MECHANISMS OF RECEPTOR-MEDIATED HYPERCALCEMIA IN HUMAN LUNG SQUAMOUS CELL CARCINOMA

DISSERTATION

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ABSTRACT

Humoral hypercalcemia of malignancy (HHM) is a debilitating syndrome seen in patients with neoplasia of squamous epithelial cell origin. The highest prevalence of HHM is associated with squamous-cell carcinoma of the lung (SCCs) and ranges from 27-66%. HHM results from increased synthesis and secretion of parathyroid hormone-related hormone (PTHrP). The precise mechanisms that activate high concentrations of PTHrP gene expression in tumors associated with HHM have yet to be identified and were investigated in these studies.

The first study evaluates the contribution of the epidermal growth factor receptor (EGFR) to HHM in the human lung SCC cell lines, RWGT2 and HARA. To test the relationship between EGFR activity and PTHrP gene expression, PTHrP mRNA levels were measured by Q-RT-PCR following treatment of lung SCC lines with the EGFR tyrosine kinase inhibitor (TKI) PD153035, anti-amphiregulin antibodies as well as with EGF-ligands. Overall, PTHrP expression was significantly increased with EGF-ligand treatment. Significant reductions of PTHrP/GAPDH mRNA ratios were noted with either anti-amphiregulin antibodies or TKI treatment. The *in vivo* relationship between EGFR and PTHrP gene expression was investigated using xenograft HARA and RWGT2 HHM models. Hypercalcemic mice were treated with the TKI, gefitinib. HARA plasma calcium

levels were reduced after the 78 h treatment whereas calcium concentrations were significantly reduced at all time points when compared to pretreatment and control values in the RWGT2 mice. In conclusion, autocrine activation of PTHrP gene expression is mediated through the EGFR in the RWGT2 line, however, our results indicated that the major mechanism of HHM induction in the HARA model was not through EGFR but rather the high concentrations of PTHrP secreted by the HARA line were significantly influenced by the tumor microenvironment. Our findings from the EGFR studies lead us to the second series of studies which investigates the role of a known regulator of calcium homeostasis in humans, the calcium-sensing receptor (CaR). Currently, it is unknown if CaR is expressed in normal human lung epithelia or in lung SCC. Our experiments evaluate evidence for the expression of the CaR in human lung SCC. As CaR can use the Ras MAPK pathway for downstream signaling and as increased PTHrP expression and secretion in squamous epithelia results from upstream Ras MAPK signaling, we examined if PTHrP secretion and HHM occurs in response to CaR stimulation in the RWGT2, HARA and BEN Australia SCCs. We find that CaR is expressed in lung SCCs and stimulation with extracellular calcium (Ca^{2+}_o) increases PTHrP mRNA expression and secretion in all the lines. Furthermore, stimulation of the lung SCCs with Ca^{2+}_o results in different patterns of cytosolic calcium oscillations dependant on the variant of CaR. Using mouse xenograft models, we determined

that CaR is necessary for the rapid development of HHM and furthermore, that overexpression of CaR in a cell line that does not induce hypercalcemia in a xenograft model will cause HHM. Finally, we present evidence that the known gain of function mutation, R990G, when expressed in an otherwise normocalcemic xenograft model predisposes to an earlier onset of hypercalcemia. Overall, our findings contribute to the understanding of the mechanisms that lead to increased PTHrP secretion and subsequent HHM in patients diagnosed with lung SCC.

Dedicated to my loving mother and father

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PUBLICATIONS

Research Publication

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FIELDS OF STUDY

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INTRODUCTION

MOLECULAR BIOLOGY OF ONCOGENES INVOLVED IN NON-SMALL CELL-LUNG CANCER

Classification of Lung Cancer

Lung cancer is the leading cause of cancer death for both men and women in the United States, killing more people than the neoplasms of breast, prostate, colon and pancreas combined: Fully 85 percent of patients who develop lung cancer die from it. In 2008, an estimated 215,020 new cases of lung cancer are expected, accounting for about 15% of total cancer diagnoses. An estimated 161,840 lung cancer deaths will occur accounting for about 29% of all cancer deaths.(1) Although the incidence rate is declining significantly in men, from a high of 102 cases per 100,000 in 1984 to 73.6 in 2004, in women, the rate is approaching a plateau at ~ 56 cases per 100,000 after a long period of increase. With half of all lung cancers in the US now diagnosed in former smokers, it is a sobering reality that tobacco control will ameliorate but not, in the foreseeable future, eliminate the problem of lung cancer. A significant proportion of non-small cell lung cancer (NSCLC) cases are detected only in the advanced stage after

the onset of metastasis, leading to a remarkably low 5-year patient survival rate of ~15%.(1) Patients with advanced NSCLC that is left untreated, have a median survival after diagnosis of 4-5 months and less than 10% of these cases will survive greater than 1-year. The meager longer term survival rate can also be attributed to the paucity of effective late-stage therapies.

Commonly thought of as a single disease, lung cancer actually represents a group of neoplasms arising from phenotypically diverse cells.(2, 3) Lung cancer is classified clinically as small cell (SCLC) (13%) or NSCLC (87%) for the purposes of treatment. Correct classification is critical because SCLC, responds well to chemotherapy and is generally not treated surgically. NSCLC is a heterogeneous aggregate of histologies. The most common malignant non-small epithelial tumor lung histologies are epidermoid or bronchioloalveolar squamous carcinoma, adenocarcinoma, and large cell carcinoma. Additional classifications of NSCLC include adenosquamous carcinoma, carcinomas with pleomorphic, sarcomatoid, or sarcomatous element, carcinoid tumor, carcinoid of salivary-gland type and unclassified carcinoma. Within each of these subcategories there are up to 6 subtypes.(4) SCLC and NSCLC show major differences in histopathologic characteristics that can now be explained by the distinct patterns of genetic alterations found in both tumor classes.

There has been a shift in lung cancer pathology over the past few decades due to changes in cigarette design and subsequent changes in inhalation patterns.(5) In the 1950's less than 1% of all commercial cigarettes had filters and by the late 1990's more than 95% had them. Prior to this change, squamous cell was the predominant cell type. Squamous cells populate the proximal and central airways. Deeper inhalation associated with the use of filtered cigarettes has lead to the development of peripheral adenocarcinoma, which has taken over as the most common NSCLC subtype. Despite the fact that the majority of lung cancers can be attributed to smoking, 10% arise in never-smokers. Definitions for the term 'never smoker' vary in the literature, but it is used in this review to refer to an individual who has had a lifetime exposure of less than 100 cigarettes. If considered in a separate category, lung cancer in never smokers would rank seventh most common cause of cancer death worldwide, ahead of cancers of the cervix, pancreas and prostate.(1) A consistent global observation is that lung cancer cases in never smokers occur more frequently in women than men. Adenocarcinoma is the commonest form of lung cancer in never smokers. Risk factors for lung cancer in never smokers include environmental tobacco smoke, residential radon, cooking oil vapors, indoor coal and wood burning, human papillomavirus strains 16/18 and genetic factors such as a family history of lung cancer, cytochrome P450 1A1 polymorphisms (Ile462Val) and polymorphisms in DNA repair genes involved in base excision repair such as *XRCC1*.(6)

The molecular events underlying the development of lung cancer and the mechanisms of resistance to chemo- and radiation therapy are still largely unknown in both smoking-induced and the never smoker forms of NSCLC. However, it is widely known that the pathogenesis of lung cancer involves genetic and molecular changes in oncogenes, tumor suppressor genes and stability or “caretaker” genes suggesting a role for multistage carcinogenesis. Theoretically carcinogenesis can be divided into four steps: tumor initiation, tumor promotion, malignant conversion and tumor progression. Tumor initiation results from irreversible genetic damage. For mutations to accumulate, they must arise in cells that proliferate and survive the lifetime of the organism. Most often exposure to a chemical carcinogen causes a genetic error by modifying the molecular structure of DNA. This commonly occurs through the formation of adducts from exposure to repeated chemical carcinogens with a nucleotide of DNA. In this phase, a susceptible stem cell acquires one or more mutations resulting in an initiated cell which has partially escaped growth control. The second phase is tumor promotion which is clonal expansion of initiated cells. These initiated cells are at risk of further genetic changes allowing them to progress on the pathway to malignancy. Tumor promoters generally are nonmutagenic, not carcinogenic alone, and often able to mediate their biological effects without metabolic activation. Many of the complex chemical mixtures in cigarette-smoke have been shown to have tumor promoting properties. The next stage is malignant conversion which results in transformation of a preneoplastic

cell into one that expresses a malignant phenotype. This process requires one of the initiated cells to undergo further genetic changes. Malignant conversion may be mediated through the activation of proto-oncogenes and inactivation of tumor-suppressor genes. The final stage is tumor progression. In this stage, the malignant cells develop a more aggressive phenotype as a consequence of genomic instability and uncontrolled growth. These phenotypic changes create a cell that is capable of metastasizing. Cells from this stage are characterized by activation of protooncogenes commonly occurring through two major mechanisms: point mutations as seen in the *ras* gene family and overexpression of multi-gene families such as *myc, raf* or *EGFR*. Overexpression of genes may arise as amplification, translocation or transcriptional upregulation. An understanding of the importance of the different mechanisms in carcinogenesis may suggest better approaches to prevention, intervention and treatment. A recent study analyzed lung cancer mortality in three cohorts, each with >1 million individuals, in relation to the multistage model for carcinogenesis.(7) Findings suggested that smoking-related promotion is the dominant mechanism associated with lung cancer mortality in all cohorts. Furthermore, smoking related initiation was less important than promotion but interacts synergistically with it.(7) The importance of smoking duration on lung cancer risk in these cohorts was a direct consequence of promotion.

While cessation of smoking may prevent promotion of premalignant cells, patients with NSCLC receive combination cytotoxic chemotherapy which often results in a modest increase in survival at the cost of significant toxicity to the patient. The advent of molecular–targeted therapeutics has therefore generated much optimism, given the perception that the limits of chemotherapy in NSCLC have been reached and that further advances in the treatment of NSCLC will need to involve radically different approaches.(8)

The main objective of this review is to summarize important molecular aspects of proto-oncogenes involved with NSCLC pathogenesis. Discovery of these molecular mechanisms have and will continue to provide the rational for the extensive development of novel small molecule inhibitors, and personalized therapy as well as constitute the impetus for clinical trials.

PROTO-ONCOGENES: ABNORMALITIES IN GROWTH REGULATORY SIGNALING PATHWAYS

Hanahan and Weinberg (9) have suggested six essential alterations in cell physiology that collectively dictate malignant growth: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of programmed cell death (apoptosis), 4) limitless replicative potential, 5) sustained angiogenesis and 6) tissue invasion and metastasis. Tumors use proto-oncogenes as a

necessary mechanism for tumor growth as these genes code for proteins that stimulate cell division. Mutated forms, called oncogenes, drive the neoplastic process by increasing tumor cell number through the stimulation of cell birth. The first oncogenes to have been identified were segments of the genomes of acutely transforming retroviruses, which cause hematological or soft tissue cancers in avian or mammalian hosts. Some of the first identified acute transforming retroviruses were Abelson murine leukemia virus and Rous sarcoma virus (RSV), which rapidly elicit leukemias, lymphomas or sarcomas after infection of their host. The first oncogene protein, v-src isolated from RSV was biochemically characterized in the late 1970's. These gene products act in a dominant manner and confer altered growth properties and morphology on specific target cells in mesenchymal tissues or the hematopoietic system without any apparent requirement for a co-carcinogen. A second group of oncogenes was identified and consisted of host proto-oncogenes whose expression was activated by insertion of the genome of slowly transforming retroviruses. An example is the retroviral insertion of v-myc gene contained in the myelocytomatosis retrovirus into c-myc host gene. Cellular proto-oncogenes become dominant acting oncogenes by additional mechanisms other than retroviral insertion and these include chromosomal translocation, gene amplification, or from subtle intragenic mutation affecting crucial residues that regulate the activity of the gene product. A mutation in an oncogene is analogous to a stuck accelerator in an automobile; the car still moves forward even when the driver removes his foot from it.

Activation of a somatic mutation in one allele of an oncogene is generally sufficient to confer a selective growth advantage on the cell. As a consequence of these alterations, their protein products become overexpressed, deregulated, overactive or mislocalized in the cell. Several genes such as NRAS, KRAS, ERBB1, and MYC orthologous to viral oncogenes have turned out to be overexpressed or mutated in human cancers. Cellular proto-oncogenes regulate cell processes that control proliferation, differentiation and survival. Proto-oncogenes act as extracellular growth factors, receptors juxtamembrane adaptors or transducers of signaling cascades emanating from growth factor receptors or other membrane receptors. Many proto-oncogene products are the protein kinases which include growth factor receptors with tyrosine kinase activity that are central to most cellular proliferation inducing stimuli. Other non-receptor cellular kinases located in the cytoplasm include non-receptor tyrosine kinases and serine/threonine kinases. A further large group of proto-oncogenes consists of transcription factors. Surprisingly, a large number of proto-oncogenes function within or interact with a single signaling network. At its core is the mitogen activated protein kinase ‘MAPK’ cascade which links growth factor signaling to transcription in the nucleus and to control of the cell cycle. However, this cascade also influences protein synthesis and the dynamics of the cytoskeleton. Normally, growth factor signaling and the MAPK cascade are tightly regulated by feedback regulation and by short half-lives of activated states. Oncogenic mutations make these proteins independent of input signals, disrupt feedback regulation or

prolong their activated state. There are few cases in which a single oncogene is sufficient to induce full malignant transformation. Rather, a single oncogene confers some aspects of the malignant phenotype and cooperates with others or with defects in tumor suppressors for complete transformation.

The Epidermal Growth Factor Receptor Family Overview

The epidermal growth factor receptor (EGFR) is historically the prototypical receptor tyrosine kinase (RTK) and is a member of the ErbB family (nomencalature based on the v-erbB oncogene derived from the avian erythroblastosis virus). It was the first of the receptor tyrosine family of transmembrane receptors to be cloned, understood to be enzymatically activated by ligand-mediated oligomerization, and established as a contributor to cancer.(10-12) The normal function of ErbBs and their ligands is to mediate cell-cell interactions in organogenesis and adulthood. With few exceptions, ErbB proteins are expressed in cells of mesodermal and ectodermal orgins. The ErbB family of RTK includes four members the EGFR (ErbB1), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4). The family is collectively referred to as ErbB or HER (human epidermal growth factor receptor) family. In the epithelium, the basolateral location of ErbBs enables them to mediate signals between the mesenchyme and the epithelium for cell growth.(13) ErbB1 is implicated in promoting proliferation and differentiation of a variety of epithelia including that of

the lung. ErbB family receptors consist of an extracellular region that contains two ligand-binding domains, an extracellular juxtamembrane region, a single hydrophobic transmembrane domain, a cytoplasmic tyrosine kinase domain and cytoplasmic tyrosine residues that serve as sites for receptor phosphorylation.(14) ErbB3 is devoid of intrinsic kinase activity,(15) whereas ErbB2 binds no ligand with high affinity.(16) Therefore, in isolation neither ErbB2 nor ErbB3 can support linear signaling. Most inter-receptor interactions are mediated by ligands, and ErbB2-containing heterodimers are formed preferentially.(17)

EGFR Ligands

Ligands that regulate ErbB receptors are classified in two main groups: the EGF agonists that activate EGFR and the neuregulins (NRG) that bind ErbB3 and ErbB4.(18) The mesenchyme serves as a store house for many ligands including the EGFR ligands and NRGs. There are at least seven different EGF agonists: epidermal growth factor (EGF), amphiregulin (AR), betacellulin (BTC), epigen (EPN), epiregulin (EPR), heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor alpha (TGF-α). Expression and processing of the precursor ligand is regulated by diverse stimuli. For example, transformation by active Ras, or exposure to steroid hormones(19) leads to increased expression of several ErbB ligands, and cleavage of ligand precursors by metalloproteinases is stimulated by activation of other receptors, such as the G-protein-coupled

receptors, lysophosphatidic acid, thrombin and endothelin and the calcium-sensing receptor.(20) Ligands such as EGF and NRG4, which bind to ErbB1 and ErbB4, respectively, have narrow specificity, whereas others such as epirregulin, NRG1β and betacellulin bind to two distinct primary receptors.(21) In addition, splice variants of NRGs and various ligand-receptor complexes also differ in their ability to recruit a partner receptor, which affects their potency and kinetics of signaling.

EGFR Signaling

The components of the ErbB signaling pathways are ancient and both the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaser* have primordial linear versions of the ErbB signaling pathway.(18) Growth factor-induced receptor dimerization is followed by intermolecular autophosphorylation of key tyrosine residues in the activation loop of the catalytic phosphotyrosine kinase (PTK) domain resulting in stimulation of PTK activity. Currently the number of tyrosine phosphorylations identified include 12 sites on EGFR, 8 on ErbB2, 11 on ErbB3 and 18 on ErbB4. Tyrosine autophosphorylation sites in other parts of the cytoplasmic domain serve as docking sites for SH2 and phosphotyrosine binding (PTB) domains of signaling proteins that are recruited, activated and couple downstream effectors to give to context specific biological responses.(22) Receptor specificity and potency of intracellular signals are determined by positive and negative effectors of ErbB proteins, as well as by the

identity of the ligand, oligomer composition and specific structural determinants of the receptors.(18) As the ErbB proteins harbor anywhere from 43 to 116 SH2 or PTB protein sites, specificity is conferred by which sites are autophosphorylated, and hence which signaling proteins are engaged, as well as the identity of the ligand and the heterodimer partner.(23) When considering all receptor activation, the Ras and Src homology 2 domain adaptor protein (shc)-activated MAPK, PI(3)K–Akt, p70S6K/p85SK and STAT pathways are downstream of most ErbB dimers. Simultaneous activation of linear cascades, such as MAPK, p70S6K/p85SK and Akt translates in the nucleus as distinct transcriptional programs that not only involve proto-oncogenes *fos, jun* and *myc*, but also zinc-finger containing transcription factors that include *Sp1* and *Egr1* in addition to Ets family members.(24) Interestingly, although the receptors share similar signaling pathways, the receptors bind to distinct signaling adaptor proteins. For example, ErbB3 cannot interact with the adaptor protein, Grb2 or the Ras-specific GTPase-activating protein, GAP, but it can associate with the adaptors Shc and Grb7.(25)

In the signaling network, the major partner of EGFR is ErbB2. When ErbB2 is overexpressed, heterodimers form preferentially. Unlike homodimers, which are either inactive (ErbB3 homodimers) or signal only weakly, ErbB2-containing heterodimers have attributes such as slow ligand dissociation, relaxed ligand specificity, slow endocytosis, rapid recycling and prolonged firing that impart

enhanced downstream signaling. This signaling leads to increased cell proliferation, increased cell migration and resistance to apoptosis.(18) Further, homodimeric receptor complexes are less mitogenic and transforming than the corresponding heterodimeric combinations, and ErbB2-containing heterodimers. Signaling provided by the ErbB2-ErbB3 heterodimer; although neither ErbB2 nor ErbB3 alone can be activated by ligand, is the most transforming and mitogenic receptor complex.(26-28)

ErbB receptor signaling is turned off by ligand-mediated receptor endocytosis with the kinetics of the process determined by the composition of the dimer: ErbB1 homodimers are targeted primarily to the lysosome for degradation; ErbB3 molecules are constitutively recycled; and heterodimerization with ErbB2 decreases the rate of endocytosis and increases recycling of its partners.(29-31) Complex dissociation leads to recycling, whereas, continuous activation of tyrosine phosphorylation in the endosome leads to recruitment of c-Cbl, a ubiquitin ligase that preferentially binds to ErbB1 homodimers and directs them to lysosomal degradation by tagging with polyubiquitin tracts.(32)

The Role of EGFR in NSCLC

Aberrant signaling from all four receptors, through misregulation of the receptors or of their ligands, has been implicated in many cancers. EGFR has emerged as a critical tumorigenic factor in the development and progression of NSCLC.

These signaling pathways impact on many aspects of tumor biology making EGFR and related receptor molecules attractive and rational targets for anticancer therapies. An increased level of EGFR gene expression is observed in many cancers and frequently confers an adverse prognosis. A retrospective analysis reported EGFR overexpression in 62% of NSCLC cases, and its expression is correlated with a poor prognosis.(33, 34) Genomic analysis has documented the amplification of chromosomal region 7p12 in NSCLC where the *EGFR* gene is located.(35)

A number of somatic mutations have been indentified in the EGFR gene in NSCLC. By and large these mutations can be classified into three major types: in-frame deletion, insertion, and missense. Although activating mutations in the ErbB receptor tyrosine kinases can be found in a variety of locations throughout the protein, kinase domain mutations represent the predominant type in NSCLC. In unselected NSCLC samples, *EGFR* mutations are present in ~10% of cases in North America and Western Europe, but ~30-50% of cases in individuals of East Asian descent, and are associated with most (over 50%) adenocarcinomas with bronchioloalveolar features that arise in non-smokers.(36-38) *EGFR* kinase domain mutations (EGFR-TK) target four exons (18-21), which encode part of the tyrosine kinase domain (the entire kinase domain is encoded by exons 18-24) and are clustered around the ATP-binding pocket of the enzyme.(37, 39-42) The EGFR-TK mutations found in NSCLC likely destabilize the inactive conformation

of the EGFR kinase domain.(10) Kinase domain mutations in EGFR have been identified in a subset (~10-30%) of NSCLC and are correlated with clinical response to selective small molecule inhibitors of EGFR kinase (gefitinib, erlotinib). Examples of kinase mutations are a specific point mutation in exon 21 at codon 858 (L835R missense mutation) and small inframe deletions in exon 19 within the EGFR catalytic pocket. These two types of mutations account for approximately 90% of mutated cases. Kinase domain mutations in EGFR are generally referred to as activating mutations, as they seem to result in the increased kinase activity of the receptor. However, this does not imply that these mutated EGFRs are necessarily constitutively or fully active, as their degree of ligand independence might be a function of experimental context.(43-45) These partially activated mutant EGFRs can be rendered fully ligand-independent, and therefore constitutively active, by second site substitutions in EGFR.(8) The mutations that display increase kinase activity couple to the phosphorylation of Akt, Stat 5 and Stat 3.(46, 47) In addition, the L835R missense mutation and small inframe deletions shift the conformational equilibrium for favor of the catalytically active confirmation, presumably accounting for the increased signaling displayed by these mutants.(46) It has been proposed that these mutations cause increase EGFR signaling that selectively engages downstream survival pathways to which tumor cells become completely dependent upon for survival or “addicted”, thus, disruption of the EGFR signaling by kinase inhibitors leads to cell death and tumor atrophy. A kinase domain drug-acquired mutation is

an EGFR T766M (also known as T790M) point mutation, which has been detected in ~50% of NSCLC that initially respond to gefitinib or erlotinib but subsequently developed resistance.(40) Interestingly, in a family with evidence of inherited predisposition to NSCLC the T766M mutation is present in the germline of affected individuals, suggesting a potential oncogenic role in addition to its role in acquired drug resistance.(48) The mechanism of resistance is conferred to a conserved threonine residue that is situated deep within the catalytic pocket and substitution with a bulky amino acids appears to reduce drug binding but yet preserve catalytic function of the tyrosine domain.(14) Interestingly, 20% of patients with resistance to gefitinib or erlotinib due to the T766M have tumor cells that display amplification of the hepatocyte growth factor receptor/met proto-oncogene (*MET)* at chromosome position 7q31.(49) Finally, an insertion mutation in the ErbB2 kinase domain (Ins776YVMA) found in NSCLC displays increase TK activity, suppresses apoptosis and possesses transforming activity.(50) Recently, Ding and colleagues (51) have found a significiant frequency of mutations in *ErbB4* after sequencing the lung cancer genomes in 188 primary lung adenocarcinomas. The discovery of nine mutations in *ErbB4*, two of which are putatively inactivating with respect to protein tyrosine kinase domain and five of which are clustered in the receptor ligand binding domain indicates the probable involvement of these mutations in lung cancer.

The Ras-MAPK pathway is downstream of EGFR and is important in cell-proliferation. Although mutations of Ras oncogenes do not seem to coexist with *EGFR,* the pathway might be important in mediating EGFR-mutant signals. NSCLC patients with *K-ras* mutations have poor sensitivity to EGFR-TKIs(52), therefore screening NSCLC patients that express EGFR for *K-ras* mutations might be helpful in avoiding the use of EGFR-TKIs and instituting alternative therapy in a timely manner.

Gene amplification is a molecular mechanism responsible for oncogene overexpression. By the production of multiple copies of a particular gene, the phenotype that the gene confers is amplified in the cell. Amplification of wild-type EGFR and other ErbB family members have been detected. Determination of the extent of EGFR amplification in NSCLC has been difficult to interpret due to the variability in different techniques used to measure copy number. Some studies have used quantitative PCR (qPCR) providing a global copy number whereas others have used FISH which evaluates copy number at the single cell level providing very divergent results. EGFR amplification, as assessed by qPCR seems to be more common in smoking-associated cancers, and does not show the same prediction towards distinct ethnic background and tumor histology.(53) High copies of EGFR amplification have been detected in approximately 30% of NSCLC patients using FISH and it has been associated with a poor clinical prognosis.(33) High *EGFR* copy number is frequently correlated with *EGFR*

somatic mutations.(54) A recent study has elegantly confirmed that mutations in *EGFR* were associated with copy number gain in lung adenocarcinomas.(51) Notably, three of six tumors with the highest *EGFR* amplification also have mutations in *EGFR*, with many cases having the mutant allele preferentially amplified.(51) Finally, mutations in *EGFR* show a negative correlation with the solid subtype of adenocarcinomas and significant positive correlation with the papillary subtype.(55) Distinct features of EGFR mutations have been correlated in smokers verses never smoker lung cancers. *EGFR* mutations are more frequently found in adenocarcinomas arising in never smokers.(6)

Decades of research have revealed multiple mechanisms that modulate the strength and duration of EGFR signal and shown the aberrant activation of this potent signaling molecule promotes growth and development of lung cancer. Analysis of these mechanisms has allowed the development of the first small molecule inhibitors. Non-smoking status is the strongest clinical predictor of benefit for the EGFR tyrosine kinase inhibitors. Compared with patients with a history of smoking, approximately fourfold higher response rates with gefitinib in never smokers with metastatic NSCLC have been reported in retrospective studies.(56, 57) The EGFR small molecule inhibitors, gefitinib and erlotinib have been and continue to be evaluated in approximately 160 clinical trials for various cancers. EGFR-targeted therapy has not been shown to have any beneficial effects in combination with standard chemotherapeutic regimens, which begs the

question of whether or not there is rationale for combination therapeutics in the treatment of NSCLC. It seems with insights into EGFR-dependent signaling over the last 40 years that crucial players in EGFR signal transduction pathway can be divided into two broad categories, the prosurvival pathways which are PI(3)K-mTOR-AKT and the proliferative pathways of Ras-Raf-MEK-ERK.(8, 58) With this categorization it seems EGFR-TKIs in combination with inhibitors that target different key components of this network might provide greater therapeutic efficacy, particularly when EGFR-TKI therapy becomes ineffective.

Overview of the RAS / RAF/ MEK/ ERK Pathway

The RAS proteins are members of a large family of low-molecular-weight GTP-binding proteins, which can by divided into families according to the degree of sequence conservation. Different families are important for different cellular processes – the RAS family controls cell growth and the RHO family controls the actin skeleton. Three members of the RAS family of protooncogenes *HRAS, KRAS,* and *NRAS* are found to be activated by mutation in human tumors and are widely expressed with *KRAS* being present in most cell types. These 3 members encode GTPase proteins that play a role in transducing growth-promoting and survival signals from membrane-bound RTKs. The activation state of RAS proteins depends on whether they are bound to GTP (in which case they are active and are able to engage downstream target enzyme) or GDP (in which

case, they are inactive and fail to interact with these effectors).(59) In normal cells, the activity of RAS proteins is controlled by the ratio of bound GTP to GDP.(60) Several RAS guanine nucleotide exchange factors have been characterized and identified as RAS activators, such as *son of sevenless* gene products SOS1 and SOS2. Following the activation of receptor tyrosine kinases such as EGFR, the autophosphorylated receptor binds to the SH2 domain of the adaptor protein growth-factor-receptor-bound protein 2 (GRB2).(60) Through its SH3 domains, GRB2 is bound to SOS, so activation of the receptor TK results in recruitment of SOS to the plasma membrane, where RAS is also localized as a result of the posttranslational modification, farnesylation. The increased proximity of SOS to RAS results in increase nucleotide exchange of RAS, with GDP being replaced with GTP, which is the predominant guanine nucleotide in the cytosol. Many other types of receptors, including G-protein-coupled receptors, like CaR, can activate RAS through stimulation of exchange factors. In some cases, this has been shown to involve transactivation of growth factor receptor tyrosine kinases.(61) Other proteins are involved in the activation of RAS, such as SHC, which can mediate between the receptor and GRB2.

Downstream signaling is a result of GTP-bound RAS's capability to bind and activate effector enzymes, and it is through these pathways that RAS controls cell proliferation, survival and other aspects of cell behavior that can contribute to the transformed phenotype. The most intensively studied effector of RAS is the

protein serine/theronine kinase RAF. GTP bound RAS binds to and contributes to the activation of the three related RAF proteins, c-RAF1, BRAF and ARAF.(62) This interaction causes RAF to be relocated to the plasma membrane which is critical for its activation.(63) Downstream of this, activated RAF phosphorylates and activates mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2)- dual specificity kinases that are capable of phosphorylating and activating the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2.(59) Substrates for ERK1/2 include cytosolic and nuclear proteins, as they can be transported into the nucleus following activation. Regulation of transcription factors is a major mechanism of cell control for these kinases. In lung cancer, regulation of ERK phosphorylates ETS family of transcription factors such as ELK1, which forms part of the serum response factor that regulates the expression of FOS; in addition, ERK phosphorylates c-JUN. This leads to activation of AP1 transcription factor, which is made up of FOS-JUN heterodimers. Ultimately, these transcription factors regulate key cell cycle regulatory proteins such as D-type cyclins, which enables the cell to progress through the G1 phase of the cell cycle.(64)

Ras has also been shown to directly activate the phoshatidylinositol-3-kinase (PI3K)-Akt pathway. Activation of AKT/PKB has strong anti-apoptotic functions by phosphorylating and inactivating several pro-apoptotic targets including Bad and the forkhead transcription factors. In addition, Akt exerts its anti-apoptotic effects

by maintaining hexokinase-mitochondrial interatctions, which appear to crucial to Akt-mediated inhibition of cytochrome c release and apoptosis.(65) Phospholipase Cε (PLC) is another RAS effector. PLC has two RAS association domains and also a RAS-GEF domain, in addition to its PLC domain, which promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol-1,4,5-trisphosphate.(65) Phospholipase Cε links RAS activation to PKC and calcium mobilization.

Normally hydrolysis of bound GTP to GDP abrogates RAS signaling, but oncogenic missense mutations in *KRAS* result in loss of intrinsic GTPase activity that is required to return RAS proteins to their inactive, GDP-bound form.(59) Therefore, the intrinsic negative-feedback control of Ras activity is lost, leaving the mutated Ras constitutively activated. In tumors activated Ras protein contributes significantly to several aspects of the malignant phenotype, including the deregulation of tumor-cell growth, programmed cell death and invasiveness, and the ability to induce new blood-vessel formation.(62)

The Role of Ras in NSCLC

The *RAS* oncogenes acquire their transforming capacity by point mutations, and these are detected in 20-30% of lung adenocarcinomas and 20% of all other NSCLC.(66, 67) Most (approximately 80-90%) are point mutations G-T transversions and are correlated with smoking.(66) Several distinctive features

characterize *KRAS* mutations in lung cancer. *KRAS* mutations are limited to NSCLC and are never present in SCLCs.(68) *KRAS* mutations also predict poor survival (69) and resistance to EGFR TKI therapy.(52) They occur more frequently in lung cancers arising in smokers, and less commonly in lung cancers from East Asia.(70) Lung adenocarcinomas with *KRAS* amplification also harbor *KRAS* mutations. In 41 lung adenocarcimoas, mutations in *KRAS* are associated with higher mRNA expression levels as well as a higher gene copy number.(51) Downstream of RAS, in 132 of 188 lung adenocarcinomas have at least one mutation in the MAPK pathway, underscoring its pivotal role in lung cancer. *EGFR* and *KRAS* mutation in lung tumors are almost entirely mutually exclusive. In addition, activation of these pathways have diametrically opposite relationships to smoke exposure such that *KRAS* mutations are almost exclusively found in adenocarcinomas in smokers, whereas EGFR mutations are more frequent in never smokers.(70) The distinct smoking-related patterns of *EGFR* and *KRAS* mutations is that in smokers the multiple potential carcinogens from tobacco might specifically induce Ras signaling pathways through mutations in *KRAS*, whereas unidentified carcinogens might selectively target the EGFR pathway to induce mutations.(6) Alternatively, the absence of activated Ras signaling might lead to activation of EGFR as the default pathway in lung cancers in never smokers. The exact role of mutations in EGFR tyrosine kinase in this independent developmental pathway in lung cancer is unknown.

Overview of MYC Family Signaling

Myc is deregulated and overexpressed in most cancer cells, where it hijacks the diverse intracellular and extracellular regenerative programs that drive normal cell expansion. The *myc* proto-oncogene family consists of *c-myc, N-myc,* and *L-myc.* MYC belongs to the basic region/helix-loop-helix/ leucine-zipper (BR/HLH/LZ) class of transcription factors. Myc-dependent transactivation requires heterodimerization with the small bHLHZ partner protein Max.(71) Max is present in stoichiometric excess to Myc, and can also form homodimers with several related proteins, known as Mad1, Mxi1, Mad3, Mad4 and Mnt. The dimers all bind directly to the same DNA sequence (CACA/GTC), which is a subset of the general E-box sequence (CANNTG) that is bound by all bHLH proteins. *In vivo*, Myc-Max complexes are often predominant in proliferating cells, whereas, Mad-Max or Mnt-Max complexes are predominant in resting or differentiated cells.(72) When bound to E-box sequences, Myc-Max heterodimers activate transcription, whereas Mad-Max and Mnt-Max heterodimers repress transcription.(73, 74) Myc proteins act as master regulators of two very distinct genetic programs: firstly, they stimulate virtually all nuclear processes leading to enhanced cell growth; and secondly, Myc proteins cancel the cell cycle arrest induced by multiple growth-inhibitory pathways. Due to this unique combination of properties, oncogenic deregulation of Myc expression generates cells possessing a tumor phenotype. In all cases, the relative amounts of Myc protein

are increased in the tumor tissue relative to the surrounding normal tissues, which indicates that the elevated expression of Myc contributes to tumorigenesis. However, the complexity and diversity of Myc gene targets has confounded attempts at identifying which of the Myc activated or repressed genes are critical for mediating tumorigenesis driven by the oncogene. Whereas the ability of Myc to drive unrestricted cell proliferation and to inhibit cell differentiation had long been recognized, more recent work shows that deregulated expression of Myc can drive cell growth and vasculogenesis, reduce cell adhesion, and promote metastasis and genomic instability.(75-77) Conversely, the loss of Myc proteins not only inhibits cell proliferation and cell growth but can also accelerate differentiation and increase cell adhesion.(78) The precise target genes through which Myc functions in each of these pathways have remained elusive in some but not all cases; one reason for this is that the number of target genes regulated by Myc is extensive and include the following biochemical categories: protein biosynthesis, metabolism, transcription factors and cell cycle, microRNAs and cyclin dependent kinases.

The physiological controls that regulate the transcription of *Myc* genes are disrupted in many human tumors, in which the expression of levels of *Myc* mRNAs are high.(79) However, the deregulated expression of *Myc* alone fails to convert either rodent or human cells into tumor cells, and tumors that arise from *Myc* transgenic mice are clonal, which implies that additional mutations are

required for tumor formation. Two main mechanisms are known to limit cellular transformation by Myc. The first network of Myc proteins is controlled post-transcriptionally by the Ras-dependent signaling pathways. Second, transformation by Myc and Myc-induced tumorigenesis are limited by the ability of Myc to induce apoptosis.(80)

Ras controls the function of Myc by three mechanisms. First, Ras control is established by phosphorylating Myc at two residues T58, and S62.(81) MAPKs phosphorylate Myc at S62, which leads to stabilization of Myc whereas phosphorylation at T58 destabilizes Myc and facilitates dephosphorylation at S62. T58 is phosphorylated by glycogen synthase kinase-3 (GSK3).(82) The ubiquitin E3 ligase SCF^{FBW7}recognizes phosphor-T58, ubiquitylates Myc and targets it for proteasomal degradation.(83) Ras inhibits GSK3 through the PI3K pathway, resulting in stabilization of Myc. Second, Myc target genes involved in cellular proliferation that are co-regulated by the FOXO family of transcription factors. FOXO factors are phosphorylated by the Akt protein kinase, which is a downstream effector of active Ras, and these factors are then exported from the nucleus, releasing the repression of myc activated genes. In their non-phosphorylated state, FOXO factors directly bind to and repress many Myc target genes by inhibiting the formation of the preinitiation complex on these genes.(78) Third, novel transcription repression by Myc is regulated through the Ras activated PI3K pathway. In human cells, repression of the expression of the

potent anti-angiogenic protein thrombospondin-1 (Tsp-1) is a critical step in the acquisition of angiogenicity and tumorigenicity. One method of achieving TSP-1 repression is via a Ras- PI3K-mediated activation of Myc. Once sufficient levels of PI3K activity are achieved they act through an unidentified signal transduction cascade, which leads through a Rho GEF to Rho and ROCK activation of Myc. Myc is phosphorylated at S71 by an unidentified presumed Rho-dependent kinase.(84) This may be achieved directly by ROCK, or alternatively, ROCK may act via a cascade of intermediary kinases to modify the Myc protein.(84) The mechanism by which Myc represses Tsp-1 transcription remains unclear. However, transcriptional repression by Myc has been demonstrated to occur in $p21^{CIP}$and p15INK4B by binding to an initiator motif.

Transformation by Myc is also limited by its ability to induce apoptosis and cellular senescence. Myc induces apoptosis through at least two distinct pathways. First, it induces the expression of $p19^{ARF}$, an inhibitor of MDM2 E3 ligase, which leads to the stabilization of p53.(85) Deletion of $p19^{ARF}$ or *p53* allows Myc to immortalize primary cells. However, $p19^{ARF}$ gene does not seem to be a direct target of Myc and the mechanism through which Myc activates $p19^{ARF}$expression is unclear. Second, Myc overexpression induces expression and/or activation of pro-apoptotic Bcl-2 proteins independent of p53 activation. Either Bax or Bak are required for activation of apoptosis through the mitochondria. Evidence suggests that Myc functionally activates each of these

proteins as well as transcriptionally activates Bax. c-Myc/Max heterodimers bind to canonical E-box elements located in the Bax promoter region, where previous analysis of Bax regulatory region mutants suggests both Myc-dependent activation as well as relief of repression through distinct E-box elements. These results suggest that c-Myc is involved in transcriptional activation of the *Bax* gene. Myc appears to have an important function in Bax activation at several levels. Recently, it was shown that, in human lung adenocarcinomas following administration of cytotoxic drugs, c-Myc cooperates with caspase 2 in activating Bax. Caspase-2 participates in the integration of Bax into the outer mitochondrial membranes, whereas c-Myc functions in oligermerization of Bax after its integration; however, it is not clear if c-Myc is directly participating in Bax activation or if c-Myc induces/activates other factors that are directly participating in Bax oligomerization. (86) Furthermore, repression of the anti-apoptotic BCL-X_L and BCL2 proteins by Myc contributes to the release of cytochrome *c*.(87) Additional data show that enhanced expression of BCL-X_L greatly facilitates Myc-induced tumorigenesis *in vivo*.(88) Finally, deregulated expression of Myc might also induce apoptosis through the induction of DNA damage as deregulated expression of Myc can override DNA-damage-induced cell-cycle arrest and the restoration of checkpoint function suppresses Myc induced apoptosis in some cell types.(89) “Myc is directly recruited to the p21(Cip1) promoter by the DNA-binding protein Miz-1. This interaction blocks p21(Cip1) induction by p53 and other activators. As a result Myc switches, from cytostatic to apoptotic, the p53-

dependent response of colon cancer cells to DNA damage. Myc does not modify the ability of p53 to bind to the p21(Cip1) or PUMA promoters, but selectively inhibits bound p53 from activating p21(Cip1) transcription. By inhibiting p21(Cip1) expression Myc favors the initiation of apoptosis, thereby influencing the outcome of a p53 response in favor of cell death." Interestingly, de-activation of Myc in established Myc-induced transgenic tumors triggers proliferative arrest and re-differentiation of tumor cells, and collapse of the tumor microenvironment and vasculature, usually resulting in rapid tumor regression.(90-92)

The Role of c-Myc in NSCLC

Overexpression and amplification of Myc family members have been reported in lung cancer cells and tumor samples.(93) Although *Myc* gene amplification is a rare event that may be induced by prior chemotherapy exposure, overexpression of Myc RNA has been reported to occur in approximately 5-10% of NSCLC and is exclusively associated with *c-myc*.(93, 94) A tentative association between the presence of the *L-myc* polymorphism and risk of lung cancer from smoking was documented in a cohort of Japanese patients with multiple different lung cancer types.(95)

Soucek and colleagues (90) have developed a mouse model that will allow evaluation of both therapeutic impact and side effects of Myc inhibition *in vivo*. The endogenous Myc function may be systemically and reversibly inhibited in

tissues of adult animals through inducible expression of the dominant interfering Myc bHLHZip dimerization domain mutant Omomyc. The Omomyc construct facilitates homodimerization with all three oncogenic Myc proteins (c-Myc, N-Myc and L-Myc) but will not allow interactions with Mad proteins or access of Myc to Max. Additionally, Myc-Omomyc heterodimers cannot bind Myc-Max E-box consensus recognition elements, therefore efficiently blocking Myc-dependent transcriptional activation. Onocomyc expression reverses Myc-induced transformation *in vitro* and Myc-driven tumorigenisis *in vivo*.(96) The Omomyc construct was used in a well established lung cancer mouse model in which tumorigenesis is initiated by onocogenic activation of endogenous *Kras*.(97, 98) Soucek and colleagues' preclinical mouse model showed that Myc inhibition triggers rapid regression of incipient and established lung tumors, defining an unexpected role for endogenous Myc function in the maintenance of Ras-dependent tumors *in vivo.* Systemic Myc inhibition also exerted profound effects of normal regenerating tissues such as the skin, testis and gastrointestinal tract. However, the effects were well tolerated and reversible.(90) Their data demonstrate the feasibility of targeting Myc as an effective, efficient tumor-specific cancer therapy.

Wnt Signaling Overview

The *Wnt* gene family encodes multi-functional ~40 kDa signaling glycoproteins that are involved in the regulation of a wide variety of normal and pathological processes, including embryogenesis, differentiation and tumorigenesis.(99, 100) The secreted Wnt proteins have been shown to activate signal transduction pathways and trigger changes in gene expression, cell behavior, adhesion and polarity via autocrine or paracrine routes. Wnt proteins comprise a family of 19 highly conserved cysteine enriched proteins. It was found that cysteine palmitoylation is essential for the function of Wnt proteins.(101) In addition, Porcupine, a protein with homology to acyltransferases in the ER, is required for secretion of WNTs.(102). Furthermore, extracellular heparin sulfate may also play a role in the intracellular transport or stabilization of Wnt proteins.(103)

Wnt Receptors, Antagonists, Canonical and Noncanonical Signaling Pathways

Wnt signaling activates at least three independent signaling pathways.(104) The best-understood Wnt signaling pathway is the canonical Wnt/β-catenin pathway which results in changes of cell fate and/or cell transformation.(105) In this pathway, Wnt ligands bind to two distinct families of cell surface receptors, the Frizzled (Fzd) receptor family and the LDL receptor-related protein (LRP) family. Fzd proteins bind Wnts through and extracellular N-terminal cysteine-rich domain, and most Wnt proteins can bind to multiple Fzd receptors and vice

versa.(106) Wnt binding to Fzd and LRP results in activation of target genes through the stabilization of β-catenin in the nucleus.(107) Wnt binding to Fzd receoptors can also signal by activating calmodulin kinase II and protein kinase C (Wnt/Ca^{2+} pathway), which involves an increase in intracellular Ca^{2+}, or the Jun N-terminal kinase (JNK) also known as the planar cell polarity pathway, This controls cytoskeletal rearrangements required for cell polarity.(108) Finally, the general structure of Fzd receptor is a seven transmembrane G protein-coupled receptor, and it appears that Fzd proteins may use heterotrimeric G proteins to transduce some Wnt signals.(109) A single-pass transmembrane molecule of the LRP family, identified as LRP5 or 6, is also required for the signaling.(110) Although not validated, it is thought that expression of both Fzd and LRP receptors on the cell surface is required to initiate the Wnt signal. (111)

The active canonical Wnt signaling pathway begins when Wnt ligands bind the Fzd/LRP corecepter. The C-terminus of Fzd binds Dishevelled (Dlv) a cytosolic protein that functions upstream of β-catenin and the kinase GSK3β. Axin, a scaffold protein translocates to the membrane and interacts with the intracellular tail of LRP or with Fzd and Dlv. This removes axin from an APC-GSK-3 associated protein destruction complex preventing β-catenin phosphorylation. In the absence of this complex, β-catenin levels increase in the cytoplasm, allowing its translocation to the nucleus, where it converts T-cell and lymphoid enhancer factors (TCF/LEF) into transcriptional activators of Wnt target genes, such as c-

Myc, MMP7, cyclin D1 and WISP.(111) In the absence of Wnt ligands or in the presence of Wnt inhibitors, the APC-Axin and GSK-3 complex binds β-catenin which phosphorylates the protein, leading to subsequent ubiquination, and degradation by proteasomes. In the noncanonical pathways, signaling is conducted independently of β-catenin, although similar ligands may be upstream of the Fzd receptors. The activity of different Wnt proteins is dependent on the receptor context, which makes strict classification of Wnts as canonical or noncanonical challenging.

Secreted inhibitory proteins can sequester Wnt ligands from their receptors. These include Frizzled-related proteins (sFRPs) and the sFRP members, WIF-1 and Cerberus bind directly to Wnt molecules and alters the ability of the Wnt ligand to bind to the Wnd/Fzd receptor complex.(112, 113) Another class of extracellular Wnt inhibitors is represented by the Dickkopf (Dkk) family, which antagonizes Wnt canonical signaling pathway through binding to the LRP5/6 component of the Wnt/Fzd receptor complex, which prevents the coreceptor from localizing in the cell membrane, resulting in the inability of Wnt to bind the Fzd receptor.(114) The sFRP class of antagonists can inhibit both the canonical and the noncanonical Wnt pathways, whereas the class of Dkk antagonists specifically inhibits the canonical pathway.(115) A tyrosine kinase receptor, Derailed has been shown to bind Wnts.(116) Derailed binds Wnts through its extracellular Wnt inhibitory factor (WIF) domain.

The Role of Wnt in NSCLC

Wnt signaling is intimately involved in tumorigenesis and cancer progression.(117, 118) Numerous reports have demonstrated aberrant Wnt activity in human cancers whereby the activation can be caused by mutations and/or deregulation of many different Wnt signaling components. Mutations in Wnt pathway components are rarely found in lung cancer. Instead, nongenetic events appear to be the major cause of aberrant activation of Wnt signaling in lung cancer which include ligand overexpression and methylation of inhibitory proteins.(111) Overexpression of Wnt1 has been demonstrated in NSCLC cell lines and primary cancer tissues.(119) Blockade of Wnt1 signaling induces apoptosis in vitro and suppresses tumor growth *in vivo*, suggesting that Wnt1 signaling is a key inhibitor of apoptosis in epithelial cancers.(120) Wnt1 expression was evaluated in 216 patients with up to stage IIIB NSCLC and was correlated to the expression of β-catenin and Wnt targets, including c-Myc, Cyclin D1, VEGF-A, MMP-7, Ki-67 and IMD.(121) The ratio of tumors with increased β-catenin expression was significantly higher in Wnt1 positive tumors than in Wnt1-negative tumors. The Wnt1 expression significantly correlated with the expression of c-Myc, Cyclin D1, VEGF-A, MMP-7, Ki-67 index and the IMD. Wnt1 status was a significant prognostic factor for NSCLC patients. Taken together the results of this study suggests that Wnt1 overexpression is associated with the expression of tumor-associated Wnt-targets, tumor proliferation, angiogenesis and a poor prognosis in NSCLCs.(121) The human Wnt2 gene, is highly

expressed in fetal lung.(122) Recently, the overexpression of Wnt2 in NSCLC has been demonstrated. A role for Wnt7a in lung cancer has been suggested. Wnt7a expression has been reported to be downregulated in most lung cancer and tumor samples.(123) It has been hypothesized that Wnt7a upregulates E-cadherin expression in lung cancer cells.(124) Wnt7a typically functions through the canonical pathway in lung cancer.(123) Further studies from the same group indicate that combined expression of Wnt7a and Fzd-9 in NSCLC cell lines inhibits transformed growth and that this antitumorigenic effect of Wnt7a and Fzd-9 is mediated through ERK5-dependent activation of peroxisome proliferator-activated receptor gamma. This pathway seems to be required for the maintenance of normal epithelia differentiation and represents noncanonical signaling by the Wnt pathway. Wnt5a's role in lung carcinogenesis has been documented. In a recent study of 123 patients with NSCLC, Huang *et al*. (125) found that Wnt5a expression in SCC was significantly higher than that in adenocarcinoma. Furthermore, Wnt5a forced overexpression produced more aggressive tumors, especially in SCC cells.

The role of Wnt antagonists in lung carcinogenesis has been identified. Downregulation of WIF-1 in has been established to occur in lung cancer.(126) Additionally, frequent hypermethylation of CpG islands in the functional *WIF-1* promoter region correlated with its transcriptional silencing in lung cancer cell lines.(127) WIF-1 expression was found to be downregulated in 83% of freshly

resected human lung cancer specimens. Therefore, it appears as though methylation silencing of *WIF-1* may be an important mechanism for aberrant activation of Wnt signaling in lung cancer. It was also found that sFRP gene promoter was hypermethylated in more than 80% of mesothelioma primary tumors (128) and in approximately 55% of primary lung tumors. More recently, it was found that promoter hypermethylation of the APC, CDH1, sFRP1 and WIF-1 genes may be able to discriminate lung primary adenocarcinomas from colorectal metastasis to the lung.(129) DKK-3 has also been shown to be downregulated in 63% of freshly resected NSCLC tissues.(130) Furthermore, overexpression of DKK-3 can inhibit cell growth.(131) Similar to other Wnt inhibitors, DKK-3 has been shown to be silenced by promoter hypermethylation in a large proportion of lung cancers.(132)

As the Wnt signaling pathway has been shown to contribute to lung carcinogenesis with many of the contributing components sufficiently characterized, this suggests numerous avenues of attractive targets for potential therapeutic agents. Examples of potential approaches are inhibition of Wnt pathways by overexpression of DKK, WIF-1, Wnt-7A, sFRP or Axin.

Overview of Src Tyrosine Kinase Signaling

Aberrant Src activation has been implicated in the development of lung cancer. Src is a member of the Src family tyrosine kinases which consists of nine known members: Src, Yes, Fgr, Yrk, Fyn, Lyn, Hck, Lck and Blk. These proteins are nonreceptor tyrosine kinases that are located within the cytosol and transduce signals between cell surface proteins, other intracellular proteins and transcription factors.(133) Most Src family kinases are expressed primarily in cells of hematopoietic origin. C-Src, c-Yes and Fyn, however, can have particularly high levels of expression in epithelial cells.(134) Src family kinases are activated in response to cellular signals that promote proliferation, survival, motility, and invasiveness, including activation of cytokine receptors, receptor protein tyrosine kinases, G-protein coupled receptors, and integrins.(134) All Src family kinases are comprised of an amino-terminal membrane localization signal, also known as the Src homology 4 or SH4 domain, a poorly conserved unique domain, SH3 domain, SH2 domain, tyrosine kinase domain, and a regulatory sequence.(135) In normal circumstances, Src is maintained in an inactive or closed confirmation state via phosphorylation of a conserved tyrosine residue (Y527 in avian c-Src), near the C-terminus of the protein which interacts with the SH2 domain.(136) Working cooperatively with the SH2/tail interaction is an association between the SH3 domain and the short stretch of amino acids linking the SH2 and kinase domains.(137) Together, these intramolecular interactions

not only limit accessibility of the kinase domain but also the binding surfaces of the SH3 and SH2 domain, thus further limiting the potential for these proteins to participate in cellular signaling. Dephosphorylation of this amino acid changes the confirmation of Src and results in the autophosphorylation of another tyrosine residue with the activation loop of the protein, resulting in a fully activated protein that is capable of interacting with other proteins. A few of the pathways Src is involved in included PDGFR, G-CSFR, ErbB, Met, IL6R, integrins, RANK and VEGFR.(138)

The Role of Src in NSCLC

Increased expression of Src has been reported in 60-80% of adenocarcinomas and bronchioloalveolar cancers and in 50% of SCCs.(139) Evaluation of 60 cancer cell lines, revealed that the NSCLC lines had the highest median Src activity.(140) Two of the most well-described Src-activated pathways involves signal transducer and activator of transcription (STAT)-3 and focal adhesion kinase (FAK) both of which are involved in tumor survival. STATs are transcription factors that mediate expression of genes involved in cell cycle progression and resistance to apoptosis. FAK is a tyrosine kinase involved in integrin signaling, and elevated FAK levels have been associated with increased cell motility, invasion and proliferation in cancer cells.(141) In NSCLC, Src-mediated constitutive activation of STAT-3 has been found and stimulation of STAT-3 by EGF, IL6 and HGF/SF all require Src activity.(142) The VEGF

pathway also requires Src activation via STAT-3 in response to hypoxia in human lung adenocarcinoma cells, thus increasing blood supply to the tumor.(143) With regards to EGFR, Src is able to hyperactivate EGFR by phosphorylating multiple sites, including tyrosine residue Y845 to promote oncogenesis via STAT-5b, independent of the MAPK pathway.(144, 145) Inhibition of Src can reverse the transformed phenotype of cells overexpressing EGFR and HER-2.(146)

As Src overexpression appears to have a role in tumor development and metastases in NSCLC, and Src mediates multiple oncogenic pathways, compounds that can inhibit Src signaling are of particular interest. Recently, the use of dasatinib, a potent, multi-targeted, oral inhibitor of Src family kinases, Bcr-Abl, Kit, platelet-derived growth factor and Eph receptors, on EGFR-dependent NSCLC cell lines resulted in apoptosis.(147) Data from preclinical studies suggets pharmacodynamic and pharmacokinetic parameters which implicate Src inhibition as a viable therapeutic target for treatment of NSCLC.

CONCLUSION

As with many cancers, NSCLC is a very heterogeneous disease that can commence with no single common molecular event that initiates tumorigenesis. This brief review on oncogenes provides an idea of the formidable obstacles to developing effective treatment. For example, the diversity, redundancy and

degree of tumor cell signaling complexity involving not only genetic but also epigenetic, and microenvironmental influences effectively demonstrates multiple mechanisms that participate in NSCLC pathogenesis. With the apparent synergy of oncogenes, for example Src with other tyrosine kinases such as EGFR, as an important mechanism for tumor growth and evidence that specific molecular aberrations may only be present in a subset of tumors, it seems reasonable the therapy must target multiple pathways to be effective. The current first-line treatments for patients with NSCLC include chemotherapy with a platinum agent in combination with a taxane or other cytotoxic agent such as gemcitabine or vinorelbine. Many patients with advanced NSCLC have only a partial response to initial chemotherapy, and even those that do respond will subsequently progress. Nonetheless, the prognosis for patients with locally advanced or metastatic NSCLC remains poor. Therefore, specific targeting of aberrant signaling or metabolic processes involved in tumorigenesis has become the new focus of NSCLC therapy. Most importantly, the continued development of biomarkers with molecular profiling techniques that accurately predict lung cancer profiles, clinical responses and outcomes of treatment will help revolutionize clinical approaches and refine personalized patient-centric therapy for an otherwise very challenging cancer with an extremely poor prognosis.

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CHAPTER 2

INHIBITION OF EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALING REDUCES HYPERCALCEMIA INDUCED BY HUMAN LUNG SQUAMOUS-CELL CARCINOMA IN ATHYMIC MICE

2.1 ABSTRACT

The purpose of this study was to evaluate the role of the epidermal growth factor receptor (EGFR) in parathyroid hormone-related protein (PTHrP) expression and humoral hypercalcemia of malignancy (HHM), using two different human squamous-cell carcinoma (SCC) xenograft models. A randomized controlled study in which nude mice with RWGT2 and HARA xenografts received either placebo or gefitinib 200 $mgkg^{-1}$ for 3 days after developing HHM. Effectiveness of therapy was evaluated by measuring plasma calcium and PTHrP, urine cAMP/creatinine ratios, and tumor volumes. The study end point was at 78 hours

The lung SCC lines, RWGT2 and HARA, expressed high levels of PTHrP mRNA as well as abundant EGFR protein, but very little erbB2 or erbB3. Both lines expressed high transcript levels for the EGFR ligand, amphiregulin (AREG), as well as, substantially lower levels of transforming growth factor-alpha (TGF-α), and heparin binding-epidermal growth factor (HB-EGF) mRNA. Parathyroid hormone-related protein gene expression in both lines was reduced 40-80% after treatment with 1 μM of EGFR tyrosine kinase inhibitor PD153035 and precipitating antibodies to AREG. Gefitinib treatment of hypercalcemic mice with RWGT2 and HARA xenografts resulted in a significant reduction of plasma total calcium concentrations by 78 hours. Autocrine AREG stimulated the EGFR and increased PTHrP gene expression in the RWGT2 and HARA lung SCC lines. Inhibition of the EGFR pathway in 2 human SCC models of HHM by an anilinoquinazoline demonstrated that the EGFR tyrosine kinase is a potential target for anti-hypercalcemic therapy.

2.2 INTRODUCTION

Humoral hypercalcemia of malignancy is a paraneoplastic disorder commonly associated with increased synthesis and secretion of parathyroid hormone-related protein (PTHrP) (1). Studies from industrialized nations in the 1970s and 1980s, as well as more recent studies from other countries, report that patients with squamous-cell carcinoma (SCC) of the lung have the highest frequency of

humoral hypercalcemia of malignancy (HHM; ranging from 27 to 66%) as compared to other tumor types (2-4). The precise mechanisms that activate high levels of PTHrP gene expression in tumors that are associated with HHM have yet to be identified. The diagnosis of HHM has a poor prognosis with a survival of less than 3 months (5). Hypercalcemia has clinical effects on multiple organs such as fatigue, psychosis, confusion, vomiting, renal failure, constipation and cardiac arrest, which become life threatening as the syndrome progresses.

The ErbB pathway genes, especially the epidermal growth factor receptor (EGFR), are frequently overexpressed in SCC of the lung (6). Lung SCCs produce a wide range of cytokines and growth factors, including those of the epidermal growth factor (EGF) family, such as transforming growth factor-α (TGF-α) and amphiregulin (AREG) (7, 8). Activation of EGFR cytoplasmic receptor tyrosine kinases regulate essential cellular functions including proliferation, survival, migration, and differentiation, and play a central role in the genesis and progression of solid tumors (9). The prominent role of EGFR signaling in many tumor types has prompted the development of pharmacological inhibitors such as the anilinoquinazolines (gefitinib and erlotinib), which disrupt EGFR kinase activity by binding the ATP pocket within the catalytic kinase domain (10). Gefitinib induced substantial clinical responses and reduced tumor burden in ~10% of patients with chemotherapy–refractory non-small cell lung cancers; however, the majority of these patients had EGFR mutations that

constitutively activated the anti-apoptotic protein AKT (11-14). Also, a phase III survival trial failed to verify the clinical benefit of gefitinib treatment for non-small cell lung cancer patients. In contrast, a phase III trial evaluating erlotinib efficacy revealed a significant overall improved survival rate for all non-small cell lung cancer patients (15). In the future, EGFR-targeted therapeutics may be routinely used in the treatment of non-small cell lung cancers, including SCCs.

Parathyroid hormone-related protein is a gene product regulated by EGFR that influences the pathogenesis of lung SCCs. Previously, we have demonstrated that autocrine stimulation of the EGFR pathway is a major activator of PTHrP gene expression in keratinocytes, and disruption of the EGFR in keratinocytes with the EGFR-tyrosine kinase inhibitor (TKI), PD153035 (PD), reduced PTHrP mRNA up to 80% (16). The role of EGFR signaling in the stimulation of PTHrP expression and subsequent development of HHM in patients with SCC of the lung is unknown. Extensive work on several human cancer lines suggests that activation of the Ras-mitogen-activated protein kinase (Ras-MAPK) pathway provides a general mechanism for activation of PTHrP gene expression (17-19). The Ras-MAPK pathway is regarded as the prototypic second messenger cascade downstream of EGFR (20).

We hypothesized that EGFR-induced Ras-MAPK signaling accounts for high levels of PTHrP expression, and contributes to HHM caused by lung SCC and the administration of EGFR receptor tyrosine TKIs would decrease total plasma calcium concentrations.

In this study we report the expression of the ErbB family of receptors and the endogenous production of EGFR ligand mRNA by two human lung SCC cell lines, RWGT2 and HARA, that have been reported previously to produce hypercalcemia in athymic mice (21, 22). We present evidence that autocrine stimulation of the EGFR pathway is a major contributor to PTHrP gene expression in both lines grown *in vitro.* Next, we compared EGFR signaling and PTHrP production in hypercalcemic mice with HARA and RWGT2 xenografts. Finally, we demonstrated that administration of gefitinib reduced hypercalcemia and decreased PTHrP production in both xenograft models.

2.3 MATERIALS AND METHODS

Cell Lines, Kinase Inhibitors, and Antibodies

RWGT2 cells were derived from a pulmonary bone metastasis of a hypercalcemic patient and obtained from G Mundy and T Guise (University of Texas Health Science Center, San Antonio, TX, USA). Cells were maintained in high glucose Dulbecco's modified Eagle's medium with GlutaMax™ (Gibco,

Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum, and Normocin™ 100 μgml^{-1} (InvivoGen, San Diego, CA, USA). The HARA cell line was obtained from Dr Haruo Iguchi (Shikoku Cancer Center, Matsuyama, Japan). The original patient had an increased PTHrP concentration and hypercalcemia, but no apparent bone metastases. HARA cells were maintained as described for the RWGT2 cells. Cells were passaged at 90% confluence. *In vitro* experiments were performed when both RWGT2 cells and HARA were ~90% confluent. For the *in vitro* kinase inhibitor assays, the TKIs, PD (Calbiochem, San Diego, CA) and gefitinib (gift from AstraZeneca, London, UK), as well as the MEK inhibitor PD98059 (Calbiochem) were dissolved in dimethylsulfoxide (DMSO), stored at -20°C at a stock concentration of 2 mM, and used at a final concentration of 1 μM. The drugs were added to cells for 6 h and control cells were treated with media containing 0.01% DMSO (vehicle). For the ligand and EGFR blocking studies, the anti-human goat AREG antibody (R&D Systems, Minneapolis, MN, USA) was resuspended in sterile phosphate-buffered saline (PBS) to yield a final concentration of 100 μgml^{-1}. Both the anti-human EGFR blocking monoclonal antibody 225 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-AREG antibody were used at 10 μgml^{-1} and cells were treated overnight. For the animal studies, gefitinib was purchased from a national corporate pharmacy.

For immunoblot analysis and/or immunoprecipitations, the following antibodies were used: Phospho-EGF receptor-Tyr1068 catalog no. 2234, EGFR no. 2232, erbB2 no. 2242, phospho-p44/42 mitogen-activated protein kinase (MAPK; Thr202/Tyr204) no. 9101, p44/42 MAPK no. 9102 (Cell Signaling, Beverly, MA, USA), 4G10 anti-phosphotyrosine no. 06-427 (Upstate USA, Charlottesville, VA, USA), erbB3 (sc-285), erbB4 (sc-283) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-*β*-actin (Sigma, St. Louis, MO, USA).

Xenografts in nude (*Foxn1*^{nu}) mice, biochemical analysis, and gefitinib administration

All experimental procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. After baseline blood and urine samples were collected, mice were preconditioned to avidly consume peanut butter by several 0.25 g offerings before tumor development. Six to eight-week-old weight-matched (24-26 g) male *Foxn1*^{nu} nude mice (Harlan, Indianapolis, IN, USA) were injected subcutaneously over the dorsal scapular area with 1x10^6 RWGT2 or HARA cells. Subcutaneous tumors were observable approximately seven days after injection.

Animals were monitored and weighed every other day and when any of the following conditions occurred: (1) tumors were greater than ≥ 1 cm^3; (2) cancer-induced loss of muscle mass; or (3) body weight decreased by ≥ 5%, total calcium concentrations were measured to determine if the mice were hypercalcemic. Blood collection was performed via puncture of the mandibular facial artery or vein with a twenty-two gauge needle and collected into a microtainer tube with lithium heparin (Becton, Dickinson and Company, NJ, USA). Plasma total calcium concentrations were measured in 10 *µ*l of heparinized plasma using the Vitros DT-60 II clinical chemistry analyzer (Johnson & Johnson, Cornilla, GA, USA). Animals were considered hypercalcemic when total plasma calcium concentrations were ≥ 12 $mgdl^{-1}$.

Once hypercalcemia was confirmed, mice were randomly allocated to treatment with: (a) 200 $mgkg^{-1}$ of gefitinib (tablet formulation; AstraZeneca, London, UK) in peanut butter daily for 3 consecutive days (0, 24, 48 h) or; (b) 0.25 g peanut butter for 3 consecutive days (0, 24, 48 h). They were randomized into two groups such that there was no difference in the mean plasma total calcium values between the two at the time treatment was initiated. During treatment, mice were placed in a cage devoid of mouse chow and bedding and observed until the mixture of drug and peanut butter or peanut butter alone had been completely consumed, approximately 2 min. Blood (75 *µ*l) was collected to measure plasma total calcium concentrations at 0, 6, 24, 52, and 78 h from

treated and control mice. As additional controls, 2 groups of nontumor-bearing mice were treated as above in (a) and (b). Urine was collected following spontaneous voiding on wax paper. To calculate cyclic AMP (cAMP):creatinine ratios, urine samples (100 μl) were collected from mice before xenografting for baseline analysis and at pretreatment and 6, 24, 52, and 78 h after treatment with gefitinib. Urine was acidified by adding 10 μl of 6 N HCl and stored at -80°C until analysis. Tumor diameters were serially measured with digital calipers, and tumor volumes were calculated by the formula: volume = width^2 x length/2. At the 78 h time point, mice were euthanized with 70% compressed CO_2 gas and tumor tissue was collected, weighed, snap frozen in liquid nitrogen, and stored at -80°C for protein analysis.

Urine cAMP and creatinine assays

Urine samples were diluted 1:2000 in 0.1 N HCl and cAMP levels were measured using a commercial enzyme immunosorbent assay kit (Assay Designs, Ann Arbor, MI, USA). Urine cAMP was normalized to creatinine concentrations, which was measured using a commercial microplate Jaffè reaction kit (Quanti Chrom Creatinine Assay, BioAssay Systems, Hayward, CA, USA).

Measurements of PTHrP concentrations

To determine the effects of gefitinib on plasma PTHrP concentrations, blood samples were collected via mandibular puncture into microtainer tubes with

lithium heparin (Becton, Dickinson and Company) on ice. A 200 *μ*l sample of plasma was obtained from individual mice for each time point. Plasma PTHrP was measured at baseline (i.e., before xenografting) (*n*=17 RWGT2 and *n*=10 HARA mice) and 6 and 78 h after the initial treatment with gefitinib or placebo.

Biologically active plasma PTHrP (1-86) was measured using a commercially available two-site immunoradiometric assay (Diagnostic Systems Laboratories, Inc, Webster, Texas, USA). The limit for detection of the assay was 0.3 pM.

RNA isolation and quantitative real-time reverse transcriptase PCR

Total RNA was prepared using the Mini RNA Isolation II kit (Zymo, Orange, CA, USA) according to the manufacturer's instructions. Reverse transcription and quantitative real-time PCR (QRT-PCR) were performed as detailed by Cho *et al* (163) for all transcripts of PTHrP, AREG, TGF-α, and HB-EGF. Data were normalized by use of the ratio of the target cDNA concentration to glyceraldehyde-3-phosphate (GAPDH) to correct for differences in RNA quantity between samples. The results represented in the figures were derived from experiments where the cDNAs were prepared at the same time and then analyzed by QRT-PCR performed on one plate.

Immunoblot analysis

Immunoblotting for erbB and extracellular signal-regulated kinase (ERK) proteins was performed as in Gilmore and Riese (23) and Foley *et al* (24). For measurement of phosphorylation of MAPK, RWGT2 cells were seeded at a density of 5 x 10^5 cells/100-mm dish 24 h before treatment with PD.

After treatment, cells were washed with ice-cold PBS, lysed with the protein extraction buffer as above for the tumor lysates, protein concentration determined, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to immunoblot analysis as described previously.

Transient transfection

Transient transfection was performed using Lipofectamine and Plus reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfections were normalized to total protein. Otherwise, details are similar to Cho *et al*, (16). Total protein was measured with the BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). Results were reported as relative luciferase units (RLUs), which represent normalized luciferase values for the Ras and Raf co-transfections divided by the normalized luciferase value of the empty vector co-transfections.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining

Apoptosis was detected utilizing the *In situ* Cell Death Fluorescein Detection Kit (Roche Diagnostics, Mannheim, Germany). Briefly, after deparaffinization, rehydration and washing in 1 x PBS, sections were treated with deoxynucleotidyltransferase (TdT) enzyme mixture, covered and incubated in a humidified slide chamber for 60 min at 37°C in the dark. After fixation, positive control slides were permeabilized with DNase I for 10 min at 20°C to induce DNA strand breaks, before labeling procedures. The negative control slides were incubated with label solution without terminal transferase. Finally, the slides were rinsed three times in PBS and analyzed under a fluorescence microscope using an excitation wavelength of 488 nm.

Statistics

Results were expressed as the mean ± s.e.m. of triplicate or quadruplicate measures. Unless specifically indicated statistical comparisons were based on two-tailed analysis of the Student's *t*-test. A probability value of $P<0.05$ was considered to be significant. Analysis of variances (ANOVAs) with repeated measures were used to analyze the time differences between groups. Plots with the variable time (baseline, pretreatment, 6, 24, 52, and 78 h) in the *x* axis are reported to show the trends over time.

2.4 RESULTS

Stimulation of the EGFR activates PTHrP gene expression in two hypercalcemia inducing SCC lines

The RWGT2 cell line was derived from a human lung SCC bone metastasis and the HARA cell line from a human primary lung SCC (21, 22). At greater than 90% confluence, RWGT2 cells produce very high levels of all transcripts of PTHrP (close to 10^6 copies per 2 μg of cDNA) as detected by QRT-PCR (Figure 1A). In comparison, HARA cells at 90% confluence produced a PTHrP/GADPH mRNA ratio that was less than the RWGT2 cells (Figure 2.1A). As shown in Figure 2.1B, both cell lines expressed high levels of EGFR and moderate levels of the ErbB2 receptor as detected by Western blots of concanavalin-A (con A)-precipitated proteins. HARA cells expressed ErbB3 protein, while ErbB4 immunoreactivity was barely detectable in either cell line (Figure 2.1B). Both cell lines were evaluated for mRNA expression of the EGFR ligands; AREG, HB-EGF, and TGF-α by QRT-PCR. The AREG/GAPDH mRNA ratios were two orders greater than HB-EGF and TGF-α in both lines (Figure 2.1C,D). To determine whether the EGFR was phosphorylated and active in the cells under normal basal culture conditions, Western blots of conA-precipitated protein extracts were probed with a general phosphotyrosine-specific antibody, 4G10. Extracts from the RWGT2 cells contained a phosphotyrosine immunoreactive band that increased in intensity in the control EGF-stimulated RWGT2 cells (left panel, Figure 2.1E). A

small amount of phosphotyrosine immunoreactive EGFR was detected in HARA cells. Despite expressing similar levels of EGFR and EGFR ligand RNA, the HARA cells expressed less PTHrP mRNA *in vitro* than the RWGT2 cells.

The role of EGFR activation and autocrine ligand production on PTHrP gene expression was further investigated using EGFR TKIs, anti-EGFR and anti-AREG antibodies in both cell lines. Additional studies evaluated the effect of EGFR ligand treatment on PTHrP gene expression by QRT-PCR. Six-hour treatments with 1 μM of the EGFR TKI, PD, decreased the ratio of PTHrP/GAPDH mRNA by ~80% in RWGT2 cells grown in basal medium (Figure 2.2A). The addition of EGFR ligand blocking and AREG neutralizing antibodies to basal culture medium reduced PTHrP/GAPDH mRNA levels 50 - 65% in RWGT2, respectively (Figure 2.2A). Neutralizing HB-EGF and TGF-α antibodies (applied at concentrations up to 10-fold greater than the AREG antibodies) failed to reduce PTHrP mRNA levels in the RWGT2 line (data not shown). Treatment of HARA cultures with PD and AREG neutralizing antibodies reduced PTHrP/GAPDH mRNA levels ~60 and 40%, respectively (Figure 2.2B). Both cell lines were treated with the EGFR ligands, EGF (100 $ngmL^{-1}$) or AREG (1 $mgmL^{-1}$) for 2, 4 and 6 h and evaluated for PTHrP mRNA levels. Addition of exogenous ligands increased PTHrP transcript levels up to 10-fold by 6 h (Figure 2.2C-D). These data support the concept that the EGFR is a potent regulator of PTHrP gene expression in RWGT2 and HARA cell lines.

The MAPK Signaling Pathway Activates PTHrP Gene Expression Downstream of the EGFR

Several of the signaling pathways activated by EGFR converge on the MAPK cascade. Activation of the MAPK cascade was evaluated by examining the phosphorylation status of the downstream MAPK (ERK1/2) in basal culture conditions and after treatment with the EGFR TKI, PD. Six-hour treatments of both RWGT2 and HARA cells with 1 μM PD resulted in a reduction of phosphorylated ERK1/2 as measured by Western blot without a change in the total ERK protein levels (Figure 2.3A). In addition, treatment with the MEK inhibitor, PD98059, reduced basal PTHrP mRNA levels in both lines to similar levels found in the EGFR TKI PD-treated cells (Figure 2.3B). Furthermore, treatment with the MEK inhibitor, PD98059, blunted the EGF- and AREG-stimulated increases in PTHrP gene expression in both lines (Figure 2.3C,D). Finally, co-transfection was used to test whether a dominant-negative Ras or Raf construct could disrupt basal expression from a human PTHrP-P3 promoter-driven reporter gene. As shown in Figure 2.3E, the dominant-negative Raf repressed reporter gene activity 50-70% in RWGT2 and HARA cells, respectively; however, the Ras construct only repressed reporter gene activity in the RWGT2 line. These results demonstrated that the effect of EGFR on PTHrP gene expression was mediated, in part, by the MAPK pathway in both hypercalcemia-inducing lung SCC lines.

Comparison of RWGT2 and HARA xenograft models of hypercalcemia

The EGFR and MAPK pathway control PTHrP mRNA expression in both lines, however, there was a 10-fold greater level of PTHrP mRNA in the RWGT2 line as compared to HARA cells *in vitro*. To determine if both cell lines would be suitable for future studies with EGFR-targeted therapeutics, we compared the onset of hypercalcemia, plasma PTHrP concentrations, tumor PTHrP mRNA expression, and EGFR phosphorylation status in subcutaneous tumors of nude mice. The HARA line produced hypercalcemia in less time after tumor cell injection than the RWGT2 cells (50% of HARA mice were hypercalcemic in 40 days *vs* RWGT2 mice in which 50% of the mice were hypercalcemic in 60 days) (Figure 2.4A). Moreover, the volume of the hypercalcemia-inducing HARA tumors was ~50% less than hypercalcemia-inducing RWGT2 tumors (Figure 2.4B). Plasma PTHrP concentrations were ~80%-fold higher (22 *vs* 12 pM) in mice with HARA as compared to RWGT2 xenografts. The RWGT2 cells grown *in vitro* or as xenografts, produced similar PTHrP mRNA levels (Figure 2.4C). In contrast, PTHrP mRNA levels were increased nearly 100-fold in the HARA xenografts compared to cells grown *in vitro* (Figure 2.4D). Tumor extracts from both lines had detectable phosphorylated EGFR immunoreactivity, but RWGT2 extracts produced more intense bands (Figure 2.4E). Therefore, both cell lines produced tumors with activated EGFR, secreted high concentrations of PTHrP, and induced hypercalcemia.

Gefitinib Reduced Hypercalcemia in Mice with RWGT2 and HARA Xenografts

The anilinoquinazolines, gefitinib, is a highly specific EGFR TKI that can be used in rodents and humans at high doses with minimal toxicity (172). Treatment with gefitinib (1 μM) was as effective as PD (1 μM) in reducing PTHrP mRNA and EGFR/ERK phosphorylation in RWGT2 cells (data not shown). To assess the ability of an anilinoquinazoline to decrease plasma total calcium concentrations *in vivo,* RWGT2 tumor-bearing mice with HHM were administered gefitinib (200 $mgkg^{-1}$) every 24 h for three treatments and plasma total calcium concentration was measured at 6, 24, 52, and 78 h after the first treatment. The total calcium concentrations for normal 6 to 8-week-old male nude mice ranged from 8-10 $mgdL^{-1}$ (Figure 2.5A). Total calcium concentrations of gefitinib-treated mice were significantly reduced at all time points when compared to pretreatment values and untreated mice at comparable time points (Figure 2.5A). The plasma total calcium concentrations of all gefitinib-treated mice returned to baseline at 52 h (P = 0.001). Moreover, when normocalcemic nude mice without tumors were given gefitinib (200 $mgkg^{-1}$) on an identical dosage schedule, treatment did not reduce plasma calcium concentrations as compared to pretreatment and baseline values (Figure 2.5A).

Stimulation of the PTH receptor by PTHrP activates adenylyl cyclase in the renal tubules so we measured the effect of gefitinib administration on urinary

cAMP:creatinine ratios. Gefitinib-treated mice with RWGT2 and HHM had a significant reduction of urine cAMP levels at 6, 24, 52, and 78 h when compared to the control mice (P = 0.02) (Figure 2.5B).

The *in vivo* response of PTHrP gene expression to treatment with an EGFR TKI was evaluated by measuring plasma PTHrP concentrations and PTHrP mRNA from tumors in hypercalcemic mice with RWGT2 xenografts. Plasma samples were collected before xenografting, 6 h after the first gefitinib treatment (6 h) as well as at the end of the trial i.e., 30 h after the third and final drug administration (78 h). Comparison of gefitinib- and vehicle-treated groups revealed a trend toward decreased PTHrP concentrations as early as 6 h after the first treatment. There was reduced PTHrP concentrations (P = 0.01) in the gefitinib-treated mice at 78 h, but the PTHrP concentrations were not decreased to baseline values (Figure 2.5C). PTHrP/GAPDH mRNA ratios were reduced ~60% in tumors from treated mice at 78 h (Figure 2.5D). These data demonstrated that gefitinib reduced PTHrP gene expression and circulating PTHrP concentrations in the RWGT2 model of HHM.

Epidermal growth factor receptor phosphorylation in tumors was measured by Western blots with the EGFR phosphotyrosine-specific antibody to residue 1068. In RWGT2 tumors harvested at 78 h from treated mice, Y1068 phosphorylation was markedly reduced compared to the RWGT2 tumor lysates

from untreated mice (Figure. 2.6A). Phospho-ERK levels were decreased in the tumors from treated mice as well. Differences in the tumor volumes at the pretreatment time point were found between the treated and untreated groups; however, there was no difference in the tumor volume between these groups of mice at the 48 or 78 h time points (Figure 2.6B). No difference in final tumor weights between treated and untreated mice were found (data not shown). Degree of tumor cell apoptosis was measured with both TdT-mediated dUTP nick end labeling (TUNEL) and hematoxylin and eosin (H&E) at the 78 h time point in both the treated and untreated mice (Figure 2.6C). There were no differences in TUNEL labeling indexes or apoptosis (H&E) of tumor cells in the treated mice compared to the untreated mice (Figure 2.6D). The gefitinib-mediated reduction in PTHrP mRNA and plasma concentrations was not accompanied by a decrease in tumor volume or an increase in tumor cell apoptosis.

In June of 2005, use of gefitinib in humans was restricted to lung cancer patients for compassionate use only and could not be purchased for research purposes. At this time, we possessed a small amount of gefitinib sufficient to treat 5 animals. This material was used to treat 5 hypercalcemic HARA xenografts using a dose and time course identical to that we used with the RWGT2 cells. Total calcium concentrations were significantly reduced by 78 h (Figure 2.7A) (P = 0.042). Urinary cAMP creatinine ratios averages were not

significantly reduced at any time point (data not shown). At 78 h plasma PTHrP levels were significantly reduced compared to untreated mice, but were not reduced to the levels observed in treated mice with RWGT2 tumors (P = 0.047) (compare Figure 2.5C to Figure 2.7B). HARA tumor PTHrP mRNA levels were decreased by 90% in the gefitinib-treated animals (Figure 2.7C).

2.5 DISCUSSION

It has been known for over a decade that exogenous EGF stimulates PTHrP gene expression in a human lung SCC and keratinocyte lines (26, 27), but it has not been determined whether EGFR signaling contributes to cancer-mediated syndromes such as HHM. We have established that the EGFR ligand, AREG, stimulated EGFR signaling and PTHrP gene expression in two human lung SCC lines that induce hypercalcemia when grown *in vivo*. As with most lung SCCs (28-30), both HARA and RWGT2 cells expressed abundant EGFR *in vitro* and *in vivo*. Phosphotyrosine immunoblotting revealed that the receptor-associated kinase was active in both lines under basal conditions. Measurements of mRNA expression of the EGF family of ligands showed that both cell lines expressed high levels of AREG and modest amounts of HB-EGF and TGF-α. The coexpression of two or more EGF-like growth factors frequently occurs in human carcinomas (31-33). The ability of neutralizing antibodies to AREG but not to TGF-α or HB-EGF to reduce PTHrP transcripts in both lines demonstrated that

AREG was the primary activator of EGFR in cell lines. In both cell lines, exogenous EGFR ligands stimulated a four- to ninefold increase in PTHrP mRNA. Epidermal growth factor receptor signaling was necessary and sufficient for increased PTHrP gene expression in the HARA and RWGT2 cells. Whether signaling by the EGFR activates PTHrP gene expression by increasing gene transcription or message stability will be addressed by future studies.

The MAPK pathway mediated the EGFR-induced activation in the RWGT2 and HARA cells. Our findings are consistent with studies that reported calcium receptor (CaR) stimulation induced EGFR-MAPK signaling in transfected human embryonic kidney cells, human prostate cancer cells and hypercalcemia-inducing rat Leydig cells (H-500) (34-36). Blockade of the Ras-MAPK pathway with a dominant-negative Raf construct or a MEK inhibitor (PD98059) reduced PTHrP gene expression in H-500 cells (17). A ras inhibitor, B-1086, was able to slow the growth of the H-500 tumor and prevented the formation of hypercalcemia *in vivo* (17, 18). Highly specific and low toxicity inhibitors of the MAPK pathway will likely prove useful to reduce PTHrP gene expression in certain cancers.

Investigations on the control of PTHrP gene expression suggest that different signaling events were responsible for the induction of hypercalcemia in the RWGT2 and HARA models. Previous studies suggested that PTHrP gene expression *in vitro* predicted the ability of a cell line to induce hypercalcemia in

nude mice (37). The RWGT2 line was part of that study and consistent with those earlier findings we determined that the PTHrP mRNA levels *in vivo* were approximately equivalent to those in the tumor (Figure 2.4). In contrast, HARA tumors taken from hypercalcemic mice demonstrated a ~100-fold increase in PTHrP mRNA levels over cells *in vitro*, indicating that host factors activated PTHrP gene expression. Activation of PTHrP gene expression *in vivo* has been attributed to circulating factors as well as tumor microenvironment-derived cytokines or growth factors. Observations based on several xenograft HHM models suggest that high plasma calcium concentrations result in CaR-mediated activation of PTHrP production (38-40). In the bone microenvironment, TGF-*β* released from bone at sites of osteoclastic resorption activates PTHrP expression in metastatic cancer cells (41). Tumor-associated stroma in soft tissues also produces TGF-*β* (42, 43).

Therefore, hypercalcemia induced by the RWGT2 model appeals to be due to an intrinsic capacity of the cancer cells to produce PTHrP, whereas the HARA line induces the syndrome as a result of extrinsic stimulation of tumor cell PTHrP gene expression.

Epidermal growth factor receptor signaling is likely to play different roles in the control of PTHrP gene expression and generation of hypercalcemia by the two cell lines. RWGT2 cells *in vitro* or in tumors exhibited relatively high levels of

EGFR phosphorylation (Figures 1,4). Consistent with a central role of the EGFR in control of PTHrP gene expression in RWGT2 cells, EGFR TKIs substantially reduced PTHrP *in vitro* as well as plasma protein levels, and this was accompanied by a rapid decline in serum calcium levels (Figures 2 and 5). In contrast, EGFR phosphorylation was barely detectable in HARA tumors, suggesting that the receptor was not active to the extent observed in the RWGT2 line. The best understood mechanism of EGFR activation involves the autocrine/paracrine release of the ectodomain of integral membrane EGFR ligand proteins (9). The shedding of the ectodomain involves activation of matrix metalloproteinases and extracellular release of EGFR ligands (44). G-protein coupled receptors such as the CaR, are able to transactivate EGFR through induction of the membrane-associated matrix metalloproteinases such as ADAM-17 (45). We have recently cloned and sequenced the CaR receptor in both the HARA and RWGT2 cells lines (data not shown), but receptor characteristics typical of neoplastic cells such as overexpression, constitutive activation and the presence of activating mutations have not been evaluated. While it is possible that signals from the CaR or other G-protein receptors might result in high levels of EGFR activity in the RWGT2 line, the lack of robust EGFR phosphorylation in the HARA tumors suggested transactivation did not account for increased PTHrP gene expression in this model. This does not eliminate the possibility that second messenger pathways directly coupled to the CaR could underlie the activation of PTHrP expression in the HARA line. The fact that gefitinib induced a decrease in

the plasma calcium levels in the HARA model suggests a role for the receptor in activation of PTHrP gene expression in these cells. We speculate that this might reflect a synergism of EGFR signaling with another growth factor pathway such as TGF-*β*. In conclusion, autocrine AREG stimulation of the EGFR may serve as the intrinsic factor that drives high levels of PTHrP gene expression in the RWGT2 line, whereas this pathway appears to play a secondary role in the extrinsic activation of the gene in HARA tumors by host factors.

The effect of gefitinib in the RWGT2 model of HHM was due in part to direct actions on tumor cells. Gefitinib treatment reduced EGFR phosphorylation at tyrosine 1068 and ERK phosphorylation in the RWGT2 tumors consistent with a blockade of EGFR kinase activity. Tumor volume, mass, and cell death were not altered in the treated mice indicating that apoptosis of the tumor cells did not contribute to the lower PTHrP concentrations. We speculate that blockade of EGFR-mediated activation of PTHrP gene expression contributed to the decreased plasma protein concentrations in both the RWGT2 and HARA models. However, recent identification of over 20 nonspecific cellular targets of gefitinib unrelated to EGFR inhibition have been identified in HeLa cells using proteomics. Therefore, we are unable to rule out the possible contribution of additional kinase inhibition in decreasing hypercalcemia in our *in vitro* studies (46).

Understanding the signaling events that regulate bone resorption has led to the development of a new generation of potential therapies for HHM. These include the soluble decoy receptor for RANKL, osteoprotegerin (OPG), anti-PTHrP antibodies, and bisphosphonates. A single injection of anti-PTHrP antibodies reduced calcium and cAMP/creatinine levels near baseline levels within hours after administration in a mouse xenograft model of HHM (47) and humanized anti-PTHrP antibodies had a similar efficacy in a nude rat xenograft model of HHM (48, 49). One treatment with OPG reduced plasma calcium concentrations to baseline within 24-48 hours in a mouse xenograft model of HHM (50). In the above models of HHM, treatment with the bisphosphonates, pamidronate, zoledronic acid, and alendronate were unable to return serum calcium concentrations to baseline (48, 50).

In summary, our findings support the concept that EGFR signaling contributes to high levels of PTHrP gene expression in lung SCCs that induce HHM. Furthermore, EGFR TKIs have the capacity to reduce hypercalcemia in two lung SCC models, and their use to treat various types of non-small cell lung cancer may prevent the development of HHM.

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Figure 2.1 RWGT2 and HARA cells express predominately EGFR and ErbB2 receptors and produce EGF-related ligand mRNA for AREG, TGF-α and HB-EGF. (**A**) Parathyroid hormone-related protein/GAPDH mRNA ratios in RWGT2 and HARA cell cultures measured by QRT-PCR. The relative ratios of PTHrP mRNA to GAPDH mRNA levels were expressed as mean ± s.e.m. of 4 cultures from a single experiment. * $P = 0.003$. (**B**) Relative epidermal growth factor family receptor expression as detected by Western blots of cell extracts from RWGT2, HARA and the breast cancer cell line MCF-7. The right side of the figure indicates the specific erbB receptor antibodies. Protein extracts were precipitated using con-A as indicated by ppt Con A on lower panel of the figure. (**C**,**D**) AREG, TGF-α, and HB-EGF/GAPDH mRNA ratios in RWGT2 and HARA cultures were measured by QRT-PCR. The relative ratios of EGF-like ligand mRNA to GAPDH mRNA level were expressed as mean ± s.e.m. of four cultures from a single experiment. Note that the *y* axis for AREG mRNA is 100-fold greater than the *y* axis for TGF-α and HB-EGF mRNA. (**E**) Phosphorylation of the EGFR was measured in extracts from cells grown in basal medium. The band labeled with phosphotyrosine antibody reactivity was increased with EGF treatment (EGF). The general phosphotyrosine antibody 4G10 and EGFR antibodies used for protein detection are indicated on the right. These experiments were repeated 4 times with cells derived from independent passages with similar results.

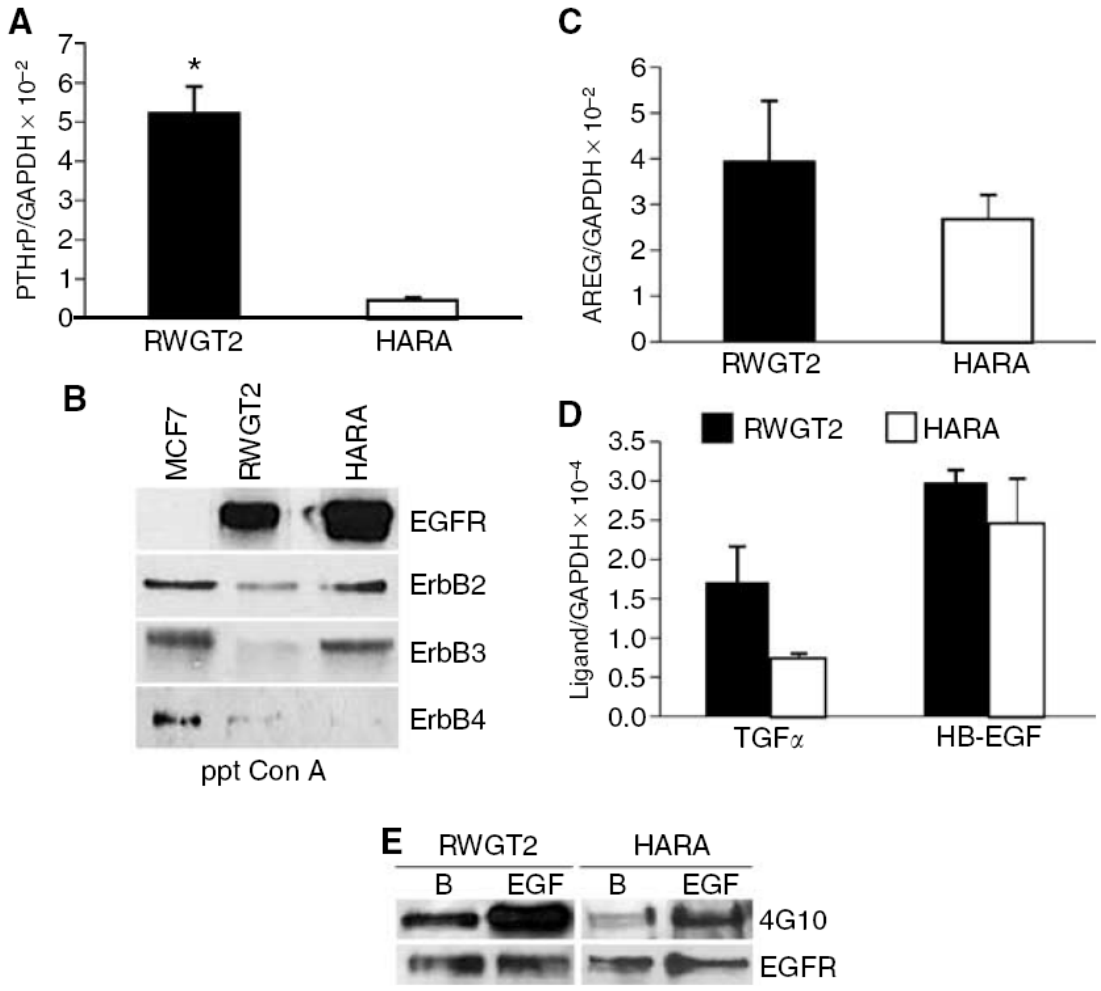

Figure 2.1 RWGT2 and HARA cells express predominately EGFR and ErbB2 receptors and produce EGF-related ligand mRNA for AREG, TGF-α and HB-EGF

Figure 2.2

Parathyroid hormone-related protein mRNA expression is reduced by EGFR inhibitors and increased by the addition of exogenous EGFR ligands. (**A**) RWGT2 cells were treated with 1 μM (PD) for 6h, a neutralizing polyclonal antibody to AREG, (10 μgml^{-1}), a ligand blocking EGFR antibody (αEGFR, 10 μgml^{-1}) or PBS (C) for 24h. Neutralizing antibodies to growth factors are represented by the symbol (α). The mRNA was harvested from four independent cultures and analyzed by QRT-PCR. PD, $P = 0.03$; αAREG, $P = 0.05$. (**B**) HARA cells were treated with 1 μM PD for 6h or a neutralizing polyclonal antibody to AREG, (10 μgml^{-1}) or PBS (C) for 24h. The mRNA was harvested from four independent cultures and analyzed by QRT-PCR. PD, $P = 0.017$; αAREG, $P = 0.034$. (**C**) RWGT2 cells were incubated with PBS (C), 100 $ngmL^{-1}$ of EGF or 1 $mgmL^{-1}$ of AREG for 2, 4 and 6h. The mRNA was harvested from four independent cultures and analyzed by QRT-PCR. Two-hour EGF, $P = 0.02$; 4h EGF, $P = 0.002$; 4h AREG, $P = 0.002$; 6h EGF, $P = 0.004$; 6h AREG, $P = 0.001$. (**D**) HARA cells were incubated with PBS (C), 100 $ngmL^{-1}$ of EGF or 1 mg/mL of amphiregulin (AREG) for 2, 4 and 6 hours. The mRNA was harvested from four independent cultures and analyzed by QRT-PCR. Four-hour AREG, $P = 0.02$; 6h EGF, $P = 0.02$, 6h AREG $P=0.013$ Values in all panels represent the mean of 4 samples from individual cultures ± s.e.m. These experiments were repeated three times with cells derived from independent passages with similar results,*$P < 0.05$ relative to C (control). Two-tailed Student's *t*-test.

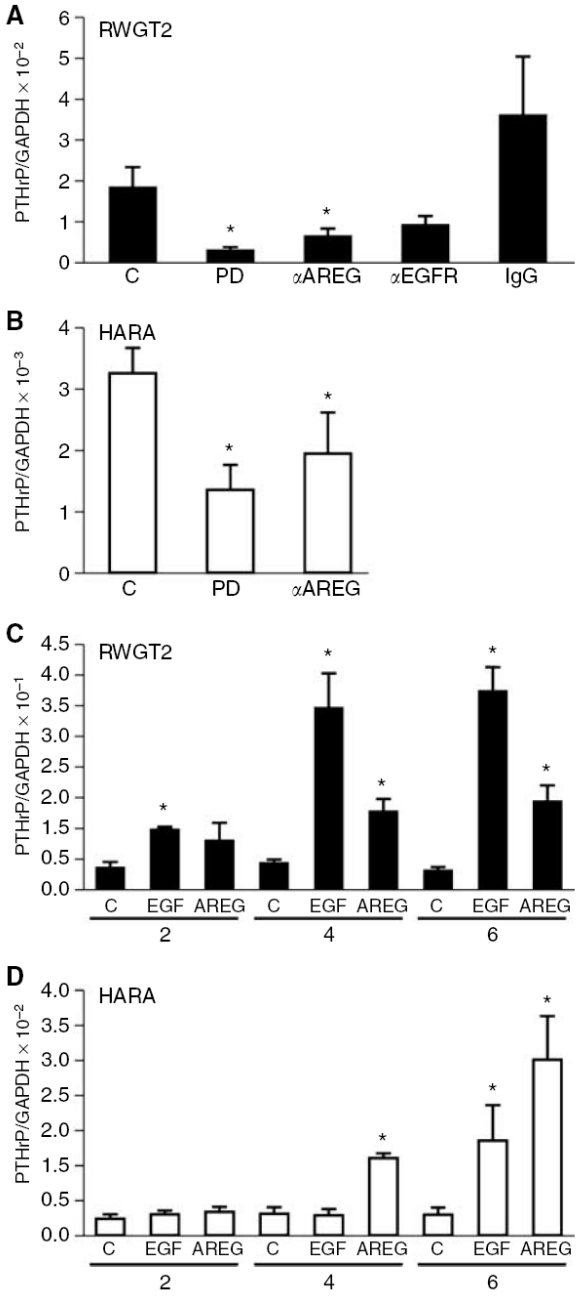

Figure 2.2 Parathyroid hormone-related protein mRNA expression is reduced by EGFR inhibitors and increased by the addition of exogenous EGFR ligands.

Figure 2.3

Mitogen-activated protein kinase signaling mediates EGFR-Induced activation of PTHrP gene expression. (**A**) Epidermal growth factor receptor and ERK phosphorylation was reduced by EGFR TKI treatment in both RWGT2 and HARA cells. Upper bands are from a Western blot of conA-Sepharose precipitated proteins probed first with the generic phosphotyrosine monoclonal antibody 4G10, then stripped and reprobed with an EGFR antibody. Lower bands are from a Western blot of 30 μg of protein probed first with a phospho-ERK 1/2 antibody, then stripped and reprobed with an antibody to ERK 1/2 and finally stripped and reprobed with an antibody to β-actin (not shown). C (control) indicates cells treated with DMSO vehicle for 6h. EGFRI indicates cells treated with PD (1 μM) for 6h. This experiment was repeated twice. (**B**) A MEK inhibitor significantly reduced basal PTHrP/GAPDH mRNA ratios in both cell lines. RWGT2 and HARA cells were treated with 1 μM DMSO (C), PD (EGFRI, 1 μM) or PD98059 (MEKI, 10 μM) for 6h. The mRNA was harvested from four independent cultures and analyzed. Two-tailed Student's *t*-test. **P* < 0.05 relative to C (control). For the RWGT2 experiments: MEKI, *P* = 0.045; EGFRI, *P* = 0.03; for HARA experiments: MEKI, *P* = 0.04; EGFRI, *P* = 0.04. (**C, D**) A MEK inhibitor blunted EGFR ligand-induced increases in PTHrP/GAPDH mRNA ratios in both cell lines. Cells were preincubated with 1 μM DMSO (C), PD (EGFRI, 1 μM) or PD98059 (MEKI, 10 μM) for 1h and then stimulated with 100 ngml^{-1} EGF (+EGF) or 1 µgml^{-1} AREG (+AREG) for 6h. B represents untreated cells in all experiments. Panels B-D represent four replicates of samples with all experiments repeated twice. Two-tailed Student's *t*-test. **P* < 0.05 relative to C (control). (**C**) EGF EGFRI, *P* = 0.01; MEKI, *P* = 0.05; AREG EGFRI, *P* = 0.02; MEKI, *P* = 0.053 not significant. (**D**) EGF EGFRI, *P* = 0.001; MEKI, *P* = 0.046; AREG EGFRI, *P* = 0.049; MEKI *P* = 0.03. (**E**) Basal reporter gene activity from a PTHrP-P3 construct was reduced by a dominant-negative Raf construct in both the RWGT2 and HARA cells. RLU represents relative luciferase units as defined in the Materials and methods. Co-transfection of the PTHrP reporter gene with the empty vector is indicated by pcDNA3, a dominant-negative Ras construct (-ras) a dominant-negative Raf construct (-raf). Values in all panels represent the mean of four samples from individual cultures ± s.e.m. These experiments were repeated three times with cells derived from independent passages with similar results. Two-tailed Student's *t*-test. **P* < 0.05 relative to C (control). RWGT2; ras, *P* = 0.019, raf, *P* = 0.033; HARA; ras *P* = 0.02.

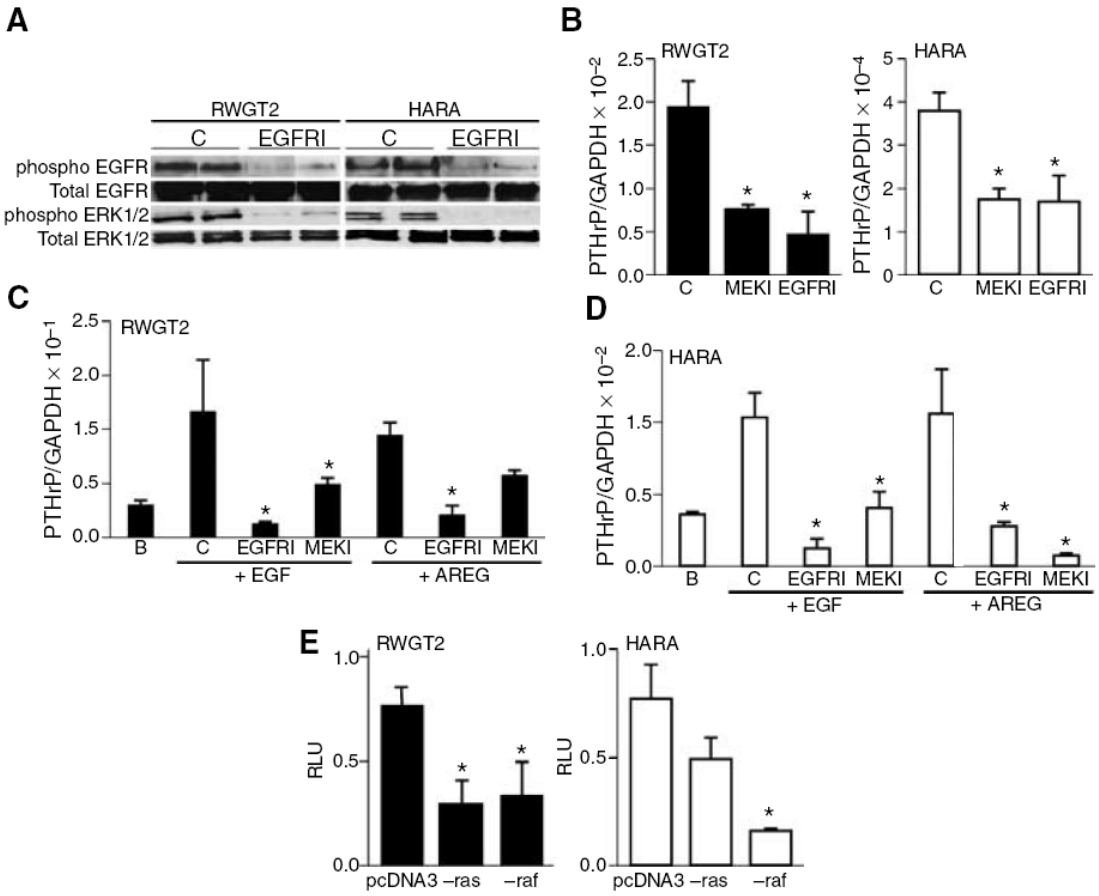

Figure 2.3 Mitogen-activated protein kinase signaling mediates EGFR-Induced activation of PTHrP gene expression.

Figure 2.4

Comparison of RWGT2 and HARA models of hypercalcemia. (**A**) Kaplan-Meir analysis of time to the development of hypercalcemia. Fifty percent of mice developed HHM 40 days after injection of HARA cells compared to the RWGT2 xenograft mice that developed HHM 60 days after injection of cells. P = 0.01. RWGT2 tumors (n=18) and HARA tumors (n=10). (**B**) Average tumor volume at time of hypercalcemia. When hypercalcemia was diagnosed, the RWGT2 (n=18) tumors were nearly twice as large as the HARA tumors (n=10) *P = 0.04 HARA compared to RWGT2. Two- tailed Student's *t*-test. (**C**) Comparison of plasma PTHrP concentrations in hypercalcemic untreated mice with RWGT2 and HARA xenografts. Plasma PTHrP concentrations were determined 78h after the development of HHM in both xenograft models. Average plasma PTHrP concentrations in the untreated mice were 80% higher (22 *vs* 12 pM) in the HARA (n=5) as compared to RWGT2-bearing mice (n=8). (**D**) Comparison of PTHrP mRNA expression between cells grown *in vitro* and *in vivo*. RNA was extracted from tumors that were removed 78h after hypercalcemia was identified. The ratio of PTHrP to GAPDH mRNA was assayed by QRT-PCR. The ratio of PTHrP to GAPDH mRNA in HARA tumors (HARA-T) was increased 100-fold compared to HARA cells (HARA-C) grown *in vitro*. The ratio of PTHrP to GAPDH mRNA in HARA tumors was 6-fold higher than RWGT2 tumors (RWGT2-T). Values in all panels represent the mean of four samples from individual cultures or tumors ± s.e.m. *P = 0.027 HARA tumors relative to HARA cells; §P < 0.05 HARA tumors relative to RWGT2 tumors. The QRT-PCR was repeated twice with similar results. Two-tailed Student's *t*-test. (**E**) Phosphorylation of the EGFR was measured in protein extracts from RWGT2 and HARA tumors by Western blotting. The general phosphotyrosine antibody 4G10 and polyclonal antibody for phosphorylated Tyr 992 was used to probe 1 mg of extracted tumor protein precipitated with conA-Sepharose. Blots were stripped and reprobed with EGFR antibodies. Four tumors from each cell line were evaluated.

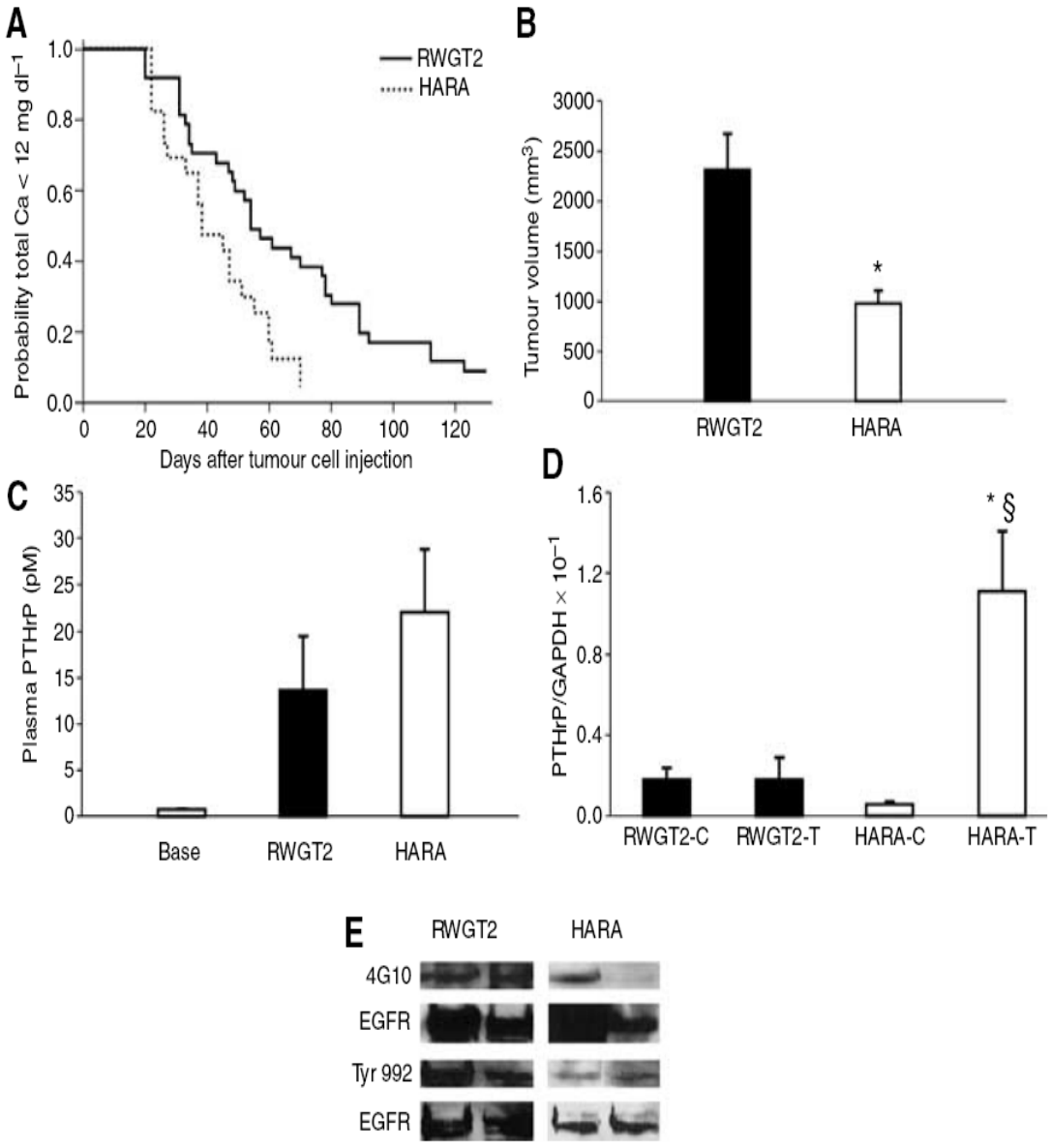

Figure 2.4 Comparison of RWGT2 and HARA models of hypercalcemia.

Figure 2.5

Gefitinib reduced plasma total calcium concentrations and urinary cAMP creatinine ratios in nude mice with RWGT2 xenografts and hypercalcemia. (**A**) Hypercalcemic mice with RWGT2 xenografts (n=9) were treated daily for 3 days with gefitinib (200 $mgkg^{-1}$, per os (p.o.)). The first treatment occurred at the onset of hypercalcemia. Treatment resulted in a statistically significant decrease in total plasma calcium concentrations compared to pretreatment values at all time points (6, 24, 52, and 78h) and compared to untreated mice (n=8). Repeated measures one-way ANOVA, Mean ± s.e.m., $*P < 0.001$. Normal nude mice (n=5) were treated daily for 3 days with gefitinib (200 $mgkg^{-1}$, p.o.) or untreated (n=5). Gefitinib did not decrease total plasma calcium concentrations compared to pretreatment values at all time points and compared to untreated mice. Repeated measures one-way ANOVA, Mean ± s.e.m. * $P = 0.001$ relative to treated; $^{\S}P = 0.001$ relative to baseline values. (**B**) Spontaneously voided urines were collected at the indicated times after the first gefitinib treatment (200 $mgkg^{-1}$). Cyclic AMP and creatinine concentrations were measured. The cAMP and creatinine ratio was expressed as $nMmg^{-1}$. Mice received additional doses of gefitinib at 24 and 48h. Values at each time point represent the mean of two measurements from eight tumor-bearing mice from both the gefitinib-treated and untreated groups, as well as, 16 nontumor-bearing mice at baseline. Cyclic AMP levels were decreased in the gefitinib-treated mice when compared to untreated mice at all time points. Repeated measures one-way ANOVA, Mean ± s.e.m. $*P = 0.02$ relative to treated; $^{\S}P = 0.004$ relative to baseline values. In all figures, the baseline and pretreatment groups are represented by the columns labeled base and pretreat, respectively. (**C**) Plasma PTHrP concentrations were measured using plasma samples collected before tumor injection (base) and at 6 and 78h after gefitinib or placebo treatment. Experiments included nine gefitinib-treated mice and eight untreated mice. Baseline PTHrP values were from the same 17 mice. In the gefitinib-treated mice (black column), there was a mild reduction at 6h followed by a marked reduction (50%) in plasma PTHrP concentrations when compared to untreated mice (white columns) at the respective time points. Untreated and gefitinib-treated mice with RWGT2 xenografts had PTHrP concentrations that were significantly greater than baseline at both 6 and 78h. $*P = 0.02$ relative to treated; $^{\S}P = 0.006$ relative to baseline values. (**D**) Gefitinib treatment reduced PTHrP mRNA levels in RWGT2 tumors. RNA was extracted from tumors that were removed 78 h after hypercalcemia was identified. The ratio of PTHrP to GAPDH mRNA was assayed by QRT-PCR. The ratio of PTHrP to GAPDH mRNA in the gefitinib treated tumors (n=6) was decreased 60% as compared to untreated (n=6). The QRT-PCR was repeated twice with similar results. $P = 0.014$.

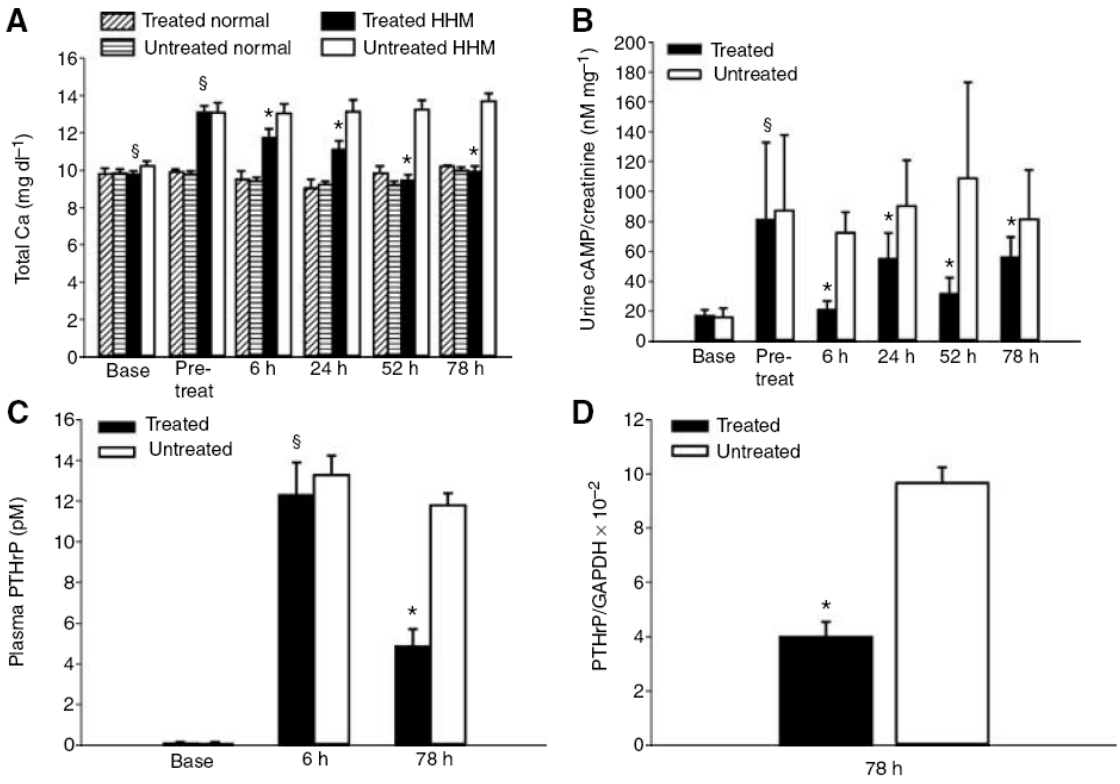

Figure 2.5 Gefitinib reduced plasma total calcium concentrations and urinary cAMP creatinine ratios in nude mice with RWGT2 xenografts and hypercalcemia.

Figure 2.6

Gefitinib treatment reduced RWGT2 tumor EGFR and MAPK phosphorylation at 78 hours but not tumor volume. (**A**) Comparison of EGFR and MAPK phosphorylation as assessed in untreated and gefitinib-treated RWGT2 tumors by Western blots. Tumors from untreated RWGT2 mice had greater phosphorylation of both EGFR phosphotyrosine residue 1068 and ERK1/2 when compared to tumor lysates from RWGT2 gefitinib-treated mice. Furthermore, all lysates had equivalent EGFR and ERK1/2 expression. Equal amounts of total protein (10 *μ*g) were separated by electrophoresis using 4-20% tris-glycine sodium dodecyl sulfate-polyacrylamide gels, transferred onto nitrocellulose membranes, and analyzed by immunoblotting using rabbit polyclonal antibodies to phosphotyrosine 1068 and phospho ERK1/2. Blots were stripped and reprobed with rabbit polyclonal antibodies against EGFR and ERK1/2. The blots were stripped a final time and reprobed with a polyclonal antibody to *β*-actin. Experiments were repeated three times, and the data from a representative blot are shown. (**B**) Tumor volumes were measured serially as described in "Materials and methods." No reduction in tumor volume was present (pretreatment, 48 and 78h) between gefitinib-treated and untreated mice at any time point. Each bar represents the mean tumor volume of eight or nine mice; bars, s.e.m. **P* = 0.048 *vs.* treated at the pretreatment time point even though the animals were randomized. (**C**) Gefitinib treatment of mice with RWGT2 xenografts did not cause an increase in apoptosis. Representative samples at 78h from gefitinib-treated and untreated tumors. H&E staining revealed similar numbers of apoptotic cells represented as shrunken cells with eosinophilic cytoplasm and karyorrhectic or pyknotic nuclei in both the treated and untreated mice (black, arrows); bar 100 *μ*m. Fluorescent TUNEL staining for apoptosis (black, arrows) revealed similar numbers of apoptotic cells throughout the tumor parenchyma. 400*x* (**D**) Comparison of numbers of apoptotic cells in gefitinib-treated and untreated RWGT2 tumors at 78h. Ten 100*x* fields from a fluorescent TUNEL slide for each mouse were counted to assess the number of apoptotic cells per field. The mean apoptotic cell number per 100x field was represented as the black bar for gefitinib-treated and white bar for the untreated. No significant differences in mean apoptotic cells in tumors were present between the gefitinib-treated *vs* untreated mice.

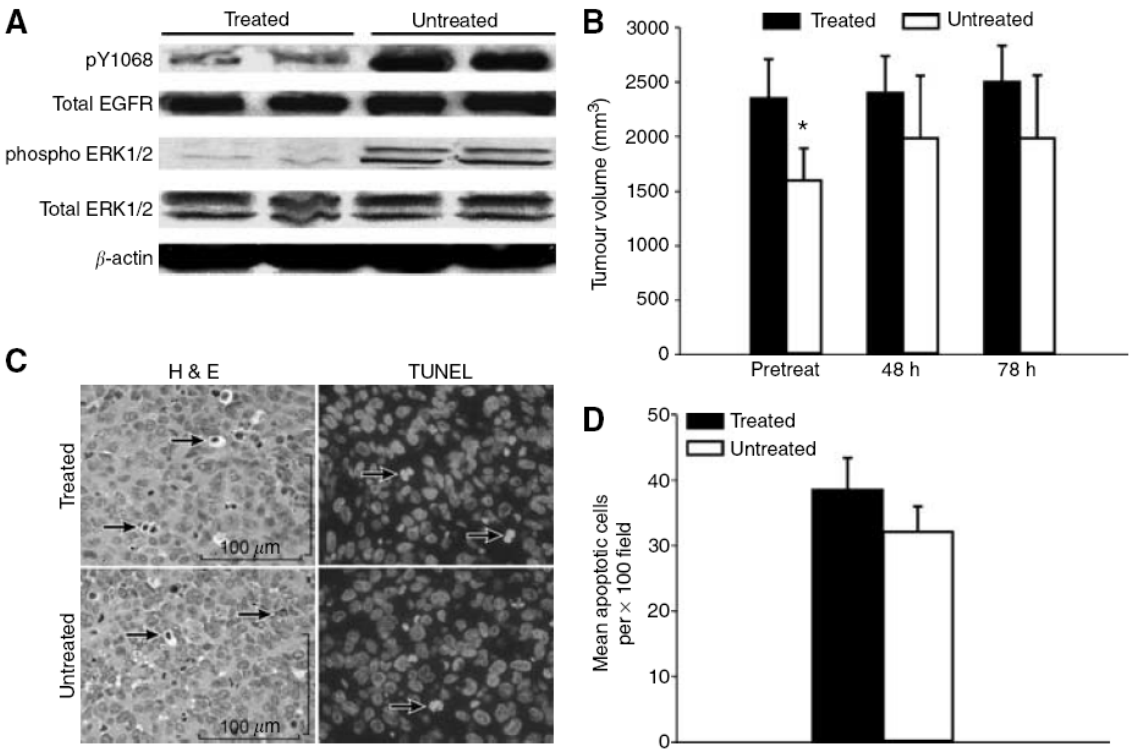

Figure 2.6 Gefitinib treatment reduced RWGT2 tumor EGFR and MAPK phosphorylation at 78 hours but not tumor volume.

Figure 2.7

Gefitinib reduced plasma total calcium and PTHrP concentrations in nude mice with HARA xenografts and hypercalcemia. (**A**) Hypercalcemic mice with HARA xenografts (n=5) were treated daily for 3 days with gefitinib (200 $mgkg^{-1}$, p.o.). The first treatment occurred at the onset of hypercalcemia. Treatment resulted in a statistically significant decrease in total plasma calcium concentrations compared to pretreatment values at 78h and compared to untreated mice (n=5). Repeated measures one-way ANOVA, mean ± s.e.m., **P* = 0.042 relative to treated; $^{\S}P$ = 0.001 relative to baseline. (**B**) Plasma PTHrP concentrations were measured using plasma samples collected before HARA cell injections (baseline) and at 78h after gefitinib or placebo treatment. Experiments included five gefitinib-treated mice and five untreated mice. Baseline PTHrP values were from the same 10 mice. In the gefitinib-treated mice (black column), there was a marked reduction (60%) in plasma PTHrP concentrations when compared to untreated mice (white columns). Untreated and gefitinib-treated mice with HARA xenografts had PTHrP concentrations that were significantly greater than baseline at 78h. **P* = 0.047 relative to treated; $^{\S}P$ = 0.001 relative to baseline. (**C**) Gefitinib treatment reduced PTHrP mRNA levels in RWGT2 tumors. RNA was extracted from tumors that were removed 78h after hypercalcemia was identified. cDNA was produced with the MultiScribe Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) and QRT-PCR performed. PTHrP to GAPDH mRNA was assayed by QRT-PCR. The ratio of PTHrP to GAPDH mRNA in the gefitinib-treated tumors (n=3) was decreased 90% as compared to untreated (n=4). The QRT-PCR was repeated twice with similar results. **P* = 0.02.

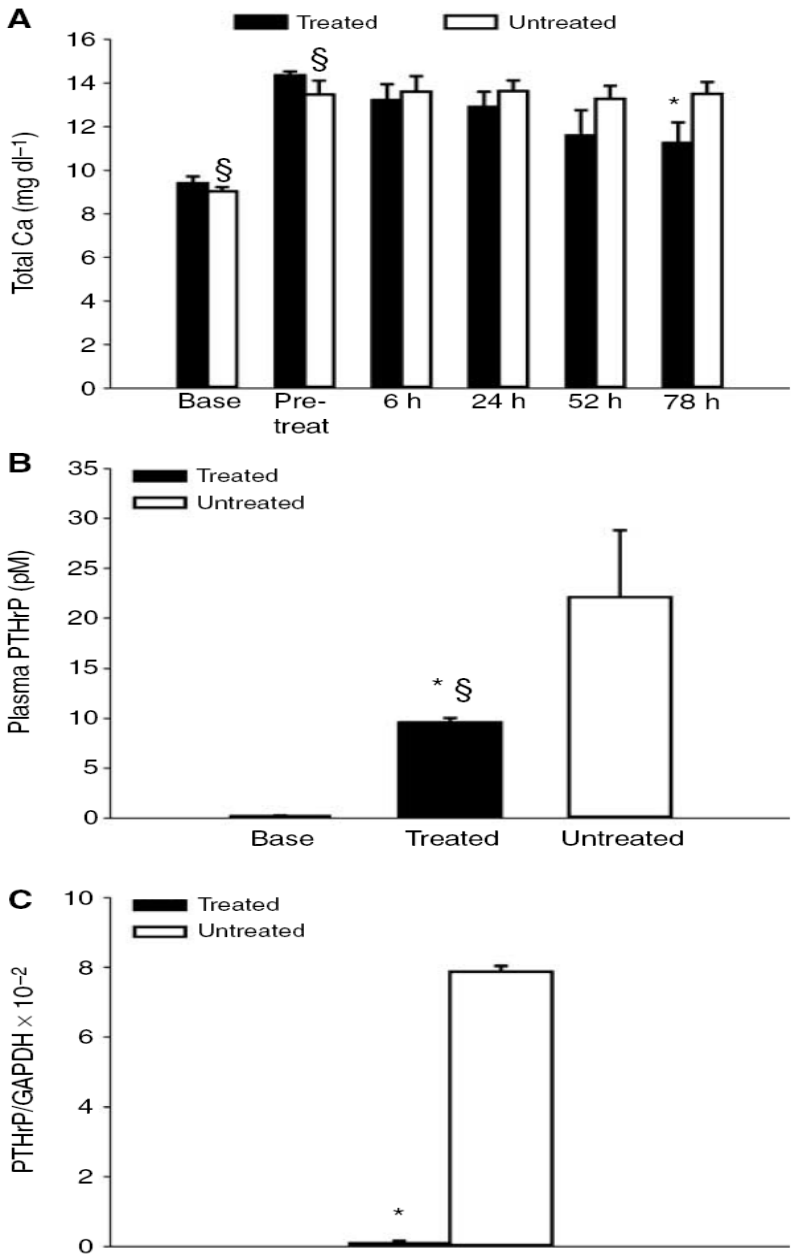

Figure 2.7 Gefitinib reduced plasma total calcium and PTHrP concentrations in nude mice with HARA xenografts and hypercalcemia.

CHAPTER 3

THE CALCIUM-SENSING RECEPTOR IS NECESSARY FOR THE RAPID DEVELOPMENT OF HYPERCALCEMIA IN HUMAN LUNG SQUAMOUS CELL CARCINOMA

3.1 SUMMARY

The calcium-sensing receptor (CaR) is responsible for maintaining physiologic extracellular calcium (Ca^{2+}_o) homeostasis. Chronic Ca^{2+} dyshomeostasis resulting from aberrant CaR activation has been shown to increase proliferation in several cancer cell lines. We report that Ca^{2+}_o activated CaR resulted in MAPK-mediated activation of PTHrP production in human lung squamous cell carcinoma (SCC) lines and induction of humoral hypercalcemia of malignancy (HHM) when SCC lines were grown *in vivo* in nude mice. A gain of function mutation in CaR changes the receptor's activation threshold, permitting signaling at reduced calcium concentrations and ultimately increased PTHrP expression.

Increasing and decreasing the expression of CaR variants with amino acid changes in the intracytoplasmic domain was both necessary and sufficient for lung SCC tumors to induce HHM.

3.2 SIGNIFICANCE

Lung cancer is the most common cause of cancer worldwide. Patients commonly develop the debilitating syndrome of HHM resulting from increased circulating concentrations of tumor-produced PTHrP and concomitant disturbances in calcium homeostasis. The basis for high levels of PTHrP gene expression associated lung cancer-induced HHM has never been adequately explained. Our findings suggest that specific CaR variants may provide a positive feed back loop by which modest tumor–induced alterations in calcium metabolism could prematurely precipitate the onset of HHM. It is likely that CaR variants could also contribute to lung SCC-associated bone metastasis. Thus, the CaR inhibitors may be of substantial clinical utility to control lung SCC-mediated diseases of bone.

3.3 INTRODUCTION

Development of hypercalcemia of malignancy (HHM) results from unregulated secretion of parathyroid hormone-related protein (PTHrP) in patients with

neoplasia of epithelial origin. The disorder is characterized by persistently increased plasma PTHrP and complicated by disturbances in calcium metabolism. Studies from developed nations in the 1970s and 80s, as well as more recent studies from newly industrialized countries, report that patients with squamous cell carcinoma (SCC) of the lung have the highest frequency of HHM (ranging from 27-66%) as compared to other tumor types.(1) The precise mechanisms that activate high levels of PTHrP gene expression in tumors that are associated with HHM have yet to be identified.

The calcium-sensing receptor (CaR) is a central regulator of extracellular calcium homeostasis. It enables many tissues to detect minute changes in extracellular Ca^{2+} (Ca^{2+}_o) and respond with an alteration in cellular function to restore Ca^{2+}_o concentrations to the normal range. The calcium ion can serve as an extracellular first messenger for CaR.(2) The biochemical properties of CaR place it in the superfamily of G-protein-coupled receptors (GPCRs) and within a subfamily of structurally related members, the C receptors. As a representative of the classical GPCR, CaR has a large 600-amino acid amino-terminal extracellular domain, a “serpentine motif” seven transmembrane-spanning domain and an intracytoplasmic C-terminal tail of greater than 200 amino acids.(3)

The CaR is activated in response to certain extracellular stimuli such as Ca^{2+}, Mg^{2+} and amino acids. Repetitive cycles of release and uptake generate oscillations of Ca^{2+} leading to specific cellular responses and gene transcription.(4, 5) Calcium binding to CaR generates intracellular signals that result in inhibition of parathyroid hormone secretion by chief cells and calcium reabsorption from the kidney thick ascending limb. Activation of CaR through ligand binding stimulates phospholipase C (PLC) enzymes to convert phosphatidylinositol 4,5 bisphosphate into diacylglylcerol and inositol 1,4,5 – trisphosphate (IP_3). Acting as a "second messenger" IP_3 interacts with IP_3 receptors situated in the endoplasmic reticulum membrane, to induce Ca^{2+} release from internal stores and increases intracellular calcium ($Ca^{2+}{}_i$.) which leads to activation of other signal transduction pathways such as mitogen-activated protein kinase- extracellular signal related kinases(MAPK-ERK) and MAPK- cJun-N-terminal kinase (JNK). (6) One mechanism of CaR regulation occurs by protein kinase C (PKC)-mediated phosphorylation of the putative PKC intracellular domain residue Thr^{888}.(7) Mutation of CaR^{T888} results in increased sensitivity of CaR to $Ca^{2+}{}_o$.(8) Furthermore, inhibition of a calyculin-sensitive phosphatase responsible for dephosphorylation of CaR^{T888} decreased CaR induced intracellular calcium oscillation frequency suggesting that constant phosphorylation of CaR^{T888} leads to decreased intracellular calcium mobilization.(7)

Therefore, phosphorylation of this PKC site inhibits activation of PLC by the CaR; thereby acting as a negative feedback system, since PKC is also activated by CaR and is often situated downstream of PLC.

The CaR is one of just a few GPCRs where genetic mutations have been linked with disease and therefore sequencing of CaR has provided insights into the molecular basis of several enigmatic inherited disorders of Ca^{2+} homeostasis in humans.(9) For example, inactivating mutations of the *CaR* gene are responsible for neonatal severe hyperparathyroidism (NSHPT). An individual with NSHPT will have two defective copies of the *CaR* gene. CaR dysregulation in NSHPT results in severe hypercalcemia due to marked hyperparathyroidism, secondary skeletal demineralization and subsequent pathological fractures.(10) Activating mutations of CaR will often cause the CaR to have a reduced set point for Ca^{2+} (enhanced sensitivity to Ca^{2+}_o) and are responsible for a form of autosomal dominant hypocalcemia. Studies analyzing genetic variability at the CaR locus in both parathyroid adenomas and pituitary tumors have failed to reveal any consistent link between mutation of the CaR and tumorigenesis.(11)

Disruptions in calcium signaling are well-established features in the genesis and progression of cancer.(12) The molecular mechanisms of dysregulated calcium homeostasis in lung SCC are incompletely understood. The CaR is expressed in a diverse array of tissues and is crucial for mineral

homeostasis One of the functions of CaR at several nonparathyroid sites appears to be the regulation of the production of PTHrP.(13) It has been reported that CaR can regulate the production of PTHrP by breast cancer cell lines *in vitro*.(14) However, it is not known if normal human pulmonary epithelia or neoplasia posses CaR and if present, whether it contributes to the regulation of PTHrP expression and secretion. Furthermore, no literature exists on CaR postreceptor signaling events, gene regulation or possible CaR mutations in lung SCC.

In this report, we identify the expression of CaR in three human lung squamous cell carcinoma lines. In addition, we document that CaR was necessary for the *in vitro* regulation of PTHrP gene expression and secretion. By using a xenograft lung SCC model of HHM with short hairpin interfering RNA against CaR we show that CaR was required for the rapid development of HHM. Finally, we demonstrated the presence of numerous CaR mutations in two lung SCC lines and HEK293T cells as well as established that either overexpression of wild type CaR or the homozygous single nucleotide polymorphism, R990G, was sufficient to induce development of HHM in an eucalcemic xenograft model of lung cancer.

3.4 MATERIALS AND METHODS

Materials

Spermine tetrahydrochloride was purchased from Sigma-Aldrich (St Louis, MO, USA). Mouse anti-CaR monoclonal antibody against a synthetic peptide corresponding to AA 15-29 at the extracellular N-terminus (Sigma, St Louis, MO, USA) was used for Western blotting and recognizes human CaR. Adult normal human kidney whole cell homogenates, the normal human kidney cytoplasmic lysates and the secondary rabbit anti-mouse HRP antibody were purchased from Abcam Inc. (Cambridge, MA, USA). Phosphorylation of CaR residue Thr^{888} was studied using a custom-made rabbit polyclonal antibody to the phosphorylated form of Thr^{888} which is within amino acids 882-896 of the human CaR sequence (KVAARA(pT)LRRSNVSR) (Invitrogen, Carlsbad, CA, USA)(203). For additional immunoblotting studies the following antibodies were used at a dilution of 1:000: Phospho-p44/42 MAP Kinase (Thr202/Tyr204) #9101, p44/42 MAP Kinase, GAPDH #2118 (Cell Signaling, Beverly, MA), and anti-β-actin was used at 1:5000 dilution (Sigma, St Louis, MO, USA).

Cell culture

The human RWGT2 SCC cell line was derived from a pulmonary bone metastasis and obtained from Drs. G. Mundy and T. Guise (University of Texas Health Science Center, San Antonio, TX, USA and University of Virginia,

Charlottesville, VA, USA). The human HARA SCC cell line was obtained from Dr. Haruo Iguchi (Shikoku Cancer Center, Matsuyama, Japan). The original patient had an increased PTHrP concentration and hypercalcemia, but no apparent bone metastases. The human BEN Australia cell line was obtained as a gift from Dr. T.J. Martin (Brisbane, Australia) and was originally established from a hypercalcemic patient with a squamous cell carcinoma of the bronchus.(15) Transformed human embryonic kidney 293 cells No. CRL-11268 (293T/17) at cell passage 12, were purchased from American Type Tissue Culture (Manassas, VA, USA). All cell lines were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) with GlutaMax™ (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C and 5% CO_2. Cells were passaged at 90% confluence. *In vitro* experiments, with the exception of intracellular calcium release studies, were performed when RWGT2, HARA, BEN Australia and HEK293T cells were ~80-90% confluent.

Reverse transcriptase-PCR

Total RNA from all lung SCCs and HEK293T cells was prepared using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using the Transcriptor Reverse Transcriptase Kit (Roche Diagnostics, Mannheim, Germany). General PCR conditions used to amplify *CaR* were an initial denaturation at 95°C for 4 min, 35 cycles of denaturation at 97°C for 30 s,

annealing at 57°C for 30 s, and extension at 72°C for 2:00 min followed by elongation at 72°C for 7 min. All amplifications used a high fidelity polymerase (Platinum Taq HiFi Polymerase, Invitrogen, Carlsbad, CA, USA).

Amplicons were resolved on a 1% agarose gel to visualize the products, and cloned directly from the PCR reaction mix into pCR®4-TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNAs from eight to 16 clones for each cell line were prepared for automated DNA sequencing.

The human specific primers used for the 481-bp *CaR* product were: forward 5'CGGGGTACCTTAAGCACCTACGGCATCTAA3' and reverse 5'GCTCTAGAGTTAACGCGATCCCAAAGGGCTC3'. The entire open reading frame of *CaR* was amplified using the following 3 sets of primers:

1) forward 5'AAACTTCTGGGAGCCTCCAAACTC3' and reverse 5'CCTCTCAGAAAGGTGTCCACAGGT3'

2) forward 5'ACCTCCAAGAAGGTGCAAAAGGAC3' and reverse 5'CGGTGTAGAGCCAGATCACACAGA3'

3) forward 5'CTGCACCTTCATGCAGATTGTCATC3' and reverse 5'ACCCCAAGAAACCTCTCTGCATTCT3'.

Plasmids and site-directed mutagenesis

A construct containing the complete human CaR open-reading frame (ORF) cloned into pCMV6-XL4 was purchased from OriGene Technologies, Inc (True

Clone cat. No. SC119946, ref. ID NM_000388). For site-directed mutagenesis, the CaR ORF was mutated using a strategy described previously(16) that involves PCR-amplification of the entire pCMV6-XL4-CaR cDNA plasmid using the Expand Long Template PCR System (Roche, Indianapolis, IN) and the following oligonucleotides that contained the desired mutations (mutated nucleotides are underlined): G158E (sense: 5'-GCTCTTCTACATTCCCCAGGTCAG-3'; antisense: 5'-TCCAGCAGATTTGCCACTGC-3'); R383G (sense: 5'-GACGGGTTTAGCAACAGCTCGACA-3'; antisense: 5'-GCCACTTTCTTCG TGACCTCTCAG-3') and R990G (sense: 5'-CCACGGGAATTCTACGCACCAGA-3'; antisense: 5'-GCCATGGCGTTCTTCTGAGG-3'). A PCR product of appropriate size (~8.5 kb) was obtained for each mutation. These PCR products were blunt-ended with T4 DNA polymerase (New England Biolabs, Ipswich, MA, USA), phosphorylated with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA), circularized with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and transformed into DH5α cells. Once clones containing the desired mutations were obtained and confirmed by DNA sequencing, subregions of the mutated CaR ORFs were subcloned back into the pCMV6-XL4-CaR cDNA parent plasmid to remove any unwanted mutations elsewhere in the plasmid that may have been caused by *Taq* DNA polymerase errors. The complete wild-type and mutated CaR ORFs were then subcloned as 3571-nucleotide *Psh*AI-*Not*I fragments into the *Swa*I-*Not*I sites of the GFP-expressing lentiviral vector pCDH-

CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA, USA). Finally, a fusion protein between the wild-type/mutant CaR ORFs and GFP was genetically engineered by replacing the CaR stop codon with a *Not*I restriction site. This modified CaR 3'-end was cloned into the lentiviral vectors as a 517-nucleotide *Bam*HI-*Not*I fragment replacing the 751-nucleotide *Bam*HI-*Not*I fragment containing the wild-type CaR 3'-end. The entire CaR ORFs of the final lentiviral vectors were confirmed by DNA sequencing.

Generation of shRNA-CaR and BEN Australia CaR-mutant cell lines

Briefly, $5x10^4$ BEN Australia, RWGT2, and HARA cells were seeded in a 12-well culture plate. Cells were transduced with SMARTvector™ shRNA Lentiviral Particles, SMARTvector™ Empty-Vector Control Particles or SMARTvector™ Non-Targeting Control Particles (ThermoScientific Dharmacon® RNA Technologies, Lafayette, CO, USA) at an MOI of 40. Lentiviral particles used were as follows: a set of three different shRNA constructs targeting CaR, GAPDH, an empty vector expressing TurboGFP and a non-targeting negative control vector (scrabbled CaR sequence) expressing TubroGFP. Seventy two hours after initial transduction, gene knockdown was assessed by RT-PCR of total cellular cDNA. Cells were cultured in the presence of puromycin for 14 days to select stable gene-silencing clones. The degree of functional CaR silencing in each of the three clones for all SCC lines was evaluated for intracellular calcium release by real-time confocal single cell microfluorometry. Additional selection for

the maximum silenced shRNA-CaR cells in all three lung SCC lines was achieved by fluorescence-activated cell-sorting (FACS) analysis from which $1x10^4$ population of cells with the greatest fluorescent reporter function were selected for use in additional studies. Generation of wild type and CaR mutation expression was initiated by cloning constructs into the pCDH-EF1-MCS-T2A-copGFP reporter vector (Systems Biosciences, Mountain View, CA, USA) see methods section on *plasmids and site-directed mutagenesis*. pCDH cDNA CaR wild type and mutant constructs were subsequently packaged into VSV-G pseudotyped lentiviral particle supernatant (Cincinnati Children's Hospital Medical Center Viral Vector Core, Cincinnati, OH, USA). BEN Australia cells were seeded at a density of 5 $x10^5$ cells/6-mm well and incubated overnight (O/N) at 37°C. Twenty-four hours later viral supernatant of each mutant and wild type construct with a MOI of five, polybrene 8 $ugml^{-1}$ and DMEM + 10% FBS were added to each well to a total volume of 2 ml. Spinoculation was performed at 32°C for 1 h @ 2700 rpm. Transduced cells expressed visible GFP within 48 h and were selected by FACS analysis.

Real-time confocal laser-scanning microscopy for determination of intracellular calcium release

Determination of $Ca^{2+}{}_o$-induced changes in intracellular free Ca^{2+} ($Ca^{2+}{}_i$) were measured by loading the lung SCC lines with Ca^{2+} -sensitive fluorescence probes, fluo-3AM or rhod-2AM for GFP-expressing cell lines (Invitrogen,

Molecular Probes, Eugene, OR, USA). Intracellular Ca^{2+} dynamics in single cells and EB were measured with an Olympus Fluoview-1000 Laser Scanning Confocal System (Olympus, LTD, Tokyo, Japan), equipped with an 60x1.4NA oil objective. Fluo-3AM or GFP fluorescence was detected after excitation with 488 nm laser line and acquired at 500-530 nm. Cells were loaded with rhod-2AM calcium dye, excited with 543 nm laser line, and fluorescence was acquired at wavelengths of >560 nm. XY fluorescence images 512x512 were recorded every 1.1 s. The magnitude of fluorescent signals was quantified in terms of F/F_0, where F_0 is background fluorescence. The solution for loading and imaging was as follows: 140 mM NaCl, 10 mM HEPES, 5.6 mM Glucose, 5.4 mM KCl, 0.5 mM $MgCl_2$, pH 7.3. Experiments were performed at 23°C. Cover slips of 30% confluent cells were incubated in normal DMEM 24 h before imaging. Lung SCCs, HEK293T or the BEN Australia lentiviral CaR mutant GFP-transduced cells were loaded with 5 μM fluo-3AM or rhod-2AM for 30 min at 37°C.

MTT assay

A colorimetric assay (CellTiter 96® Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI) was used according to the manufactures instructions to determine cell number and/or viability after 24 h of treatment with various calcium chloride concentrations or spermine in the three lung SCC lines and HEK293T cells. Briefly, 5000 cells of each cell line were seeded in a well of a 96-well culture plate in 100 μl of normal growth media, and were incubated at 37°C in

humidified 5% CO_2 for 24 h. The following day, normal growth media was replaced by 100 μl $CaCl_2$-free DMEM supplemented with L-glutamine and 10% dialyzed FBS (0.15 mM Ca^{2+}). Calcium chloride was used as the active agent for $[Ca^{2+}]_o$ delivery and was added to the calcium-free DMEM to give final total calcium concentrations of 0.5, 1.0, 3.0 or 5.0 mM. Medium supplemented with the polycationic CaR agonist, spermine (2 mM), was used as a positive control. Twelve wells of each cell type were used for each calcium concentration and spermine treatment. Cells were incubated with CaR agonist treatments at 37°C in humidified 5% CO_2 for 24 h. Fifteen μl of the dye solution was added to each well and cells were incubated as above for 4 h to convert water-soluble MTT to insoluble formazan, which occurs only in viable cells. After 4 h, 100 μl of solubilization/stop solution was added to each well to solubilize formazan and the plates were incubated at 37°C overnight (O/N). The absorbance at 570 nm wavelength was recorded using a 96-well plate reader.

PTHrP secretion studies

For studying the effects of $Ca^{2+}{}_o$ and the polycationic CaR agonist, spermine, on PTHrP secretion, the three lung SCC lines and their respective shRNA-CaR SCC lines were seeded in triplicate at a density of 3 x 10^6 cells/100 mm in polystyrene tissue culture dishes (Falcon®, Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 10 ml normal growth medium. After 48 h, the medium was removed from each plate, cells were washed with Dulbecco's phosphate-buffered saline

(DPBS) and the medium was replaced with 10 ml of sodium pyruvate and calcium chloride-free high glucose DMEM® (Gibco®/Invitrogen, Grand Island, NY, USA) supplemented with 10% dialyzed triple 0.1 μm sterile-filtered FBS (HyClone®, Logan, UT, USA) and 4 mM L-glutamine alone (0.15 mM) or supplemented with $CaCl_2$ to a final concentration of 0.5, 1.0, 3.0 or 5.0 mM or 2 mM spermine. Medium total calcium concentrations were precisely measured using a calcium analyzer (Stat Profile® Critical Care Xpress, Nova Biochemical, Waltham, MA, USA). At 2, 4, 8 or 24 h after the addition of $CaCl_2$ or spermine, the conditioned medium was removed and biologically-active plasma PTHrP (1-86) was measured using a commercially available two-site immunoradiometric assay (IRMA) (Diagnostic Systems Laboratories, Inc, Webster, TX, USA). The limit for detection of the assay was 0.3 pM. Parathyroid hormone-related peptide concentrations were calculated by using a standard curve generated from the standards provided in the assay. At 2, 4, 8 or 24 h after the addition of CaR agonists, the cells that generated the conditioned medium were harvested for RNA isolation.

RNA isolation and quantitative real-time reverse transcriptase PCR

Total RNA was prepared using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. TaqMan® Reverse Transcription kits were used to make cDNA for quantitative real-time PCR (QRT-PCR) analysis of all transcripts of PTHrP. The unlabeled primers and MGB FAM

dye-labeled probe set for PTHrP QRT-PCR was from a Pre-Developed TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA). Absolute quantification was performed by interpolating quantities of unknown sample from a standard curve derived from known concentrations of PTHrP and GAPDH dilutions. Data were normalized by use of the ratio of the target cDNA concentration to GAPDH to correct for differences in RNA quantity between samples. The results represented in the figures were derived from experiments where the cDNAs were prepared at the same time and then analyzed by QRT-PCR performed on one plate when possible.

Immunoblot analysis

For measurement of CaR and actin proteins in lung SCC and HEK293T cells, cells were grown to 90% confluency in 100 mm dishes subsequently placed on ice, rinsed with ice-cold DPBS, and lysed with a cold solution containing 20 nM HEPES (pH 7.4), 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5% Triton X-100, protease inhibitors (Halt protease inhibitor cocktail kit, Pierce, Rockford, IL, USA), 1 mM Na_3VO_4, 2 mM NaF, and 50 mM PMSF. Cells were scraped and the lysates were incubated in the buffer for 15 min on ice, and centrifuged for 20 min at 16,000 x *g* at 4°C. The supernatants were collected and protein concentration determined by a modified Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For immunoblot analysis, 3X Laemmli sample buffer with 1 mM beta-mercaptoethanol, was added to 40 *μ*g of protein

extract (35-200 μg) at a final concentration of 33%, and the samples were heated at 100°C for 5 min. Cell protein extracts were fractioned on a pre-cast 7% NuPAGE® TRIS-acetate SDS gel (Invitrogen, Carlsbad, CA, USA) followed by electrophoretic transfer to nitrocellulose membranes (Pall Life Sciences, East Hills, NY, USA). The membranes were incubated O/N at 4°C with 0.2% Tween-20 in TBS (TBST) and 2% BSA with rabbit monoclonal and polyclonal antibodies. The blots were incubated with secondary anti-rabbit IgG horseradish peroxidase-linked antibody in TBST and 2% non-fat milk for 1 h at room temperature (RT) (Cell Signaling, Beverly, MA, USA). The custom phosphoCaR antibody was used at a 1:75 dilution. Immunoreactive bands were detected using a combination product that detects both chemiluminescence and fluorescence (Amersham ECL Plus Western Blotting Detection Reagents, GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

TOPO TA cloning and sequencing

Standard PCR was used to generate *Taq* polymerase-amplified PCR products for cloning into the pCR®4-TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA) and subsequent sequencing. Plasmids were sequenced through the cloned region using BigDye™ Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were run on an Applied Biosystems 3730 DNA Analyzer automated sequencer.

Signaling pathway analysis

Signaling pathways were investigated using Proteome Profiler™ arrays (R&D Systems, Minneapolis, MN). For analysis of MAPK phosphorylation, a human phospho-MAPK antibody array was used. The human phopho-MAPK antibody array is a nitrocellulose membrane with 21 different anti-kinase antibodies (Abs), including three positive controls and six negative controls, which are in duplicate. Positive controls are phosphorylated proteins, which are recognized by the anti-kinase Abs. BEN Australia and HARA lung SCC lines and their respective shRNA-CaR SCC lines were seeded in duplicate at a density of 3 x 10^6 cells/100 mm polystyrene tissue culture dish (Falcon®, Becton Dickinson Labware, Franklin Lakes, NJ, USA) in 10 ml of normal growth medium. After 24 h, cells were washed twice with DPBS and the culture medium was replaced with 10 ml of a sodium pyruvate and calcium chloride free high glucose DMEM® (Gibco®/Invitrogen, Grand Island, NY, USA) supplemented with 10% dialyzed triple 0.1 μm sterile-filtered FBS (HyClone®, Logan, UT, USA) and 4 mM L-glutamine alone (0.15 mM) or supplemented with $CaCl_2$ to a final concentration of 3.0 mM for 30 min. Total protein from whole cells was harvested using Nonidet P-40 lysis buffer. Cell lysates were gently rocked for 30 min at 4°C and then centrifuged at 14,000 x *g* for 5 min (4°C). The supernatants were collected and protein concentration measured by the modified Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 300 µg of protein was used for each array. To prevent nonspecific protein binding, arrays were blocked using

2% BSA in PBS for 1 h at RT. Subsequently, cell lysates were diluted with PBS containing 2% BSA and the arrays were incubated with the diluted cell lysates O/N on a rocker at 4°C. The arrays were then washed three times for 10 min with wash buffer as specified by the manufacturer. Arrays were incubated with a biotinylated detection antibody mixture for 2 h at RT, followed by a washing step and incubation with streptavidin-horseradish peroxidase conjugate for 30 min at RT. Arrays were developed by chemiluminescence (Amersham ECL Plus Western Blotting Detection Reagents, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The array immunoreactivity was quantified using Typhoon™9410 and ImageQuant™ TL Array Analysis software version 7 (GE Healthcare, Piscataway, NJ, USA).

Xenografts in nude *(Foxn1^{nu}/Foxn1^{+})* mice and biochemical analysis

All experimental procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. After baseline blood samples were collected, seven-week-old weight-matched (29-30 g) male *Foxn1^{nu}*/Foxn1^{+} nude mice (Harlan, Indianapolis, IN, USA) were injected subcutaneously over the dorsal scapular area with either 3x10^{6} HARA or HARA shRNA-CaR – GFP expressing cell lines (n=9/group). Subcutaneous tumors were observable seven days after injection and weekly tumor volume measurements in mm^{3} were recorded using automated calipers. Additional xenograft studies used the same age, sex and strain of mice; however, mice

were injected subcutaneously over the dorsal scapular area with either $3x10^6$ BEN Australia cells transduced with GFP wild type CaR (n=15), $3x10^6$ BEN Australia cells transduced with GFP expressing CaR that posses the R990G single nucleotide polymorphism (n=16) or $3x10^6$ BEN Australia cells (n=10).

Animals were monitored and weighed every other day and when any of the following conditions occurred: (1) tumors were greater than ≥ 1.0 cm^3, (2) cancer-induced loss of muscle mass or (3) body weight decreased by ≥ 5%, total calcium concentrations were measured to determine if the mice were hypercalcemic. Blood collection was performed via puncture of the mandibular facial artery or vein with a twenty-two gauge needle and collected into a microtainer tube with lithium heparin (Becton, Dickinson and Company, NJ, USA). Plasma total calcium concentrations were measured in 10 μl of heparinized plasma using the Vitros DT-60 II clinical chemistry analyzer (Johnson & Johnson, Cornilla, GA, USA). Animals were considered hypercalcemic when total plasma calcium concentrations were ≥ 12 $mgdl^{-1}$.

Statistics

Results were expressed as the mean±s.e.m. of triplicate or quadruplicate measurements with the exception of the PTHrP secretions studies in which the results are expressed as mean±s.d. Statistical comparisons were made using the two-tail analysis of the Student's *t*-test for paired groups. A probability value of

P<0.05 was considered to be significant. Analysis of variances (ANOVAs) with repeated measures were used to analyze the time differences between groups. Plots with the variable time (2, 4, 8, and 24 h) in the x-axis are reported to show the trends over time.

3.5 RESULTS

Calcium-sensing receptor mRNA and protein were expressed in human lung squamous cell carcinoma lines

To analyze if CaR mRNA and protein were present in lung SCC, RT-PCR and Western blots were performed. Structure-function relationships of CaR have been traditionally studied using CaR-transfected HEK293 cells. The widespread use of this model system resulted from literature that reported that HEK293 cells do not express endogenous CaR.(17-20) Therefore, before using a derivative of HEK293 cell line as a negative control for CaR, we explored CaR expression in a HEK293 cell line that was transformed with a temperature-sensitive gene for SV40 T-antigen and is highly transfectable (HEK293T/17). In addition, we evaluated cells designated as HEK293 obtained from two different labs for the presence and sequence analysis of CaR. The cells obtained from the different labs had been originally purchased from ATCC. The breast cancer line, MDA-MB-231, PC-3 human prostate cancer line, whole human kidney tissue and an additional HEK293 line were used as positive controls for CaR expression.(14) Primers designed to amplify a 481-bp human CaR product were used for RT-

PCR reactions. The CaR product was amplified from the cDNA of all lung SCC lines as well as MDA-MB-231 and HEK293T cells (Figure 3.1A). DNA sequence analysis revealed > 99.8% homology with the corresponding region of the human parathyroid CaR cDNA.

Expression of CaR protein was analyzed by immunoblotting with an anti-CaR polyclonal antibody that recognizes the extracellular domain of the receptor. Figure 3.1B demonstrates the presence of strong immunoreactive bands observed at 130 kDa which are representative of the mannose-modified intracellular form of CaR present in human whole kidney lysate (positive control), HEK293T cells and all lung SCC lines (Figure 3.1B). These results demonstrate that CaR mRNA and protein are present in lung SCC and HEK293T cell lines. A suitable negative control cell line for CaR is still under investigation.

Treatment of lung SCC with increasing extracellular calcium chloride concentrations for 24 h does not decrease viability

A colorimetric (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (MTT) assay was used to assess cell viability of the three lung SCCs lines and HEK293T cells after 24 h exposure to increasing $[Ca^{2+}]_o$ and spermine.

There were no significant effects on cell viability of any of the lung SCCs (Figures 3.2B-D); however, a dose-response decrease in cell viability was seen in the HEK293T cells, reaching statistical significance ($P < 0.5$ *vs.* 0.15 mM) after 24 h spermine treatment (Figure 3.2A).

Increasing $[Ca^{2+}]_o$ induces PTHrP mRNA expression and secretion by lung SCC cells in vitro

To determine whether increasing $[Ca^{2+}]_o$ and spermine act as CaR activators in lung SCC, we selected three different lung SCC lines, two derived from primary pulmonary tumors and one derived from a bone metastasis. All lines have been reported to express moderate to high levels of PTHrP.(21-23) Cells were treated with increasing calcium concentrations (0.15 = no added calcium chloride, 1.0, 3.0, 5.0 mM for the expression studies and 0.15, 0.5, 1.0, 3.0, 5.0 and 2.0 mM spermine for the secretion studies) for 2, 4, 8 and 24 h. The concentrations of Ca^{2+}_o used in this study were selected to range from the serum ionized calcium of a normal patient, 4 $mgdl^{-1}$ (1.0 mM) through calcium concentrations equivalent to a patient with HHM, 12 $mgdl^{-1}$ (3.0 mM). The $[Ca^{2+}]_o$ of 5.0 mM corresponds to an ionized calcium value of 20 $mgdl^{-1}$ which is incompatible with life.

Quantitative real time RT-PCR for PTHrP gene expression showed treatment of cells with 3.0 mM $CaCl_2$ for 2, 4, 8 and 24 h resulted in statistically significant increases in PTHrP/GAPDH mRNA ratios as compared to control

(0.15 mM) at all time points in all cell lines except BEN Australia at 2 h (*P< 0.05 *vs.* 0.15 mM) (Figures 3.3A-C).

Extracellular calcium up to the addition of 3.0 mM and the CaR agonist, spermine, stimulated PTHrP secretion from all three lung SCC lines. A statistically significant stimulation of PTHrP secretion at 3.0 mM *vs.* 0.15 mM was present in all cell lines at all time points (Figures 3.4A, 4C, 4E) with the exception of RWGT2 at 8 h (Figure 3.4C) and BEN Australia at 2 h (Figure 4A). PTHrP secretion after treatment with 3.0 mM $CaCl_2$, which correlates to 12 $mgdl^{-1}$ serum ionized calcium, was greater than the non-physiologic calcium concentration of 5 mM (20$mgdl^{-1}$) in all cell lines at all time points except RWGT2 at 8 h (Figures 3.4A, 4C, 4E). Interestingly, treatment with 5.0 mM calcium chloride consistently decreased PTHrP expression and secretion in all cell lines (Figures 3.3A-C and Figures 3.4A, 4C, 4E). These data demonstrated that activation of CaR may have regulated PTHrP expression and secretion from the three lung SCC lines.

shRNA-CaR abrogated $[Ca^{2+}]_o$ – mediated PTHrP secretion in lung SCC lines

As our previous experiment indicated that extracelluar Ca^{2+} may have activated CaR-induced PTHrP secretion in the lung SCC lines, we determined if the induction of PTHrP was occurring through CaR; therefore, the time course experiment analyzing conditioned media for PTHrP after treatment with

increasing $[Ca^{2+}]_o$ and spermine was repeated using BEN Australia, RWGT2 and HARA shRNA-CaR cell lines. When comparing PTHrP secretion from non-silenced to the CaR-silenced BEN Australia, RWGT2 and HARA cells at all time points and all $CaCl_2$ treatments, the overall reduction of PTHrP secretion was 31% (23-44%), 55% (48-63%) and 74% (57-80%), respectively (Figures 3.4A-F). BEN Australia PTHrP secretion was maximally silenced to 57% after the 4 h spermine treatment (Figure 3.4A *vs*. Figure 3.4B). Similarly, treatment of shRNA-CaR RWGT2 cells with spermine showed the overall greatest reduction of PTHrP secretion at 63%. In addition, the maximum inhibition for a single time point and concentration was at the 24 h spermine treatment which resulted in a 70% reduction of PTHrP secretion when compared to non-silenced RWGT2 (Figure 3.4C *vs*. Figure 3.4D). Silencing of CaR in the HARA line was the most robust. Not surprisingly, maximum inhibition of PTHrP secretion of 91% was found at 2 h after treatment with calcium-free media (Figure 3.4E *vs*. Figure 3.4F). These results definitively demonstrated that lung SCCs require CaR in the presence of extracellular calcium to secrete high levels of PTHrP.

Inhibition of CaR expression reduced Ca^{2+}_i mobilization and gene expression

To further characterize the effect of inhibiting CaR expression on Ca_i^{2+} stores, we knocked down endogenous CaR using a lentiviral short-hairpin interfering RNA (shRNA-CaR) and examined gene expression for CaR using reverse

transcriptase PCR. In addition, the biophysical impact of CaR knockdown on Ca_i^{2+} release after stimulation with Ca_o^{2+} was evaluated using real-time confocal microscopy. Transduction of shRNA-CaR reduced the 481-bp CaR product detectable by RT-PCR in all lung SCC lines when compared to non-transduced cells, cells transduced with scrabbled CaR sequence or an empty vector construct (Figure 3.5A). The traces shown in Figures 5B-D represent the average $Ca^{2+}{}_i$ release after inactivation of the endogenous CaR with shRNA. Markedly decreased $Ca^{2+}{}_o$-induced $Ca^{2+}{}_i$ responses were recorded in all shRNA-CaR lung SCC lines. The traces show that raising the external [Ca^{2+}] from 0.15 mM to 3.0 mM resulted in a reduction in cellular responsiveness to $Ca^{2+}{}_o$ in all lung SCCs as represented by a relatively non-oscillatory state of fluorescence decay (Figure 3.5B). This data supports that the effects of increasing $[Ca^{2+}]_o$ on Ca_i^{2+} oscillations are Ca^{2+} dependent through the activation of CaR.

Analysis of the MAPK signaling pathway in BEN Australia, BEN Australia shRNA-CaR HARA and HARA shRNA-CaR lines

In order to identify the molecular pathway involved in coupling the reported PKC activation to CaR-activated PTHrP secretion we used a human phosphoMAPK array to assess the relative level of phosphorylation of MAPKs in the BEN Australia and HARA cell lines and shRNA-CaR lines. The phospho-MAPK array detected strong phosphorylation of ERK1 (MAPK3) ERK2 (MAPK1) and JNK2 in both cell lines in response to calcium stimulation as well as a decrease in the

phosphorylation of both ERK1/2 in the companion shRNA-CaR lines under the same treatment conditions (Figures 3.6A and B). Thus, both ERK and JNK modules are activated to different extents in BEN Australia and HARA lines.

We next examined the temporal activation of the MAPKs ERK1/2 which have been implicated in mediating the CaR postreceptor signaling events leading to PTHrP expression and secretion. As shown by Western blot in Figures 6C and D and quantitative densitometry in Figures 6E and F, treatment of BEN Australia and HARA cells with 3.0 mM $CaCl_2$ for a period of at least five minutes resulted in the induction of ERK1 and ERK2 phosphorylation. An overall greater phosphorylation of ERK2 was present in both cell lines. Maximum ERK2 phosphorylation occurred at 5 minutes in the HARA line and 10 minutes in the BEN Australia line. Phosphorylation in the BEN Australia cell line began to decease at 15 minutes whereas phosphorylation at 10, 15 and 30 minutes in the HARA cell line appeared to wax and wane resulting in prolonged phosphorylation. These results demonstrated that the effect of CaR on PTHrP secretion was mediated, in part, by the MAPK pathway in the BEN Australia and HARA SCC lines.

In Vivo HARA shRNA-CaR silencing increases time to development of HHM

We further investigated if CaR was required for the development of lung SCC-induced HHM by comparing the time to development of HHM between a HARA

SCC xenograft mouse model of HHM (n=9) and a xenograft model created with the HARA shRNA-CaR SCC line (n=9). Kaplan-Meir log rank survival analysis revealed a significant difference (*P*=0.023) between the groups when the onset of hypercalcemia was determined. The HARA line produced hypercalcemia in less time after tumor cell injection than the HARA shRNA-CaR cells (50% of the HARA mice were hypercalcemic in 29 days *vs.* HARA shRNA-CaR in which 50% of the mice were hypercalcemic in 49 days) as shown in Figure 3.7A. Moreover, there were no differences found in the mean tumor volume at the time of euthanasia between the HARA and HARA shRNA-CaR groups (Figure 3.7B), which suggests that the differences in time to development of HHM was not due to differences in tumor volume. No differences in final tumor weights between the two groups of mice were found (data not shown). The average plasma PTHrP concentrations in the HARA mice were ~187% higher (23 *vs.* 8 pM) compared to HARA shRNA-CaR xenografts, however, these data did not reach statistical significance (P=0.11) (Figure 3.7C). Since knockdown of CaR in the HARA cells did not completely abrogate the development of HHM and the endpoint for these studies was the development of hypercalcemia, it was not surprising that there were no differences between total plasma calcium concentrations at euthanasia between the HARA and HARA shRNA-CaR groups (Figure 3.7D). The results suggest that CaR is necessary for the rapid development of HHM in the HARA xenograft model.

Our data to this point indicates that the CaR of lung SCCs regulates PTHrP secretion through the MAPK pathway. Since the frequency of Ca^{2+}_i oscillations have been linked to changes in gene expression (24, 25) we hypothesized that differential stimulation of CaR and the resulting Ca^{2+}_i release as represented by either oscillation frequency or amplitude may account for the differences observed in PTHrP expression and secretion and the development of HHM. We tested this hypothesis by using a series of increasing $CaCl_2$ concentrations to measure the range of $[Ca^{2+}]_o$ from which a release of Ca ions from intracellular calcium stores could be observed using real-time confocal microscopy in the three lung SCC lines and HEK293T cells. For each increment in $[Ca^{2+}]_o$, BEN Australia cells had gradually increasing peaks in Ca^{2+}_i (Figure 8A), whereas RWGT2 cells responded to each incremental increase in Ca^{2+} with low indiscrete peaks of Ca^{2+}_i until stimulation with 1.0 mM Ca^{2+}_o where there was abrupt increase Ca^{2+}_i (Figure 3.8B). The HARA cell line displayed vigorous oscillations manifested as transient discrete peaks of Ca^{2+}_i when challenged with each calcium concentration reflecting concomitant changes in Ca^{2+}_i with a maximum F/F_0 intensity ratio of approximately 2.5 occurring when stimulated with 1.0 mM Ca^{2+}_o (Figure 3.8C). Most importantly, when the HARA cells were treated with just 0.5 mM Ca^{2+}_o a F/F_0 intensity ratio of 2.0 was obtained, which indicated that intracellular calcium release occurred with minimal calcium stimulation at an intensity that was greater than the maximal F/F_0 ratio achieved by stimulation of either the BEN Australia or RWGT2 lines. Finally, the HEK293T cells

demonstrated minimal increases in Ca^{2+}_i with increasing Ca^{2+}_o stimulation until the treatment with 3.0 mM $CaCl_2$ when a dynamic release of Ca^{2+}_i occurred (Figure 3.8D). These data show that the threshold for stimulation of Ca^{2+}_i oscillations by Ca^{2+}_o occured at 0.5 mM for HARA and BEN lung SCC cells, and 1.0 mM for RWGT2 and HEK293T cells. Maximum Ca^{2+}_o evoked changes in Ca^{2+}_i occurred at 3.0 mM for BEN Australia and HEK293T, and 1.0 mM for the RWGT2 and HARA cells. In the HARA SCC line, the Ca^{2+}_i release occurs with significantly greater intensity at a lower Ca^{2+}_o. Therefore, the intracellular calcium release can be described as shifted to the left when compared to the Ca^{2+}_i release from BEN Australia and RWGT2 cells. The shift in Ca^{2+}_i response curve to the left represents an overall gain of function status for the HARA SCC line when its responses are compared to the other two cell lines.

RWGT2 and HARA lung SCC have multiple CaR mutations

To further understand the differences in intracellular calcium responses in each cell line after Ca^{2+}_o stimulation, automated sequencing of up to 15 bacterial clones for each cell type was performed. Sequencing identified five novel missense and one nonsense mutation in the RWGT2 line and four missense mutations in the HARA lung SCC lines, as well as, 10 missense and one nonsense mutation in HEK293T cells. The BEN Australia line had no mutations in its' CaR. Interestingly, the HEK293T cells had only heterozygous mutations that were confined to the extracellular and transmembrane domains of *CaR*. All

of the mutations found in the RWGT2 line were located in the extracellular domain and included a homozygous mutation that encoded a premature stop codon (W458X). This stop codon sequence was also present as a heterozygous mutation in the HEK293T cells. The HARA line had one heterozygous and three homozygous mutations that were located in both the extracellular and intracytoplasmic regions of the receptor and contained a R990G mutation in exon seven which had been previously characterized in genomic DNA isolated from human peripheral blood cells as a single nucleotide polymorphism that results in a gain-of-function of the CaR and increased susceptibility to primary hypercalciuria.(20) Table 1 and Figure 3.9.

Functional assessment of selected CaR mutations

To define the mechanisms of action of several of the novel missense CaR mutations identified in the HARA lung SCC on Ca^{2+} signaling, we measured Ca^{2+}_i oscillations after stimulation with incremental increases Ca^{2+}_o in BEN Australia cells transduced with pCDH cDNA constructs containing the individual CaR mutations G158E, R383G, R990G or with a combination of all three mutations. These mutations were selected for additional study due to the nonconservative amino acid substitution predicted to impact receptor properties. In addition, BEN Australia cells were transduced with the pCDH cDNA lentiviral construct containing the coding region of human CaR (BEN CaR^{WT}). Overexpression of BEN CaR^{WT} in its parent cell line did not substantially increase the cytosolic Ca^{2+}_i

response when stimulated with Ca^{2+}_o compared to the Ca^{2+}_i response in the non-transduced BEN Australia cell line (Figures 3.10A-B). Conversely, ectopic expression of BEN CaR^{G158E} or BEN CaR^{R383G} resulted in profound changes in the cytosolic Ca^{2+}_i release oscillations when compared to BEN CaR^{WT} (Figures 3.10C-D *vs.* 3.10B). The amplitude of Ca^{2+} oscillations were markedly reduced when compared to the BEN CaR^{WT} cells. Surprisingly, overexpression of the BEN CaR^{R990G} mutation did not result in either a robust or a left shift in the cytosolic calcium oscillation curve after treatment with Ca^{2+}_o but rather the response was similar the BEN CaR^{WT} cells after treatment with increasing $CaCl_2$ concentrations. To further understand how the mutations were contributing to the apparent gain of function seen in the HARA SCCs, we ectopically expressed the three nonconservative CaR mutations, G158E, R383G and R990G in the BEN Australia line and evaluated the intracellular cytosolic calcium response. The resulting Ca^{2+}_i oscillations recapitulated the findings in the HARA line, although with less fluorescence intensity (Figure 3.10F). A discrete short transient Ca^{2+}_i release was detected immediately after the addition of 0.5 mM $CaCl_2$ and concommently with each treatment. Release of cytosolic calcium from Ca^{2+}_i stores after stimulation with 0.5 mM $CaCl_2$ was not evident with any of the other CaR mutations.

Overexpression of BEN CaR^{WT} or BEN CaR^{R990G} was sufficient to cause HHM in a normocalcemic lung SCC xenograft model

We reasoned that if CaR was necessary for the development of hypercalcemia that overexpression of the wild type CaR in a normocalcemic lung SCC xenograft model, would induce HHM. Although our *in vitro* functional assessment of the CaR^{R990G} variant did not suggest the presence of an activating mutation, the CaR^{R990G} has been associated with familial clinical diseases of calcium dysregulation. Therefore, we evaluated the ability of CaR^{R990G} to predispose to early onset of HHM. Kaplan-Meir log rank survival analysis revealed a significant difference (P<0.001) between the groups when the onset of hypercalcemia was determined. The BEN CaR^{R990G} line produced hypercalcemia in less time after tumor cell injection than the BEN CaR^{WT} cells (50% of the BEN CaR^{R990G} mice were hypercalcemic in 36 days compared to BEN CaR^{WT} mice where 50% of the mice were hypercalcemic in 49 days) as shown in Figure 11A. Moreover, there were no differences in the mean tumor volume at the time of euthanasia between the BEN CaR^{R990G} and BEN CaR^{WT} groups (Figure 3.11B), which suggests that the differences in time to development of HHM was not due to differences in tumor volume. However, a significant difference in tumor volume was found between the BEN CaR^{R990G} and the BEN Australia xenograft models with the later having an appreciably larger tumor volume reaching the defined tumor volume endpoint for an otherwise normocalcemic model (P<0.05).

The results demonstrate that overexpression of CaR is sufficient for the development of HHM in the BEN xenograft model and acquisition of the CaR^{R990G} mutation predisposes to early onset of HHM.

3.6 DISCUSSION

In this study we have shown that lung SCCs express the CaR and that stimulation of CaR by Ca^{2+}_o results in downstream activation of the MAPK pathway and upregulated PTHrP mRNA expression and secretion. In addition, CaR variants have been identified that when ectopically expressed in the BEN Australia SCC line alters intracellular calcium release. The CaR is a critical factor necessary for the development of HHM since either overexpression of CAR^{WT} or the single nucleotide polymorphism (SNP) CaR^{R990G} is sufficient to induce HHM in a normocalcemic lung SCC xenograft model. Therefore, Ca^{2+}_o acts as a malignant stimulus by activating CaR in lung SCC.

Understanding the effects of CaR variants on both the *in vitro* and *in vivo* calcium threshold has contributed to the understanding of abnormal calcium homeostasis in patients with lung SCC. Cellular expression of different combinations of membrane Ca^{2+} influx and Ca^{2+} release channels, coupled with Ca_i^{2+}-releasing stimuli gives rise to numerous mechanisms for increasing intracellular Ca^{2+} signaling. We have established that lung SCCs respond to

increasing Ca^{2+}_o stimulation in a stimulus-specific manner resulting in concentration-dependent upregulation of PTHrP mRNA expression and secretion. Recognizing that changes in $[Ca^{2+}]_o$ can regulate the gating properties of plasma membrane channels, have non-specific effects on cell excitability, alter cyclic nucleotide-gated channels, or influence responses of sodium and potassium channels, it was important to clarify whether extracellular calcium induced PTHrP responses in the SCCs were mediated by the CaR. Neither Ca^{2+}_o, or spermine had any significant effect on cell viability of the SCC lines, indicating that CaR agonist-stimulated PTHrP secretion was not the result of altered cell number and/or viability. The incremental changes in calcium concentration and resulting *in vitro* SCC PTHrP production correlated well with expected PTHrP concentrations in patients that develop squamous-cell carcinoma-induced HHM. For example, PTHrP expression and secretion were greatest at all time points when cells were treated with 3.0 mM $CaCl_2$ and treatment of SCC lines with amounts of $CaCl_2$ conducive to patient mortality (5.0 mM) caused considerable decreases in both PTHrP mRNA and protein as early as 4 h after treatment. When considering PTHrP production and calcium concentration relevance in the SCC cell lines, Silver *et al.* (26) reported $[Ca^{2+}]_o$ of *in situ* resorbing osteoclasts in osteoporotic bone fragments ranged from 8 mM to a maximum of 40 mM in the erosion sites which was associated with a decrease in the local pH to 4.7. In our studies, both primary pulmonary tumor-derived and the pulmonary bone metastasis-derived cell lines decreased calcium-stimulated

PTHrP when subjected to 5.0 mM $CaCl_2$. These results suggest that extremely high calcium concentrations found in Howship's lacuna with activated osteoclasts or bone surfaces with significant osteoclastic resorption are not needed for the production of CaR-induced PTHrP in SCC cells.

We used short hairpin antisense RNA to silence CaR expression and examine its' effect on PTHrP secretion. The decrease in PTHrP with the shRNA-CaR SCC lines confirmed CaRs' involvement in the activation of GPCR-related intracellular signaling cascades leading to increased PTHrP production, albeit, complete abrogation of PTHrP did not occur. This may reflect the transduction or shRNA efficiency, or indicate involvement of other factors contributing to the control of PTHrP gene expression. Consistent with a central role of the CaR in control of PTHrP in SCC, the shRNA-CaR HARA xenograft model had substantially reduced PTHrP plasma protein concentrations and a 20-day delay in the onset of HHM when compared to the HARA xenografts. The continued ability of the HARA shRNA-CaR model to develop hypercalcemia suggests potential contribution of other humoral factors such as 1,25-$(OH)_2$ vitamin D_3, tumor necrosis factor-α, IL-1, IL-6 and/or tumor microenvironment-derived cytokines in the xenograft model. Certainly transactivation of EGFR by CaR induction of the membrane-associated matrix metalloproteinases, such as ADAM-17, has been reported to occur in the HHM-inducing prostate PC-3 and Leydig H-500 cell lines (27, 28); however we did not see increased EGFR

tyrosine kinase phosphorylation in HARA xenografts relative to cells grown *in vitro*, suggesting that CaR-EGFR transactivation did not significantly contribute to PTHrP production in this model of HHM.(29) Alternatively, the development of HHM may result from CaR signaling through $G\alpha_{q11}$ and PLC, which raises the possibility that PTHrP secretion could be induced by activation of other GPCRs such as the metabotropic pyrimidine-activated P2Y receptor family. P2Y receptors are found on airway epithelia and are activated by the nucleotides, ATP and UTP, in an autocrine or paracrine fashion and use the ERK1/2 signaling pathway. This receptor controls lung epithelial Ca^{2+}_i oscillations (30) and has been shown to regulate proliferation of the NSCLC tumor line A549 in concert with EGF. (31) Future studies will be needed to determine the role of P2Y receptors and CaR for inducing lung SCC PTHrP expression.

The identification of multiple CaR mutations in the SCC and HEK293T lines demonstrates that receptor variants can influence signaling in cancer cells consistent with those discovered in endocrine diseases, as well as through novel mechanisms. Previous studies have suggested that CaR may consist of at least two separate functional domains, one comprising the ECD and the other including the intracellular transmembrane domain 3 (i3) and the carboxyl-tail (C-tail) of the CaR.(32) Reconstitution of receptor-dependent signaling can be achieved by co-transfecting mutants that provide both functional domains(32), which was consistent with our findings in the HARA cell line. However, in the

HARA cells our functional analysis revealed that coexpression of two CaR mutations in ECD with another mutation in the i3 and/or C-tail resulted in a gain of function in relation to calcium threshold. One mechanism for the gain of function is functional complementation in which cells with a heterozygous mutation in one domain swap for a normal domain of the dimerization partner allowing signaling to occur as proposed by Gouldson and Reynolds (33). This mechanism; however, does not explain the activating mutation phenotype seen with homozygous CaR mutations in the HARA cells. Additional studies will be needed to understand the contribution of the HARA homozygous mutations in the ECD and C-tail to receptor activation. Ectopic expression of CaR^{G158E} or CaR^{R383G} in BEN Australia SCCs resulted in severely attenuated amplitudes of extracellular-induced calcium oscillations when compared to BEN Australia CaR^{WT}. This demonstrated a pronounced dominant-negative effect of the mutants on the release of cytosolic Ca^{2+} stores. The dominant- negative effect of the extracellular domain (ECD) point mutations could negatively affect the ligand binding affinities for Ca^{2+}_{o} and/or the cooperatively between the dimerization partner leading to reduced Ca^{2+}_{i} responses.(32) However, the interpretation of our findings in light of previous functional evaluations of CaR ECD mutations are challenging since all published functional CaR studies have been performed in HEK293 cell lines expressing mutant CaR constructs and the reported functions may have been influenced by multiple mutations in the CaR of HEK293 cells similar to the ones found in the HEK293T cell line. Interestingly, the R990G SNP

of the *CaR* gene was associated with primary hypercalciuria in patients with and without renal calculi.(20, 34) Vezzoli *et al.*(20) performed functional studies to evaluate the increase in Ca^{2+}_i caused by increased Ca^{2+}_o in HEK293 cells with the 990G variant allele and determined that the 990G allele resulted in a gain of function of the CaR (although weak when compared to most activating mutations that can produce EC_{50} values up to 1mmol/l).(20, 35) In our studies, BEN Australia cells transduced with the CaR^{R990G} SNP did not have an EC_{50} intracellular calcium release consistent with an activating mutation when compared to BEN Australia cells transduced with CaR^{WT}. This may suggest that mutant receptor dimerization with the endogenous non-mutated CaR could negate any apparent gain of function for this otherwise homozygous mutation. Taken together, the presence of multiple missense mutations within the two domains of CaR had a complex impact on the calcium signaling milieu in lung SCC lines.

The presence of the RWGT2 CaR homozygous nonsense mutation, CaR^{W458X}, was surprising as the full-length monomer protein under reducing conditions was detected by Western blot analysis. Furthermore, treatment with incremental $[Ca^{2+}]_o$ increased PTHrP expression and secretion in concentrations that were comparable to the other SCC lines without the premature stop codon mutation. When considering the Ca^{2+}_o induced intracellular calcium release for this cell line, it was apparent that the RWGT2 CaR was nonresponsive until

treated with 1.0 mM of $CaCl_2$, whereas the BEN Australia SCCs with the normal CaR had a response that began at 0.50 mM $CaCl_2$ with gradual releases of intracellular calcium after each incremental increase in calcium. Since the delayed responsiveness to Ca^{2+}_o was not present in the HARA cell line, the contribution of the homozygous nonsense mutation to deferred receptiveness of the CaR can not be discounted. The presence of the full length, active RWGT2 CaR protein may have resulted from the induction of stop codon readthrough. Promotion of stop codon readthrough in human cells can be induced by three types of factors: aminoglycoside antibiotics, suppressor tRNAs, and factors decreasing termination efficiency, with the greatest levels of readthrough obtained by prolonged treatment with aminoglycosides and suppressor tRNAs.(36) Readthrough efficiency is dependent on the mutation, on the nucleotide context of the stop codon and on other unknown factors involved in readthrough regulation.(37-39) The RWGT2 cell line was cultured in antibiotic-free media, therefore we speculate that codon readthrough in this line may be attributed to the presence of nonsense suppressors which are normal cellular tRNAs that read their cognate sense codons and are able to recognize stop codons through unconventional codon-anticodon base pairings, since natural suppressor tRNAs are scarce in mammalian cells.(40) Alternatively, translation termination initiation may be suppressed by misincoporation of an amino acid at the stop codon.

When the altered codon enters the ribosomal A site for peptide release, continued translation in the original reading frame occurs and results in synthesis of a longer protein.

Deciphering the rules dictating read-through efficiency and how it is influenced by the sequence context of the nonsense codon could be of pharmacological importance in tailoring specific treatments for a patient with HHM since inactivation of high readthrough efficiencies in neoplastic CaR would be desirable.

When considering the clinical relevance of the mutations of CaR in relation to the time of development of HHM in the xenograft models, the mutations in the HARA SCC line provide a collaborative gain of function suggesting that CaR may be activated by normal physiological extracellular calcium concentrations leading to PTHrP production at a much earlier stage of disease. Disruption of CaR function contributed to altered pathophysiology of lung squamous carcinoma cells. Therapeutic strategies directed at restoring normal CaR function may contribute to lessening the sequelae of cancer, particularly HHM, and could play a supporting role in cancer treatments. While the use of negative allosteric modulators (calcilytics) of the CaR which decrease receptor activation seems appealing for use in the patient with HHM, potential limiting factors for their use may be the lack of specificity tumor-associated CaR. For example, a patient with

HHM treated with a calcilyitc may experience unintended consequences of stimulation of endogenous PTH secretion and renal calcium re-absorption with a resultant elevation or unchanged serum calcium concentration. In addition, the role of CaR mutations on the function of these drugs may be important since mutated residues comprising allosteric binding sites for the inhibitors may negate or change the desired effect. Alternatively, blocking these effects by targeting a proximal downstream signaling molecule of the CaR may be a future approach.

In conclusion, lung SCCs posses CaR and stimulation of CaR by Ca^{2+}_{o} leads to enhanced PTHrP release through the MAPK pathway. The CaR is necessary and sufficient for the rapid development of HHM and may predispose a patient to an advanced onset of this debilitating syndrome.

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Figure 3.1 Calcium-sensing receptor mRNA and immunoreactivity in BEN Australia, RWGT2 and HARA cells.

(**A**) Reverse transcription-PCR (RT-PCR) amplification of CaR from HEK293T embryonic cells (HEK) (historical negative control for CaR expression) (lane2), BEN Australia (BEN), RWGT2 (RW) and HARA (HA) lung SCC lines (lanes 3-5) and an additional HEK293T (HEK) cell line (lower lane 2) as well as the positive controls (+Cntrls) consisting of human kidney tissue (KID), the human PC-3 prostate carcinoma cell line (PC-3), and the breast cancer cell line MDA-MB-231(MDA) (lanes 3- were visualized by ethidium bromide staining. Lane 1 is the ladder showing the 500 bp mark. CaR-specific human primers, amplified a single 481-bp product in all cell lines, including the historical negative control HEK293T cells obtained from ATCC and an additional laboratory. DNA sequence analysis revealed >99% identity with the corresponding region of human parathyroid CaR cDNA. (**B**) Relative CaR expression by western blots of cell extracts from adult human kidney (KID) (+ control) lane 1, HEK293T cells (HEK) from ATCC lane 2, and lung SCC lanes 3-5 (BEN, RW, HA). Equal amounts of total protein (30 μg) were separated by electrophoresis using SDS-PAGE gels, transferred onto nitrocellulose membranes, and detected with rabbit anti-human CaR polyclonal antibody. Blots were stripped and reprobed with a polyclonal antibody to β-actin. Experiments were repeated three times and data from a representative blot are shown.

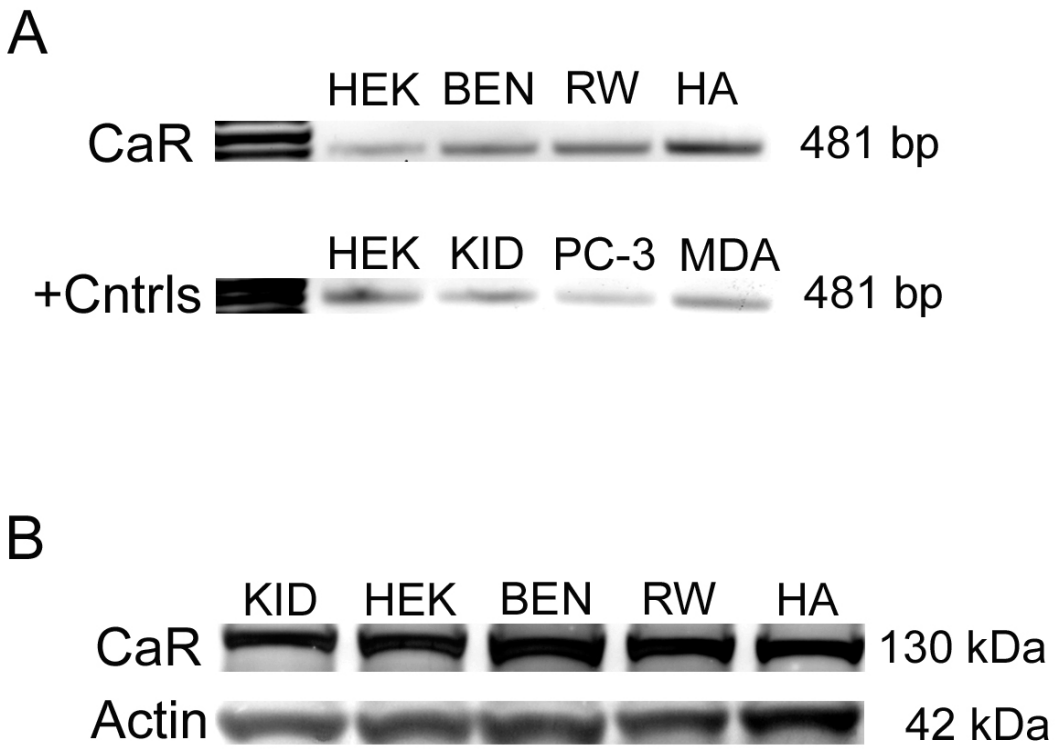

Figure 3.1 Calcium-sensing receptor mRNA and immunoreactivity in BEN Australia, RWGT2 and HARA cells.

Figure 3.2 Cell viability decreased for HEK293T cells but not lung SCCs when treated with increasing extracellular calcium concentrations.
(**A-D**) MTT assays were used to measure the cytotoxicity of elevated extracellular calcium concentrations and 2 mM spermine on HEK293T and lung SCC lines after 24 hours of treatment. (*P<0.05 *vs.* 0.15 mM)

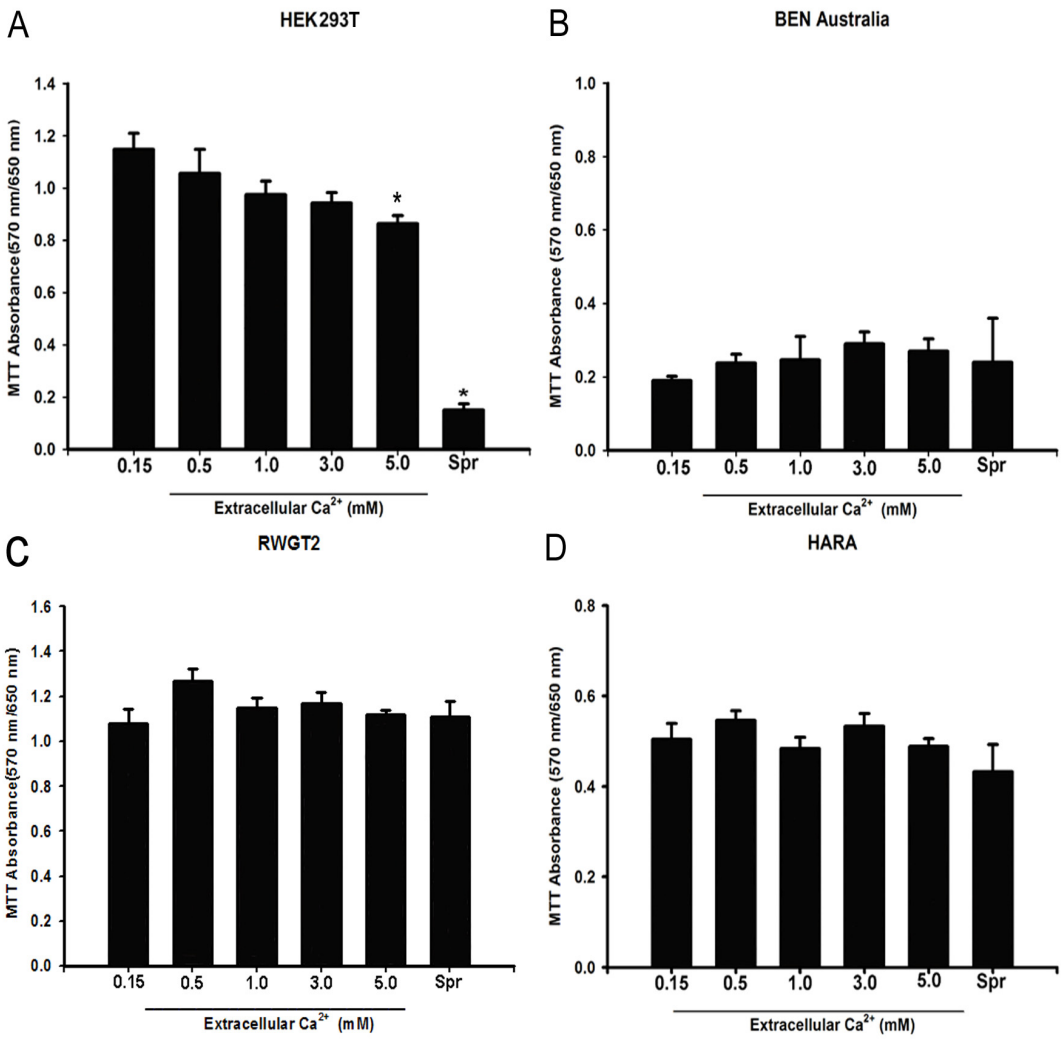

Figure 3.2 Cell viability decreased for HEK293T cells but not lung SCCs when treated with increasing extracellular calcium concentrations.

Figure 3.3 Escalating extracellular calcium concentrations increased PTHrP mRNA levels of lung SCC lines measured by quantitative real-time RT PCR. (**A-C**) Addition of 3.0 mM $CaCl_2$ to lung SCC cultures for 2, 4, 8 and 24 h resulted in significant increases in PTHrP/GAPDH mRNA ratios as compared to controls (0.15 mM) at all time points in all cell lines except BEN Australia at 2 h. The relative ratios of PTHrP mRNA to GAPDH mRNA levels were expressed as mean±s.e.m. of three cultures per time point and per concentration (*P< 0.05 *vs.* 0.15

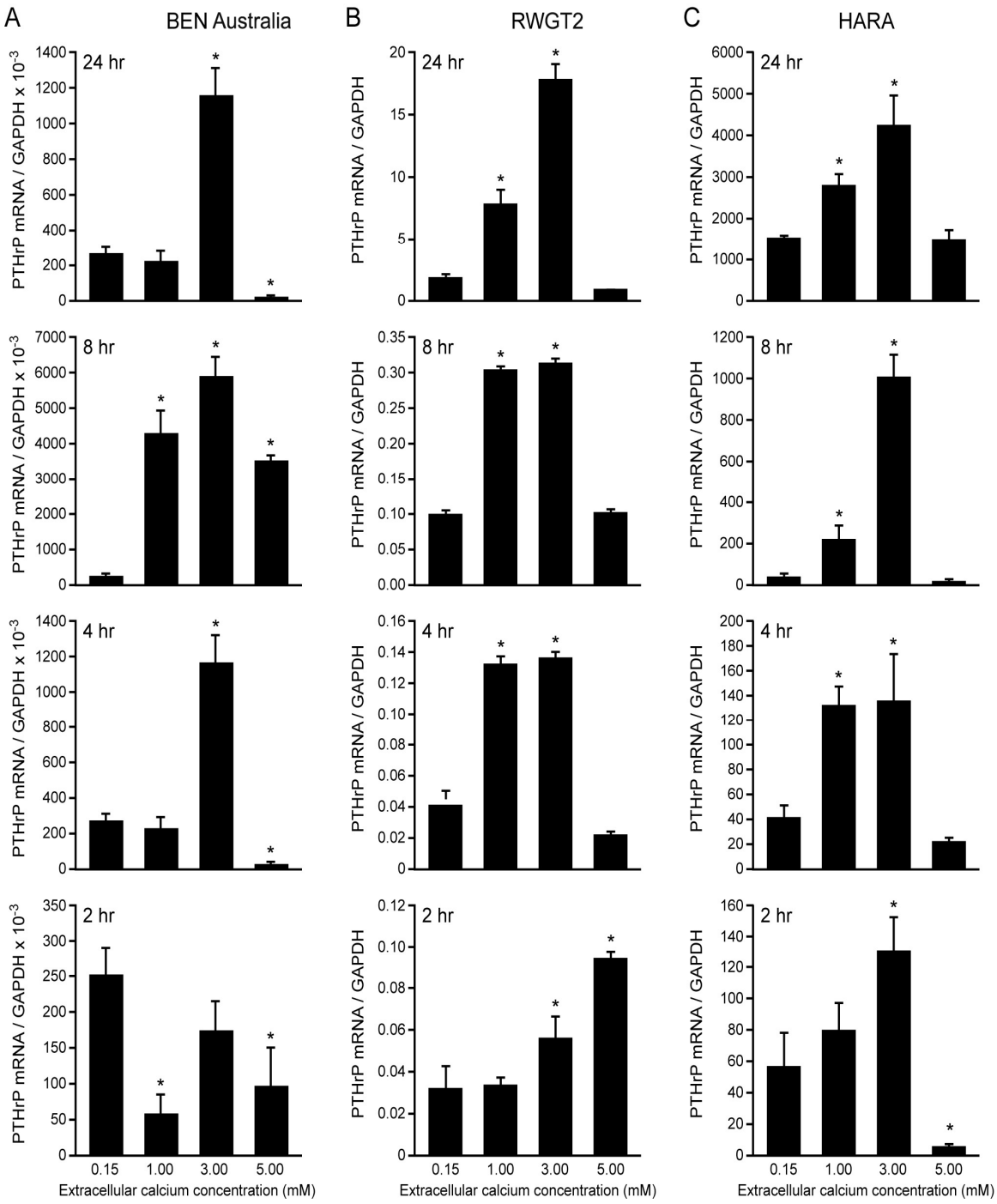

Figure 3.3 Escalating extracellular calcium concentrations increased PTHrP mRNA levels of lung SCC lines measured by quantitative real-time RT PCR.

Figure 3.4 Effect of $[Ca^{2+}]_o$ on secretion of PTHrP from lung SCC and cells transduced with CaR shRNA.

PTHrP concentrations were measured in conditioned media harvested from BEN Australia, RWGT2 and HARA SCCs (**A, C** and **E**) exposed to increasing concentrations of Ca^{2+} or spermine (2 mM) for 2, 4, 8 and 24 h. CaR activation increased PTHrP concentrations at all time points with an EC_{max} of approximately 3.0 mM $CaCl_2$ for all lung SCCs at all time points with the exception of RWGT2 at 8 h and excluding the positive control treatment of spermine. Each PTHrP value is the mean±s.d. of three experiments. * denotes a significant difference between treated and cells grown in 0.15 mM (**P*<0.05 *vs.* 0.15 mM)

The knockdown of *CaR* in lung SCC using shRNA (shRNA-CaR) reduced PTHrP secretion from all lung SCC lines (panels **B, D** and **F**). The conditioned media was collected under the same conditions as for the companion cell line. When comparing PTHrP secretion from non-silenced to the CaR-silenced BEN Australia (**A** *vs.* **B**), RWGT2 (**C** *vs.* **D**) and HARA (**E** *vs.* **F**) cells, the overall (all time points and all $CaCl_2$ treatments) reduced of PTHrP secretion was 31% (23-44%), 55% (48-63%) and 74% (57-80%), respectively.

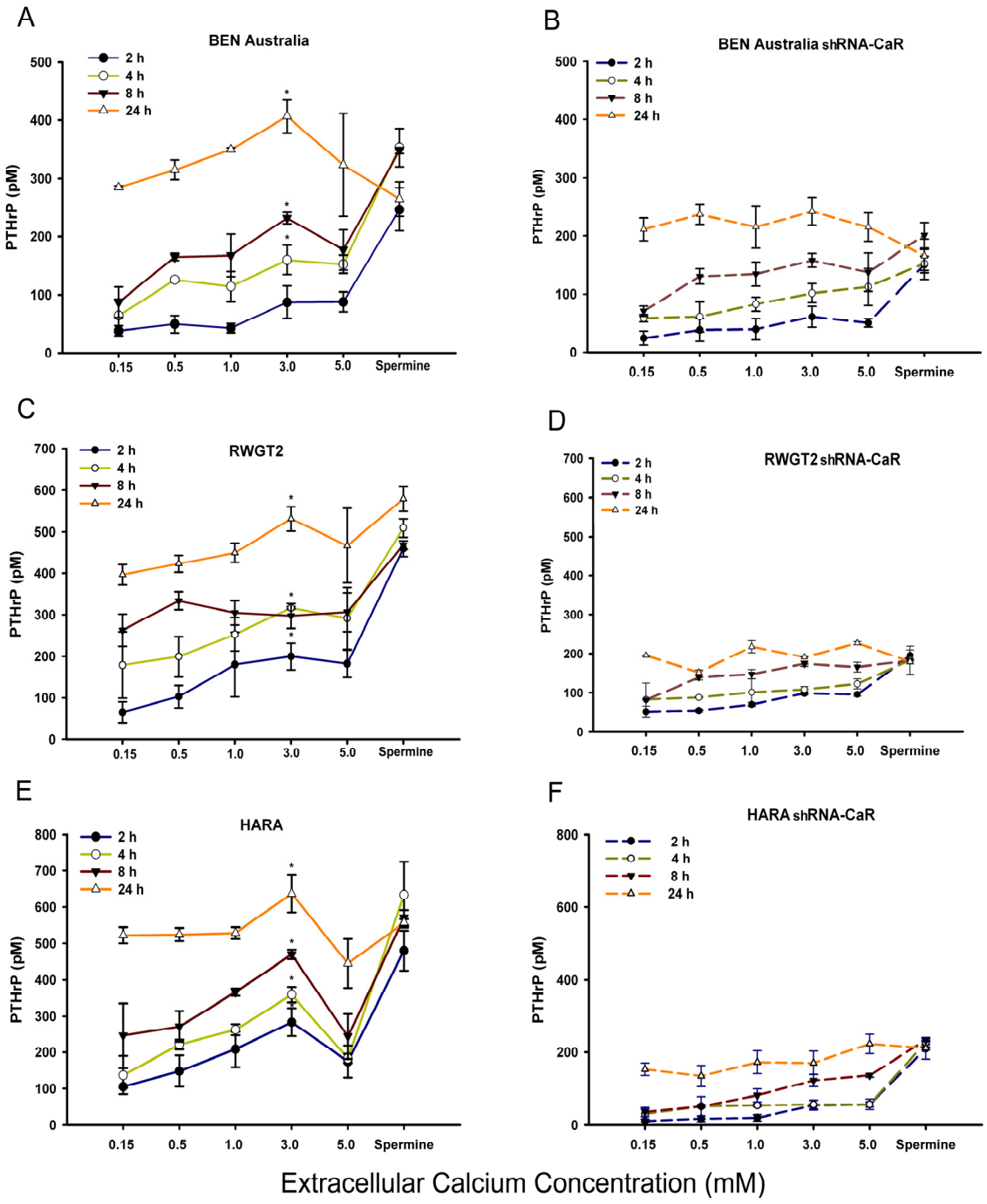

Figure 3.4 Effect of $[Ca^{2+}]_o$ on secretion of PTHrP from lung SCC and cells transduced with CaR shRNA.

Figure 3.5 Lung SCCs transduced with shRNA-CaR had decreased receptor mRNA expression and diminished intracellular calcium release.
(**A**) Total cellular RNA was collected from three lung SCCs transduced with lentiviral RNA interference knockdown for CaR (Si2) and harvested for RT-PCR amplification. Additional control cells included non-transduced lung SCC lines (NC), cells transduced with scrabbled-control RNA (SC), and cells expressing the empty lentiviral vector (EV).
(**B**) The graphs are representative of rhod-2 fluorescent intracellular calcium responses observed in typical experiments using each lung SCC shRNA-CaR line. A series of increasing incremental $CaCl_2$ concentrations were used to assess the range of $[Ca^{2+}]_o$ from which a discrete release of Ca^{2+} ions from intracellular calcium stores could be observed using real-time confocal microscopy. Intracellular calcium baseline was measured in 0.15 mM Ca^{2+} bathing solution, and $Ca^{2+}{}_i$ oscillations were initiated by the addition of 0.50 mM Ca^{2+}. The two panels below intracellular calcium response curves labeled (**a**, **b**) are confocal microscopy images depicting rhod-2 loaded cells at baseline before stimulation (**a**) and subsequent emittance of fluorescence upon stimulation with 3.0 mM $Ca^{2+}{}_o$ in the same cell, (**b**) the third panel depicts green fluorescent protein (GFP) expression in the same cell. 10X

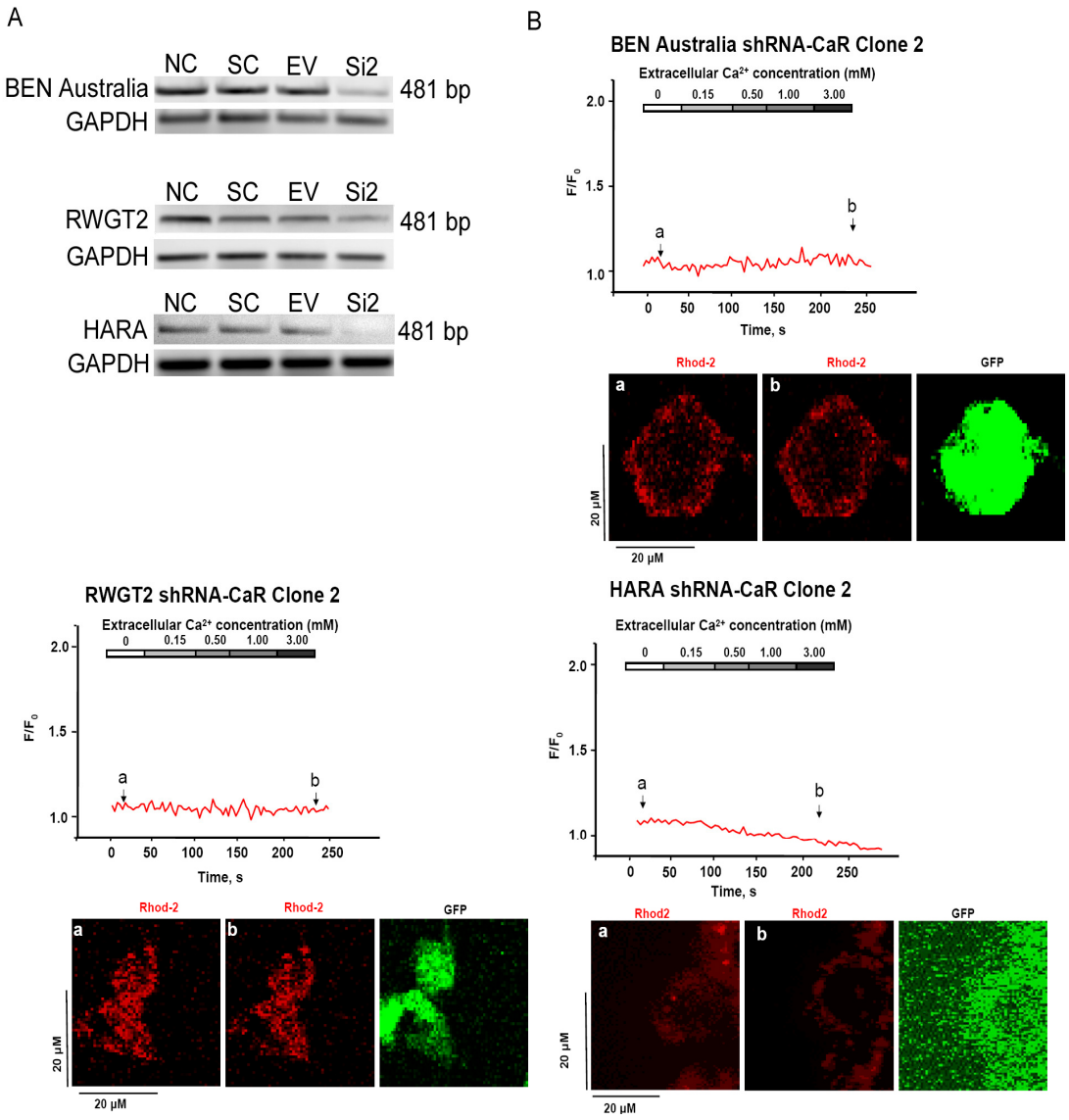

Figure 3.5 Lung SCCs transduced with shRNA-CaR had decreased receptor mRNA expression and diminished intracellular calcium release.

Figure 3.6 Treatment with 3.0 mM Ca^{2+}_o induced ERK1/2 phosphorylation in BEN Australia and HARA cells *in vitro*.

The human phospho-MAPK array was used to detect multiple phosphorylated kinases in human lung SCCs with either an intact CaR or a silenced CaR after stimulation with $CaCl_2$. BEN Australia, BEN shRNA-CaR and HARA, HARA shRNA-CaR cells, $3x10^6$ cells/100 mm-plate were seeded in duplicates and grown to ~80% confluency. Twenty-four hours after seeding, growth medium was changed to 0.15 mM calcium-free DMEM supplemented with 10% dialyzed FBS and L-glutamine for 4 hours. Cells were washed with DPBS and medium changed to DMEM with 3.0 mM $CaCl_2$, 10% dialyzed FBS and L-glutamine for 30 min. Cells were harvested. Arrays were incubated with 300 *u*g of cell lysate and were performed in duplicate. Panels **A** and **B** depict the phosphorylation profile of BEN Australia and HARA lines as represented by normalized pixel volumes. (**C** and **D**)Proteins extracted from BEN Australia and HARA SCCs at 0, 1, 5, 10, 15 and 30 min after stimulation with 3.0 mM $CaCl_2$ were analyzed by Western blot using an antibody against ERK1/2 phosphorylation, total ERK and GAPDH as a loading control. E and F. Quantitative analysis of Western blots by densitometry. The levels of phosphorylated ERK1/2 (p-ERK1/2) were normalized to total ERK (t-ERK) (top) and GAPDH (bottom) in both BEN Australia and HARA lines. Cell lines were treated with 3.0 mM $CaCl_2$ supplemented media for the indicated times. Results were generated from three independent experiments. *Bars*, S.E.M. * ERK2 and ** ERK1, $P < 0.05$, compared to 0 time point (t-test).

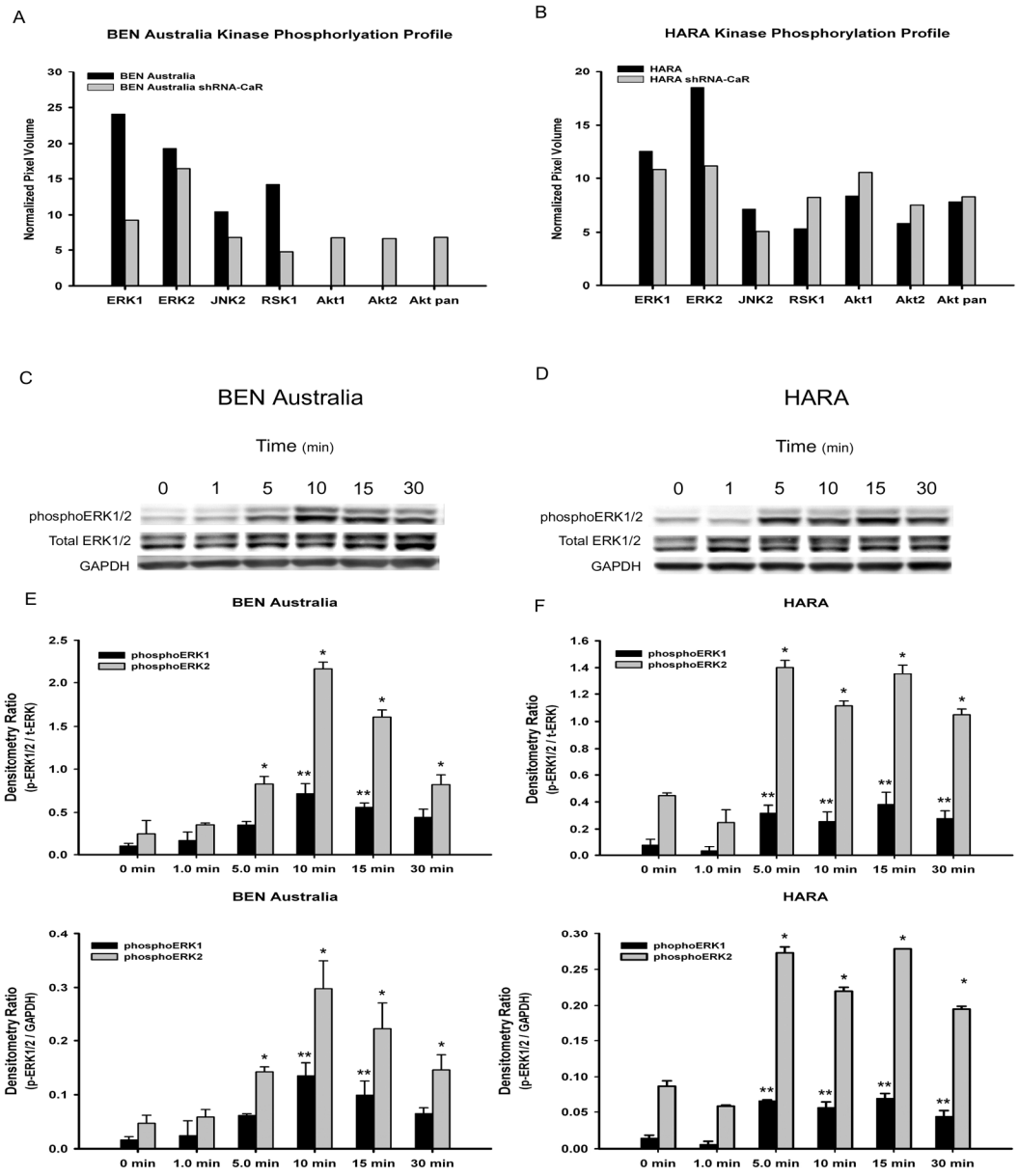

Figure 3.6 Treatment with 3.0 mM Ca^{2+}_o induced ERK1/2 phosphorylation in BEN Australia and HARA cell lines *in vitro*

Figure 3.7 The calcium-sensing receptor was necessary for the rapid development of HHM in the HARA xenograft model.
(**A**) Kaplan-Meir analysis showed a statistically significant difference in the time to development of HHM between the HARA (black) and HARA shRNA-CaR (blue) with fifty percent of HARA mice developing HHM 29 days after injection compared to the HARA shRNA-CaR xenograft mice that developed HHM 49 days after injection of the cells. (*P*=0.023) HARA (n=9) tumors and HARA shRNA-CaR (n=9). (**B**) Average tumor volume at time of hypercalcemia. When hypercalcemia was diagnosed, the HARA (n=9) tumor volume was not statistically different from the HARA shRNA-CaR (n=9) tumor volume and tumor volume did not reach early removal criteria. (**C**) Comparison of plasma PTHrP concentrations in hypercalcemic mice with HARA (black) (n=9) and HARA shRNA-CaR (blue) (n=9) xenografts. Plasma PTHrP concentrations were measured at the time of development of HHM and before xenografting (baseline). Average plasma PTHrP concentrations in the HARA mice were 187% greater (23 *vs*. 8 pM) compared to the HARA shRNA-CaR mice. (*P*=0.11) (**D**) Comparison of plasma total calcium concentrations in HARA and HARA shRNA-CaR mice at the time of hypercalcemia and before xenografting (baseline). No difference in total calcium concentrations were found between the two HARA xenograft groups.

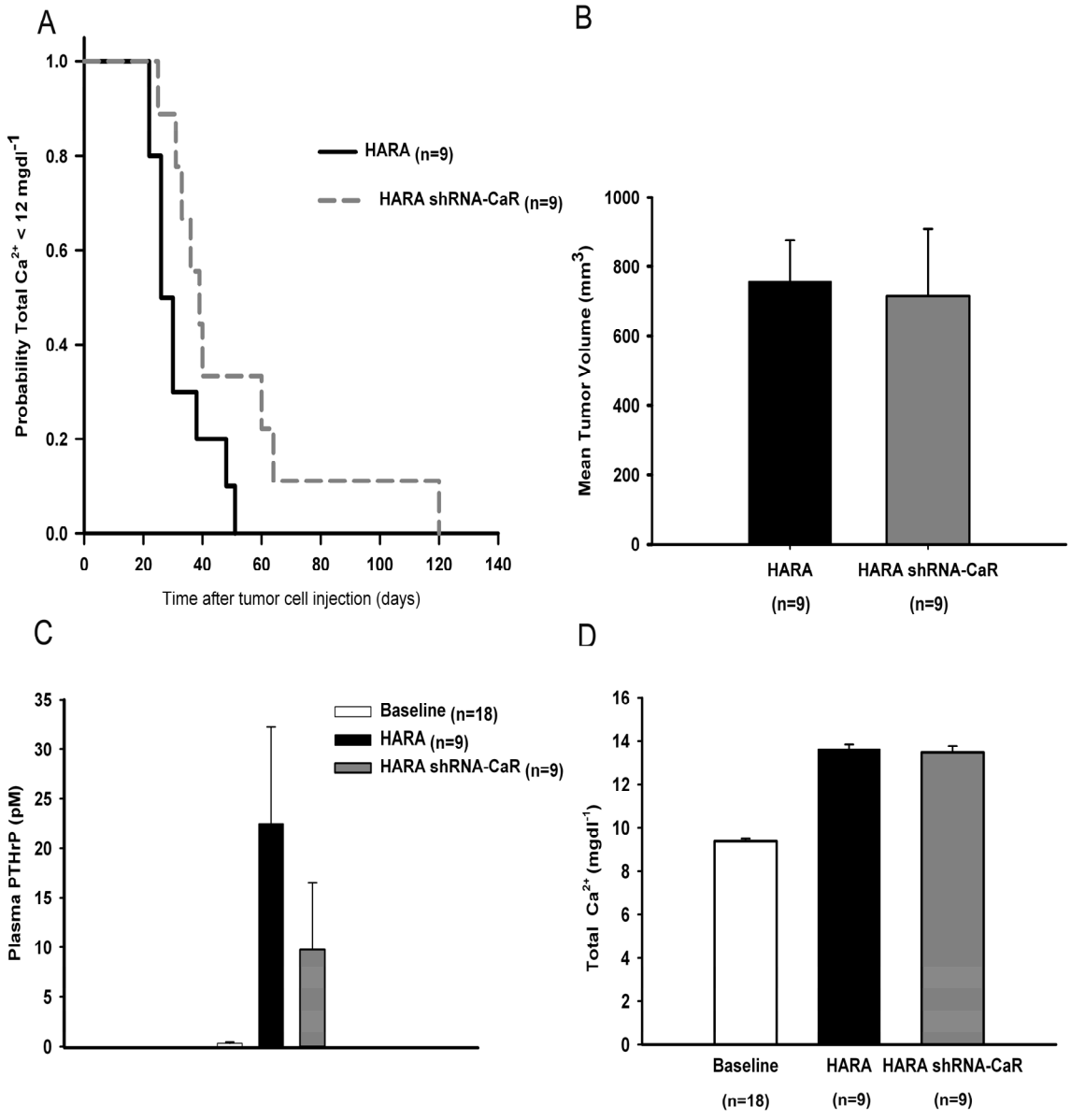

Figure 3.7 The calcium-sensing receptor was necessary for the rapid development of HHM in the HARA xenograft model.

Figure 3.8 Treatment of lung SCCs with increasing $[Ca^{2+}]_o$ induced a biphasic Ca^{2+}_i rise in BEN Australia, RWGT2 and HARA cells .
(**A-D**) The graphs are representative of fluo-3 fluorescent intracellular calcium responses observed in typical experiments using each lung SCC line and HEK293T cells. Intracellular calcium baseline was measured in 0.15 mM Ca^{2+} bathing solution, and Ca_i oscillations were initiated by the addition of 0.50 mM Ca^{2+}. The two panels below each intracellular calcium response curve labeled (**a**, **b**) are confocal microscopy images depicting fluo-3 loaded cells at baseline (**a**) and subsequent emittance of fluorescence upon stimulation with Ca^{2+}_o (**b**). 10X

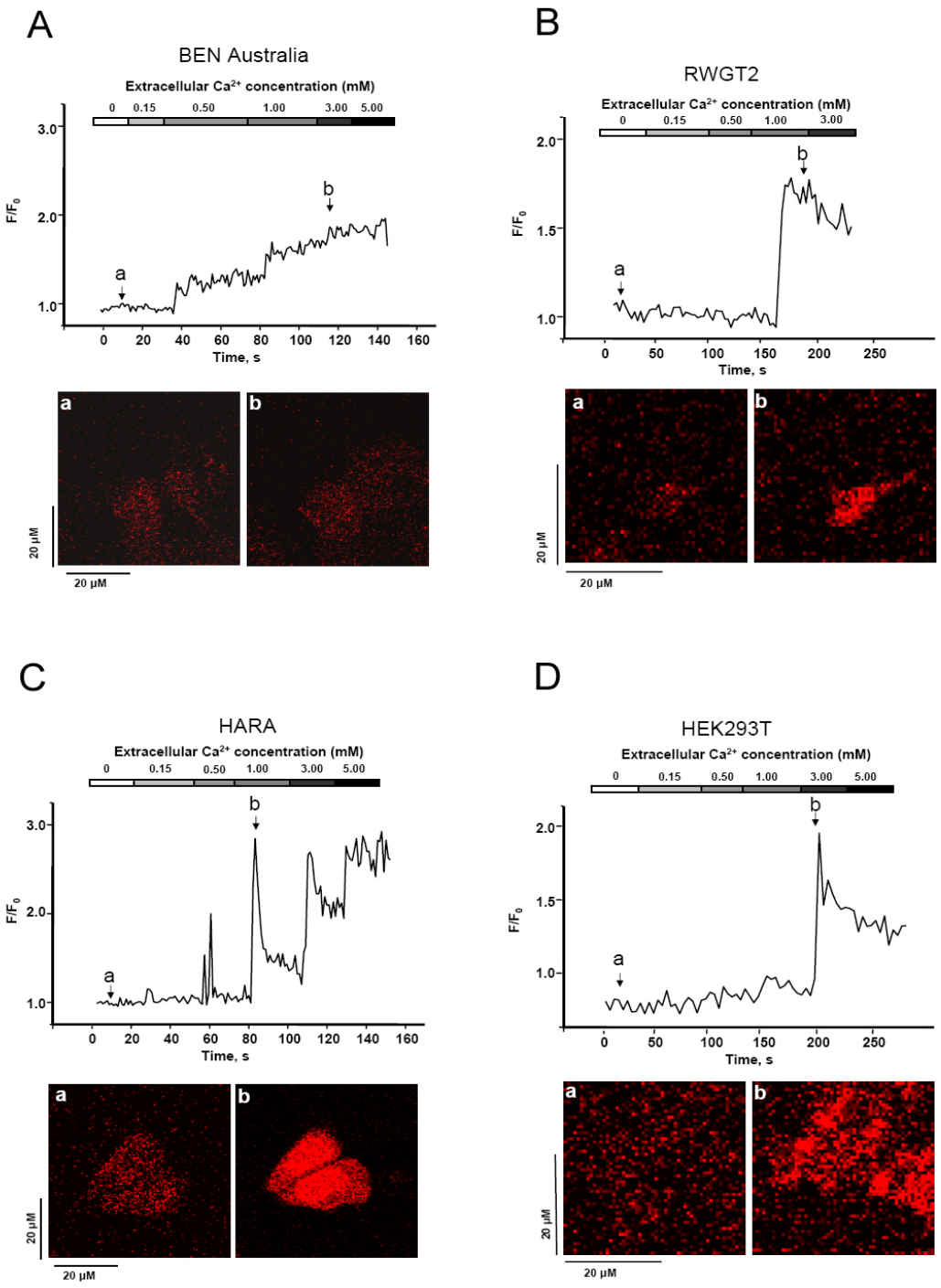

Figure 3.8 Treatment of lung SCCs with increasing $[Ca^{2+}]_o$ induced a biphasic Ca^{2+}_i rise in BEN Australia, RWGT2 and HARA cells .

Domain	Amino Acid Change	Exon	Codon Change	Function	Cell Type			
					HEK293T	BEN Aus	RWGT2	HARA
Extracellular	G35E	2	GGG→GAG	Unknown			Heterozyg	
	I75V	3	AUA→GUA	Unknown			Heterozyg	
	G158E	3	GGG→GAG	Unknown				Heterozyg
	R383G	4	AGG→GGG	Unknown	Heterozyg		Homozyg	Homozyg
	I427V	4	AUU→GUU	Unknown	Heterozyg		Homozyg	Homozyg
	W458X	4	UGG→UGA	Unknown	Heterozyg		Homozyg	
	V504A	5	GUG→GCG	Unknown	Heterozyg			
	C542R	6	UGC→CGC	Unknown	Heterozyg			
	C582R	6	UGU→CGU	Unknown	Heterozyg			
	N583D	7	AAC→GAC	Unknown	Heterozyg		Homozyg	
	F612L	7	UUU→CUU	Unknown	Heterozyg			
Transmembrane	L659P	7	CUC→CCC	Unknown	Heterozyg			
	Q735R	7	CAG→CGG	Unknown	Heterozyg			
	F792Y	7	UUC→UAC	Unknown	Heterozyg			
Cytoplasmic	R990G	7	AGG→GGG	Gain				Homozyg

Table 3.1

Mutations present in HEK293T and lung SCC lines.

Figure 3.9 Schematic of the calcium-sensing receptor in human SCC cells demonstrating the locations of mutations.
(**A-D**) Topographical representation of the human CaR showing the position of the indentified mutations. Individual amino acids are represented by red circles; blue circles represent mutations. Heterozygous or homozygous mutations are denoted (het) and (hom) respectively.

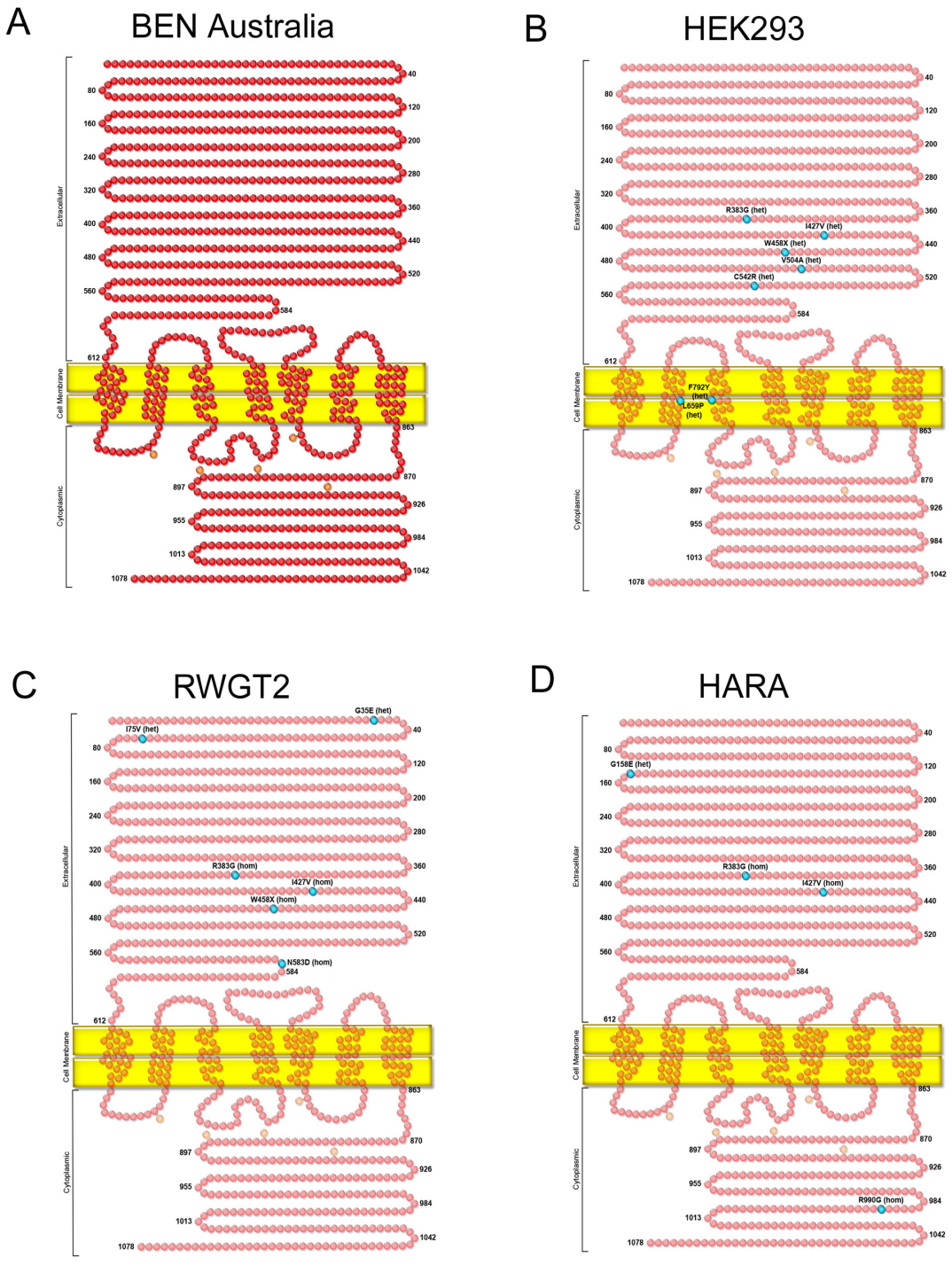

Figure 3.9 Schematic of the calcium-sensing receptor.

Figure 3.10 Extracellular calcium stimulated intracellular calcium release in BEN Australia or BEN Australia SCC transduced with *CaR^{WT}*, *CaR^{G158E}*, *CaR^{R383G}*, *CaR^{R990G}* or *$CaR^{G158E, R383G, R990G}$*.

(**A-F**) The graphs are representative of rhod-2 fluorescent intracellular cytosolic calcium oscillations observed in BEN Australia SCC, and BEN Australia SCC lines stably transduced with a GFP-expressing construct for either CaR wild type receptor (CaR^{WT}), or the CaR mutations, CaR^{G158E}, CaR^{R383G} CaR^{R990G}, or $CaR^{G158E, R383G, R990G}$. Intracellular calcium baseline was measured in 0.15 mM Ca^{2+} bathing solution, and Ca_i oscillations were initiated by the addition of 0.50 mM Ca^{2+}. The two panels below the intracellular calcium response curves labeled (**a, b**) are confocal microscopy images depicting rhod-2 loaded cells at baseline (**a**) and subsequent emittance of fluorescence upon stimulation with Ca^{2+}_o (**b**). The third panel in Figures **B-F** depicts GFP expression from BEN Australia cells with the GFP–CaR fusion protein. 10X

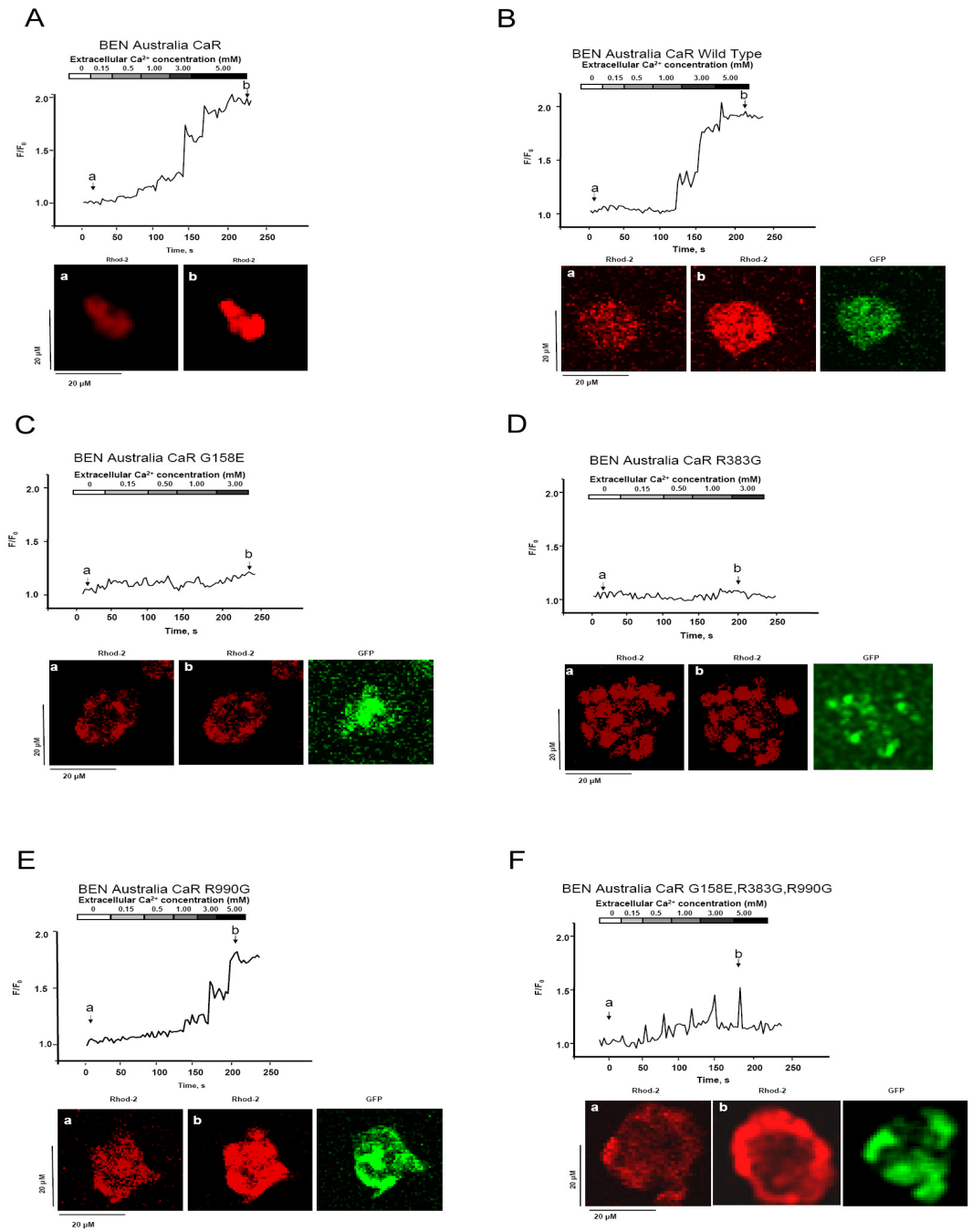

Figure 3.10 Extracellular calcium stimulated intracellular calcium release in BEN Australia or BEN Australia SCC transduced with *CaR*^{WT}, *CaR* ^{G158E}, *CaR* ^{R383G}, *CaR* ^{R990G} *or CaR*$^{G158E, R383G, R990G}$.

Figure 3.11 Comparison of the xenograft models BEN Australia overexpressing *CaR^{WT}* and BEN Australia overexpressing *CaR^{R990G}*.
(**A**) Kaplan-Meir analysis showed a statistically significant difference in the time to development of HHM between the BEN CaR^{WT} (green) and BEN CaR^{R990G} (blue) with fifty percent of BEN CaR^{R990G} mice developing HHM 36 days after injection compared to the BEN CaR^{WT} xenograft mice that developed HHM 49 days after injection of the cells. (P<0.001) BEN CaR^{WT} (n=15) and BEN CaR^{R990G} (n=16). (**B**) Average tumor volume at time of hypercalcemia. When hypercalcemia was diagnosed, the BEN CaR^{WT} (n=15) tumor volume was not statistically different from the BEN CaR^{R990G} (n=16) tumor volume, however, a statistal difference in tumor volume was present between BEN CaR^{R990G} and the BEN Australia control group (n=10) (P<0.05).

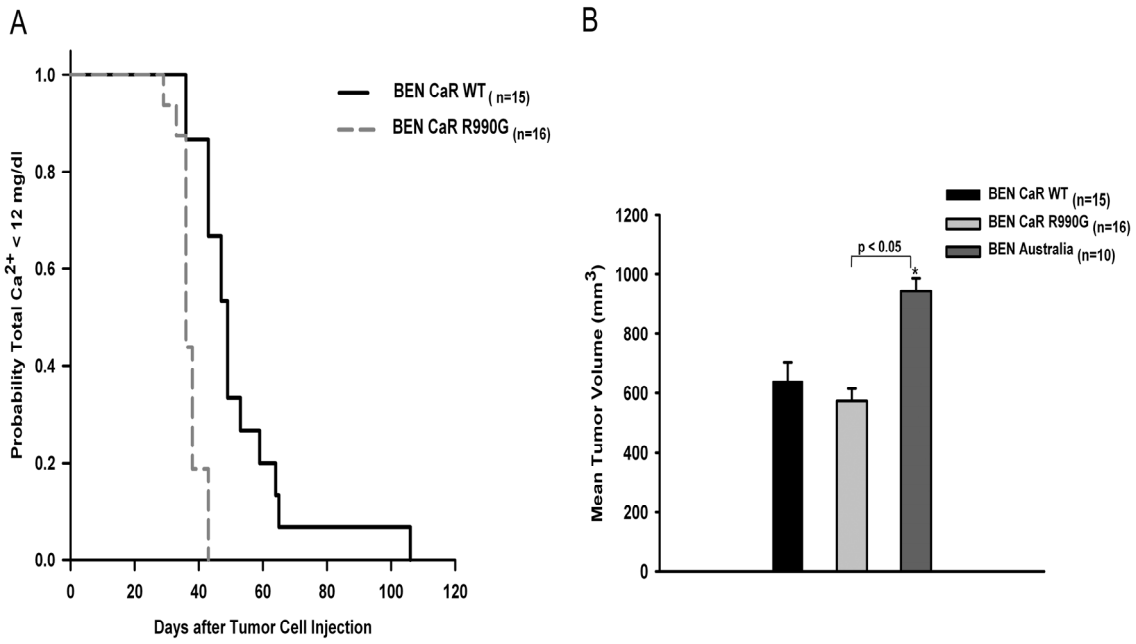

Figure 3.11 Comparison of the xenograft models BEN Australia overexpressing *CaR*^{WT} and BEN Australia overexpressing *CaR*^{R990G}.

CHAPTER 4

SYNOPSIS AND FUTURE DIRECTIONS

Calcium ions (Ca^{2+}) are critical to multiple biological and physiological functions. Therefore, it is not surprising that humans have developed a complex homeostatic system that maintains near constancy of the level of free extracellular calcium (Ca^{2+}_o). Maintaining Ca^{2+}_o homeostasis involves the carefully orchestrated control of calcium's movements into and out of the body via the parafollicular cells of the thyroid, the gastrointestinal tract, the kidney and bone, as well as ensuring that the calcium needs of intra- and extracellular functions are met. Calcium homeostasis is regulated through a balanced release of parathyroid hormone, vitamin D, PTHrP and calcitonin to maintain plasma calcium levels within tight physiological limits. Alternatively, calcium homeostasis can be defined at the cellular level as the appropriate balance between proliferation, differentiation and apoptosis, depending on the overall cellular environment. Neoplasia, particularly of squamous cell origin, is accompanied by

loss of both hormonal and cellular regulatory mechanisms of calcium homeostasis. Humoral hypercalcemia of malignancy (HHM) represents a clinical syndrome of calcium dyshomeostasis. This debilitating disorder is consistently observed in several types of cancer, including lung cancer. The proximal cause of HHM is increased circulating levels of PTHrP, however, the exact mechanisms for high levels of PTHrP gene expression associated lung cancer induced HHM has never been adequately explained.

Experiments presented in this dissertation demonstrate that the epidermal growth factor receptor (EGFR) contributes to high levels of PTHrP gene expression in lung squamous cell carcinoma that induce HHM via the MAPK pathway. Interestingly, different activators of signaling mechanisms in the HARA and RWGT2 lung SCC lines were responsible for PTHrP secretion. The RWGT2 cell line produced abundant amounts of the EGF-ligand mRNA for amphiregulin, and moderate amounts of TGFα and HB-EGF. Increased production of EGF-ligands, and robust phosphorylation of EGFR tyrosine 992 in RWGT2 tumors and cell lines suggested an autocrine loop of activation for production of PTHrP. In contrast, the HARA cell line produced decreased amounts of EGF ligand mRNA and had minimal EGFR tyrosine phosphorylation in vitro and in vivo but curiously the HARA xenografts had plasma PTHrP concentrations that were 80% higher than RWGT2 xenograft and tumor PTHrP/GAPDH mRNA ratios that were sixfold greater than the RWGT2 tumors, suggesting an alternative mechanism of EGFR

activation or an alternative mechanism for PTHrP production. These observations lead to the following unanswered questions outlined below.

Does signaling by the EGFR activate PTHrP gene expression by increasing gene transcription or message stability?

Heath *et al*. (1) have shown that steady-state levels of PTHrP mRNA and secreted PTHrP were increased 10-fold by maximally effective concentrations of EGF in the immortalized human keratinocyte cell line, HaCaT. EGF increased both PTHrP gene transcription and PTHrP mRNA stability. The major effect was seen on the abundance of transcripts initiated by P1 and P2, with less marked regulation of P3-initiated transcripts. Thus EGF regulation of PTHrP gene expression in HaCaT cells is multifactorial and the combination of its actions at the 5' and 3' ends of the gene favors the accumulation of subpopulations of PTHrP mRNA containing exons I, VII and VIII. While EGF is not a primary ligand in lung SCCs, our studies in chapter 2 have documented an essential role for the EGF-ligand amphiregulin in EGFR activation and subsequent PTHrP mRNA expression. Considering the role of EGF in PTHrP regulation in a squamous epithelial line, it would be reasonable to assume that amphiregulin may play an important role in PTHrP stability in both the HARA and RWGT2 cell lines.

Conversely does PTHrP increase EGFR gene expression and message stability? Both calciotropic hormones PTH and calcitriol increase EGFR receptor expression in UMR 106-01 osteoblast-like cells by different mechanisms. Treatment with calcitriol resulted in a time and dose dependent increase in EGFR mRNA levels in confluent cultures of UMR 106-01 osteoblast-like cells and was found to prolong the half life of EGFR mRNA, however, PTH was found to increase EGFR transcription but have no effect on EGFR mRNA stability.(2) As PTHrP is also considered to be a calcitopic hormone it seems plausible that PTHrP in lung SCCs may contribute to an additional autocrine/paracrine loop of activation resulting in up-regulation of EGFR expression. This scenario may be difficult to demonstrate as although evidence for a novel receptor for the N-terminal PTHrP in squamous carcinoma lines exists (3) no receptor has been identified.

Does CaR transactivate EGFR through triple membrane pass signaling in the RWGT2 lung SCC line?

An emerging body of evidence indicates that some GPCRs transactivate receptor tyrosine kinases such as EGFR and PDGF receptors. The initial transactivation process involves the stimulation of matrix metalloproteinases which results in the extracellular release of latent membrane-spanning precursors of the family of ligands known to activate these groups of receptors.(4,5) Ligands such as HB-EGF then secondarily activate EGFR to phosphorylate specific tyrosine residues

residing on their own intracellular domain, thereby activating downstream proteins such as MAPKs.(6-9) Our studies in chapter 2 have clearly demonstrated that EGFR stimulation of the MAP kinase pathway plays a key role in PTHrP secretion in RWGT2 and HARA lines and in chapter 3 we show that abrogation of the CaR with shRNA decreases MAP kinase activation. As a EGFR autocrine activation loop is clearly present in the RWGT2 cell line and the cell line is responsive to addition of Ca^{2+}_{o} through the CaR, determining if signaling is occurring through transactivation of EGFR is necessary for the further development of individualized comprehensive therapeutic plans for patients with lung SCC.

Our studies in chapter 3 aimed to identify an alternative signaling mechanism that could explain the differences in PTHrP-induced hypercalcemia between the RWGT2 and HARA models. When considering regulators of calcium homeostasis and PTHrP, we investigated the role of the CaR as it had previously been shown to regulate PTHrP in breast, prostate, cervical epithelial, testicular, meningioma and astocytoma cancer cells but had not been identified in lung cancer cell lines or in human lung tissue.

One of the key players in extracellular calcium homeostasis is the CaR. The two major functions of this receptor in maintaining the calcium state of equilibrium is to inhibit PTH release from the parathyroid gland and to inhibit

renal absorption of calcium. Besides being expressed in the chief cells of the parathyroid gland and along the nephron, the CaR has also been found to be expressed in tissues not related to calcium homeostasis including some cancer cells. In chapter 3, we have identified CaR expression in the NSCLC lines, HARA, RWGT2 and BEN Australia. Additionally, we show that CaR is a critical factor necessary for the development of HHM as either overexpression of CAR^{WT} or the single nucleotide polymorphism (SNP) CaR^{R990G} is sufficient to induce HHM in an eucalcemic lung SCC xenograft model. Therefore, Ca^{2+}_{o} acts as a malignant stimulus by activating CaR in lung SCC. Many studies have documented diverse roles for CaR in tissues which are not directly involved with calcium homeostasis. A few of the cellular processes include but are not limited to the following: 1) *homing of stem cells*, hematopoietic stem cells (HSCs) from CaR knockout mice were highly defective in localizing anatomically to the endosteal niche, behavior that correlated with defective adhesion to the extracellular matrix protein, collagen I. CaR has a function in retaining HSCs in close physical proximity to the endosteal surface and the regulatory niche components associated with it, (10) 2) *regulation of proliferation* in neoplastic testis Leydig cells (240) and 3) *differentiation* of keratinocytes (241) and mammary epithelial cells (13). When considering these three known functions of CaR, many additional questions regarding its role in the context of lung cancer biology remain.

Cancer stem cells arise from either normal stem cells or from differentiated cells that have reverted to a stem cell-like phenotype and are resistant to many anticancer agents, thus allowing unlimited survival in a nearly quiescent status that may then progress to produce recurrences and metastases of disease. Definitive human lung cancer stem cells have yet to be identified, however, in NSCLC a rare undifferentiated population of cells expressing CD133+, an antigen present in the cell membrane of normal and cancer-primitive cells of the hematopoietic, neural, endothelial and epithelial lineages, were able to grow indefinitely as tumor spheres on soft agar and readily generated tumors when engrafted. Upon differentiation, lung cancer CD133+ cells acquire specific lineage markers while loosing the tumorigenic potential with CD133+ expression. As CaR has been demonstrated to contribute to stem cell homing in hematopoietic stem cells, we should ask the question is CaR present the CD133+ population of NSCLC cells and if so does it have a metastasis role that could be abrogated by shRNA therapy to CaR?

It is well known that by altering the levels and activities of transcription factors, MAPK leads to altered transcription of genes that are important for the cell cycle. We have documented that CaR signals through the MAPK pathway in both the BEN Australia and HARA SCCs. Activation of the MAPK pathway in our cells resulted in increased PTHrP production, however, in our studies we have not addressed if stimulation of CaR and temporal ERK1/2 activation results

induced proliferation or differentiation. Murphy *et al*. have demonstrated that stimulation of EGFR induced prolonged activation of ERK1/2 and induced proliferation, whereas stimulation of PDGF activated ERK1/2 for a much shorter time and did not produce any proliferatory response.(14) The question we need to ask in future studies is do the differences in the ERK1/2 activation kinetics seen in the BEN Australia and HARA lines translate to distinct biological responses, in particular induction of immediate early genes that would alter rates of proliferation or differentiation?

In conclusion, our current understanding puts EGFR and CaR as molecules that can promote the development of HHM in lung SCCs. However, there is much to learn about their potential roles in lung SCC metastatsis and within the microenvironment of both tissue and bone. Comprehensive studies addressing these points will allow better understanding of the potential consequences attributed to these receptors and therefore patient treatment.

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