK phosphorylation was increased in cells containing PTHrP. This finding was the crux of the experiments presented, where the data presented replicated the increased ERK phosphorylation. To understand the role of the ERK phosphorylation in PTHrP positive non-small cell lung carcinoma, changes in cell activity were examined when the levels of ERK phosphorylation were altered with siRNA. These experiments examined the changes in cell proliferation, integrin subunit expression and the cells' increased sensitivity to apoptosis.

Overall, these experiments found that PTHrP inhibits cell proliferation in NSCLC through an ERK independent pathway. Rather than contributing to the growth inhibition, ERK activation in PTHrP positive cells increases cell proliferation as seen when ERK2 knockdown decreased proliferation. The presence of ERK1 was seen to be necessary for proliferation to occur. On the contrary, ERK1 knockdown increased proliferation, suggesting a feedback mechanism between ERK1 and ERK2 or that ERK1 and ERK2 compete for upstream substrate binding.

ERK contributing to proliferation is not surprising since ERK is reported in many studies to cause proliferation. What is interesting is that PTHrP manages to decrease proliferation despite activating a pro-proliferative protein. PTHrP's alternative pathway for inhibiting proliferation overwhelms the ERK pathway. A localization experiment of activated ERK was performed and found that pERK is generally located in the nucleus as a result of ectopic PTHrP expression. Because both pERK1 and pERK2 were found in the nucleus, the differing effects of pERK1 and pERK2 are not because of their subcellular localization and due to another unknown factor.

PTHrP is expressed in roughly two-thirds of lung cancers. It has the potential to improve prognosis of NSCLC patients with its growth inhibitory effects. Because ERK activation by PTHrP

can increase proliferation of these cells, ERK may be a potential target to improve PTHrP's positive effects on patients.

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