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Differential growth regulation by beta-adrenergic/cyclic amp signaling in phenotypically different human pulmonary adenocarcinomas cell lines that express the epidermal growth factor-mediated pathway

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University of Tennessee

To the Graduate Council:

I am submitting herewith a thesis written by Hibret A. Adissu entitled "Differential growth regulation by beta-adrenergic/cyclic amp signaling in phenotypically different human pulmonary adenocarcinomas cell lines that express the epidermal growth factor-mediated pathway." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Hildegard M. Schuller, Major Professor

We have read this thesis and recommend its acceptance:

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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Provost and
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Hibret A. Adissu
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DEDICATION

This thesis is dedicated to my wife, Genet Asmelash, for her indescribable love and sacrifice.

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ABSTRACT

Human pulmonary adenocarcinoma (PAC), which originates from bronchiolar epithelial/Clara cells (PACC) or the alveolar type II cells (PAC type II), constitutes one of the most rapidly rising lung cancer types. The occurrence of PAC in smoking and non-smoking individuals suggests that other factors besides smoking contribute to the development and progression of PAC.

Earlier studies showed the presence of beta-adrenergic/cAMP growth pathway in PACC. Consequently, beta-adrenergic stimulants in various drug formulations that are used in the management of chronic respiratory diseases have been proposed as potential risk factors for the development of PAC. On the other hand, little is known about the role of this pathway in the proliferation of PAC type II. Using thymidine incorporation, this study showed that the two PAC phenotypes have differential response to the beta-adrenergic/cAMP stimulation. Isoproterenol (broad-spectrum beta-adrenergic agonist) and forskolin (adenylyl cyclase stimulator) induced significant proliferative response in NCI-H322 cell line (PACC). Both beta-1 and beta-2 adrenergic receptors and cAMP are involved in this proliferative response as shown by selective receptor and enzyme inhibitors. On the other hand, A549 cells (PAC-type II) were inhibited by forskolin while being unresponsive to beta-adrenergic stimulation. Accordingly, forskolin caused significant and persistent activation of the extracellular signal regulated kinase (ERK1/2) in NCI-H322 cells in contrast to A549 cells, in which inhibition was observed.

Cyclic AMP immunoassay of basal and stimulant-induced cAMP amount showed marked difference between the two cell lines. The basal cAMP content in A549 cells was significantly lower than that of NCI-H322 cells. Moreover, isoproterenol had no effect while forskolin had significant but moderate rise of cAMP. By contrast, both isoproterenol and forskolin induced a marked and significant accumulation of cAMP in NCI-H322 cells.

In both cell lines, significantly higher proliferative response was observed in low serum than in high serum culture condition. The epidermal growth factor receptor (EGFR)-mediated growth pathway is common to both cell lines as demonstrated by the use of AG1478 (EGFR-specific tyrosine kinase inhibitor). Furthermore, AG1478 inhibited isoproterenol but not forskolin-induced proliferation in NCI-H322 cells, implying the existence of cAMP dependent and independent growth pathways and transactivation of the EGFR.

The present study demonstrated an important difference between two cancer cell phenotypes that are generally grouped as PAC. The implication of this distinction in relation to cancer chemoprevention approaches and chronic management of respiratory diseases with beta-adrenergic stimulants was emphasized. On the other hand, the EGFR growth pathway is commonly expressed and plays a central role in the proliferation of both PAC phenotypes. Consequently, the EGFR pathway may provide a common chemotherapeutic target in broad family of cancer types and phenotypes with diverse growth pathways.

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LIST OF ABBREVIATIONS

AA: Arachidonic acid

AC: Adenylyl cyclase

AKT (PKB): Protein kinase B

AMP: Adenosine monophosphate

ATF-1: activating-transcription-factor-1

β-AR: Beta-2-adrenergic receptor

BCA: Bichinchoninic acid assay

BSA: Bovine serum albumin

cAMP: cyclic-adenosine monophosphate

c-fos: Nuclear phosphoprotein gene (transcription factor)

c-jun: Nuclear phosphoprotein gene (transcription factor)

c-myc: Nuclear phosphoprotein gene (transcription factor)

Ca^{2+}: Calcium ion

CDK: Cyclin dependent kinase

COS: Simian fibroblast cells

COX: Cyclooxygenase

CPM: Count per minute

CRE: c-AMP response element

CREB: c-AMP response element binding protein

CREM: c-AMP response element modulator

DAG: Diacylglycerol

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EPAC: Exchange protein directly activated by cAMP

ERE: Estrogen response element

ERK: Extracellular regulated kinase

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

GRK: G-protein inwardly rectified potassium channel

GPCR: G-protein coupled receptor

Grb2: growth factor receptor bound protein 2 (adaptor protein)

GAP: GTPase activating protein

HCl: Hydrochloric acid

HER/ERB: human epidermal growth factor receptor

HGF: Hepatocyte growth factor

IGF: insulin growth factor

IL: Interleukin

IP3: Inositol 1,4,5, triphosphate

JAK: Janus kinase

JNK: c-jun N-terminal kinase

Kip: CDK inhibitory protein

LcK: Src family tyrosine kinase

LOX: Lipoxygenase

MAPK: Mitogen activated protein kinase

MEK: MAPK/ERK kinase

MMP: Matrix metalloprotease

mRNA: Messanger ribonucleic acid

Na^+: Sodium ion

NaOH: Sodium hydroxide

NCI: National Cancer Institute

NIH: National Institute of Health

NNK: 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone

NSCLC: Non-small cell lung carcinoma

PAC: Pulmonary adenocarcinoma

PACC: Pulmonary adenocarcinoma of Clara cell phenotype

PAC-II: Pulmonary adenocarcinoma of alveolar type II cell phenotype

PAGE: Polyacrylamide gel

PBS: Phosphate buffered saline

PDGF: Platelet derived growth factor-A/B

PGE: Prostaglandin E

PKA: Protein kinase–A

PKB (AKT): protein kinase-B

PKC: Protein kinase-C

PI: phosphatidylinositol

PIK-3: phosphatidylinositol 3-kinase

PDK: phosphoinositide-dependent kinase

PLA: Phospoholipase-A

PLC: Phospholipase-C

PMSF: Phenylmethylsulfonyl fluoride

Raf-1: Cytoplasmic serine / threonine protein kinase

Rap1:Ras-related G-protein

Ras: Membrane associated GTP protein kinase

RIPA: Cell lysing buffer

RTK: Receptor tyrosine kinase

SAPK: Stress-activated protein kinase

SCLC: Small cell lung carcinoma

SDS: Sodium dodecyl sulfate

SH2: Src homology 2 adaptor protein

Shc: Src homology containing adaptor protein

STAT: Signal transducers and activators of transcription

SOS: Son of sevenless (guanaine exchange factor)

Src: Non-membrane associated tyrosine kinase

TBS: Tris buffered saline

TGF-α: transforming growth factor alpha

TGF-β: transforming growth factor beta

I. INTRODUCTION

General Overview

It is estimated that around 1.3 million new cases of cancer will be diagnosed and approximately half a million people will die from cancer in the United States in the year 2002 (Jemal et al., 2002). Besides, the total annual cost of cancer care in the United States including direct and indirect costs has been estimated at more than $96 billion (Schuette et al., 1995).

Lung cancer, which is one of the most common cancer types, has become a major epidemic of the 20th century in the developed world from a very rare disease at the beginning of the century (Janssen-Heijnen and Coebergh, 2001). It is the most frequently diagnosed cancer type and the most common cause of cancer deaths in males and females in the world. This trend is also expected to continue for many years to come (Travis et al., 1996). Lung cancer mortality after 1950 may reflect the early impact of teenage cigarette smoking on lung cancer risk in people under the age of 45 years (Jemal et al., 2001). Unlike some types of cancers, lung cancer is usually diagnosed after metastasis and acquisition of drug resistance (Malkinson, 2001). Despite improvement in diagnosis and treatment, mortality rate is higher than 95% within 1 year of diagnosis (Parker, 1997; Kane and Bunn, 1998).

The four major pathological types of lung cancer are adenocarcinoma (35-40%), squamous cell carcinoma (25-30%), large cell carcinoma (10-15%), and small cell lung carcinoma (20-25%). Because of their similar clinical behavior, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma are commonly grouped as non-small cell lung cancer (NSCLC)(Kang et al., 2000).

Pulmonary adenocarcinoma is the fastest rising lung cancer type (Malkinson et al., 2000). There has been a dramatic increase in the incidence of PAC in the last 10 to 15 years (Wynder and Hoffmann, 1994; Hoffmann et al., 1997) and this trend is expected for a wider geographical region in the near future. Janssen-Heijnen and Coebergh (2001) predicted a worldwide increase in mortality from adenocarcinoma in contrast to squamous cell carcinoma and small-cell carcinoma, for which a decrease is expected. Adenocarcinoma may replace squamous cell carcinoma as the most frequent type of lung cancer among men (Devesa et al., 1991). This trend has been largely attributed to deeper inhalation after the introduction of filtered low tar cigarettes with consequent high concentration of carcinogens in the peripheral airways (Wynder and Hoffmann, 1994; Hoffmann et al., 1997). Consequently, the decrease in lung cancer mortality after 1990 due to the alleged long-term benefits of reductions in tobacco carcinogens in cigarettes around 1960 (Jemal et al., 2001) might not apply to PAC. Moreover, PAC is common in both smoking and non-smoking individuals implying that additional factors contribute to the continued rise of this cancer type. Park et al. (1995) demonstrated a novel role of β-adrenergic mitogenic signal transduction in pulmonary Adenocarcinoma and Schuller et al. (1999) showed that the tobacco-specific carcinogen NNK (4-(methylnitrosamino)-1-

(3-pyridyl)-1-butanone)) is a potent stimulator of this proliferative pathway. These researchers pointed out that chronic treatment of existing respiratory diseases such as asthma by beta-adrenergic stimulators might play a significant role in the PAC epidemic. For instance, chronic obstructive pulmonary disease (COPD), which encompasses both chronic bronchitis and emphysema, is one of the most common respiratory conditions of adults in the developed world (Szelenyi and Marx, 2001). The major clinical management of COPD and other obstructive diseases utilizes the beta-adrenergic agonists or cAMP elevating agents to relieve airway obstruction. The possible contribution of this phenomenon to the spatial co-distribution of PAC and chronic respiratory diseases has been suggested (Park et al., 1995). In fact, various studies have shown that COPD precedes lung cancer. Consequently, this association warrants that patients with COPD should be monitored carefully for lung cancer (Weiss, 1991). Such findings underlie the necessity of characterizing the growth-regulating pathways in the various types of lung cancers and identifying hitherto unknown risk factors. Moreover, assessment of the presence of differential pathways would provide information on the applicability of such risk factors across the major types of lung cancers.

Current cancer chemotherapy is mainly based on cytotoxic drugs and radiation. Narrow safety margins and resistance has rendered several of the chemotherapeutic agents ineffective (Ciardiello, 2001), and chemotherapy of NSCLC is considered to have reached its plateau of efficacy. This has made targeting pathways unique to these cancer cells the best approach (Ferreira et al., 2002). Therefore, assessing the differential growth pathways in the various NSCLC is deemed an important step towards this goal.

Cellular origin and physiology

Pulmonary adenocarcinoma (PAC) arises from the lung periphery and exhibits either phenotypic feature of bronchiolar Clara cells (PACC) or alveolar type II cells (PAC-II). Immunohistochemical evidence showed that about 50% of PAC expresses alveolar type II phenotype whereas the remaining 50% exhibit Clara cell phenotype (Linnoila, 1998)

Alveolar type II cells

The alveolar epithelium, which forms a lining of the alveolar spaces of the lung, consists of alveolar type I and alveolar type II cells. Alveolar type I cells which constitute about 90% of the alveolar surface form most of the gas exchange surface. The alveolar type II cells cover the remaining 10% of the alveolar surface (Sutherland et al., 2001). Alveolar type II cells differentiate and replace type I alveolar epithelial cells. Alveolar type II cells are involved in the transportation of Na^+ from the apical to the basolateral surface to minimize excess alveolar fluid. This function, which is under beta-adrenergic control, facilitates alveolar fluid clearance (Dumasius et al., 2001).

Alveolar type II cells synthesize and secrete pulmonary surfactant that reduces the surface tension and prevents alveolar collapse after expiration (Pian and Dobbs, 1995; Malkinson et al., 1997). Surfactant secretion is regulated, among others, by a β-

adrenergic/cAMP pathway (Brown and Longmore, 1981; Isohama et al., 2001). Cyclic AMP (cAMP, 3': 5'-cyclic adenosine monophosphate) increases the total amount of saturated phosphatidylcholine, a lipid component of pulmonary surfactant, as well as the incorporation of [^{3}H] choline into surfactant phosholipid in a human lung cancer cell line with phenotypic features of alveolar type II cells (A549) (Niles and Makarski, 1979). Smith (1977) showed that adrenergic agents stimulated saturated phosphatidylcholine secretion by A549 cells and that this was enhanced by cortisol. Smith (1977) reported that media that supported the most rapid rate of growth in A549 cells were least optimal for saturated phosphatidylcholine synthesis. Saturated phosphatidylcholine production was most efficient when growth was slow or arrested, whereas choline was incorporated into unsaturated (structural) phosphatidylcholine when A549 cells were in an active proliferative state. Cortisol-induced upregulation of B-adrenergic receptors with subsequent differentiation and growth inhibition of A549 cells was also reported by (Nakane et al., 1990). Unless there is a lung injury, most alveolar type II cells are in cell cycle arrest. Proliferative and pathologic appearance of hyperplasic alveolar type II cells is induced by growth factors including the epidermal growth factor (EGF) and transforming growth factor–α (TGF-α) (Mason, 1991). In summary, the β-adrenergic signaling pathway may have a dual regulatory function in normal alveolar type II cells, where it positively regulates many of the physiological processes while suppressing cell proliferation.

Clara cells

Clara cells and ciliated cells are the two main functional cells of the bronchiolar epithelium. The Clara cells, which are sometimes called the non-ciliated bronchiolar secretory cells, are cuboidal to columnar epithelial cells lining the most distal conducting airways (Plopper, 1991). They are secretory cells and may be involved in mucus production (Malkinson et al., 1997), a function that is under beta-adrenergic control. Beta-adrenergic agonists such as isoproterenol and adrenalin and cAMP analogues stimulate secretion whereas antagonists like propanolol inhibit it (Plopper, 1991). Clara cells can differentiate into mucin producing goblet cells. This differentiation is mediated through non-proliferative epidermal growth factor receptor (EGFR) activation (Nadel, 2001). This is in contrast with their tumorigenic counterparts where EGFR activation induces cell proliferation (Schuller et al., 1994; Al Moustafa et al., 2002). Clara cells also give rise to other Clara cells as well as ciliated cells. In steady state conditions, the turnover of the Clara cell population is very low and less than 1% of the cells incorporate radiolabeled thymidine. Upon injury to bronchial epithelium, however, proliferation is stimulated with more than 90% of the Clara cells incorporating radiolabeled thymidine (Plopper, 1991).

Clara cells have a high content of xenobiotic-metabolizing enzymes. Their active detoxifying function may selectively expose Clara cells to cell injury or chemical carcinogens such as nitrosamines, which are components of cigarette smoke (Widdicombe, 1982). The human pulmonary adenocarcinoma cell line with Clara cell

phenotype, NCI-H322, metabolizes the polycyclic aromatic hydrocarbon benzo(a)pyrene to various metabolites particularly to the reactive 7,8-diol-BP (Kiefer et al., 1988). Activated intermediates of phase-I metabolism, such as diol epoxides are electrophilic carcinogens that covalently bind to DNA and other macromolecules (Annas et al., 2000). Clara cells of rabbit origin have a lower DNA excision repair than alveolar type II cells, suggesting an additional risk factor for mutation and subsequent malignant transformation of these cells (Deilhaug et al., 1985). Consequently, Clara cells may serve as the origin of distal airway tumors (Komaromy and Tigyi, 1988).

Major growth regulating signaling pathways

The epidermal growth factor receptor (EGFR)

The EGFR signaling pathway demonstrates remarkable versatility in mediating a host of signaling pathways that regulate diverse physiological and pathological processes. The EGFR belongs to the family of Receptor Tyrosine kinases (RTKs). The RTKs have intrinsic enzymatic activity in the cytoplasmic region that is directed against the receptor itself and downstream signaling molecules (Hubbard, 1999). Most RTK ligands are soluble polypeptide ligands including insulin, epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and platelet-derived growth factor (PDGF). The RTKs are involved in a range of cellular physiology including fertilization, proliferation, cell migration and apoptosis. In addition, they play key roles in various pathological processes including cancer (Ostman and Bohmer, 2001).

The EGFR is expressed in a variety of cell types and regulates a range of functions including cell growth, differentiation, de-differentiation, and apoptosis, depending on the context of density, type of matrix, and other cytokines (Wells, 2000); (Baselga, 2000). The EGFR family consists of four closely related receptors namely the EGFR (erbB1), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). The EGFR (erbB1) binds EGF, TGF-alpha, amphiregulin, heparin-binding EGF like growth factor, betacellulin, and epiregulin. These ligands are synthesized as transmembrane precursors and are cleaved by the matrix metalloproteases to be released as mature growth factors (Gschwind et al., 2001).

EGFR (erbB1) is a 170 KD glycoprotein composed of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain, which has Tyrosine kinase activity (Hsieh et al., 2000). Binding of ligand to the monomeric EGFR, induces EGFR dimerization, activation of intrinsic EGFR Tyrosine kinase activity, and transphosphorylation of the dimerized receptor monomers (Maudsley et al., 2000). Receptor autophosphorylation of Tyrosine residues out of the catalytic portion provides a docking site for other signaling molecules via src homology-2 (SH2). Phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), growth factor receptor-bound protein 2 (GRB-2), and GTPase activating protein (GAP) are among the many signaling molecules that use these docking sites to assemble signaling complexes (Nystrom and Quon 1999; Hackel et al., 1999) (Figure 1)

The Ras-mediated mitogen activated protein kinase (MAPK) cascade is one of the best-characterized signaling pathways initiated by activated EGFR. The Ras-mediated EGFR-MAPK cascade is initiated when the Grb-2 associated Sos (Ras guanine nucleotide exchange factor) is recruited to phosphotyrosine residues of activated EGFR. This association allows the formation of a complex that mediates the exchange of GTP for GDP, hence activation of the small G protein Ras. This is followed by membrane-recruitment and activation of the serine-threonine kinase Raf (MAPK kinase kinase), which in turn phosphorylates and activates MEK (MAPK kinase). The dual-specific MEK (MAPK kinase) phosphorylates and activates the serine/threonine mitogen activated protein kinases (MAPKs) (vanBiesen et al., 1996; Maudsley et al., 2000). Activated MAPK dimerizes and translocates to the nucleus to activate nuclear transcription factors such as c-fos, c-jun, and c-myc that are involved in mitogenesis and transformation (Lieberman et al., 1996b). The best-studied MAPKs are the extracellular signal-regulated kinases (ERK1/p44^{mapK} and ERK2/ p42^{mapK}). Depending upon the interacting signaling molecules, MAPKs mediate a range of cellular functions in the membrane, nucleus, cytoplasm and cytoskeleton (vanBiesen et al., 1996), eliciting a wide array of responses such as cell division, differentiation, and secretion (Seger and Krebs, 1995).

Besides the classical Ras-mediated kinase cascade, various signaling molecules could also undergo phosphorylation and activation upon binding to phosphotyrosine residues on activated EGFR. Activated Phospholipase C (PLC) hydrolyses membrane bound phosphatidylinositol yielding diacylglycerol (DAG) and inositol phosphates (Graves and

Lawrence, 1996). Inositol phosphate regulates intracellular calcium, which together with DAG, activates protein kinase C (PKC). Protein kinase C (PKC) causes potent activation of ERK through the direct phosphorylation and activation of Raf-1 in a Ras-independent manner (Kolch et al., 1993). On the other hand, DAG can be metabolized to arachidonic acid (AA), which in turn is metabolized by two enzyme families, the cyclooxygenases (COX) and lipoxigenases (LOX), into metabolites that mediate various cellular functions, including proliferation (Rao et al., 1994; Liu et al., 2000).

Phosphatidylinositol 3-kinase (PI3K) binds to phosphorylated Tyrosine residues of RTKs (Stover et al., 1995) and Shc-Grb2 (Saleem et al., 1995) and catalyzes the phosphorylation of inositol phospholipids (vanBiesen et al., 1996). Phosphorylated inositol phospholipids (Phosphatidylinositols) are required for the membrane localization of phosphoinositide-dependent kinase (PDK). Membrane-localized phosphoinositide-dependent kinase (PDK) phosphorylates and activates protein kinase B (Akt), an enzyme involved in cell proliferation and inhibition of apoptosis (Kim et al., 2001). Recently, Akt/PKB was identified as a promoter of cancer cell invasion through increased motility and metalloproteinase production (Kim et al., 2001). The requirement of PI3K for the induction of DNA synthesis in quiescent cells was demonstrated by (Roche et al., 1994).

Among the proteins that become phosphorylated by EGFR is the Janus kinase 1 (JAK 1) (Shual et al., 1993) and the signal transducers and activators of transcription (STAT) (David et al., 1996). Activated JAK phosphorylates STAT, which dimerizes and translocates to the nucleus for gene activation (Shual et al., 1993; Frank, 2002).

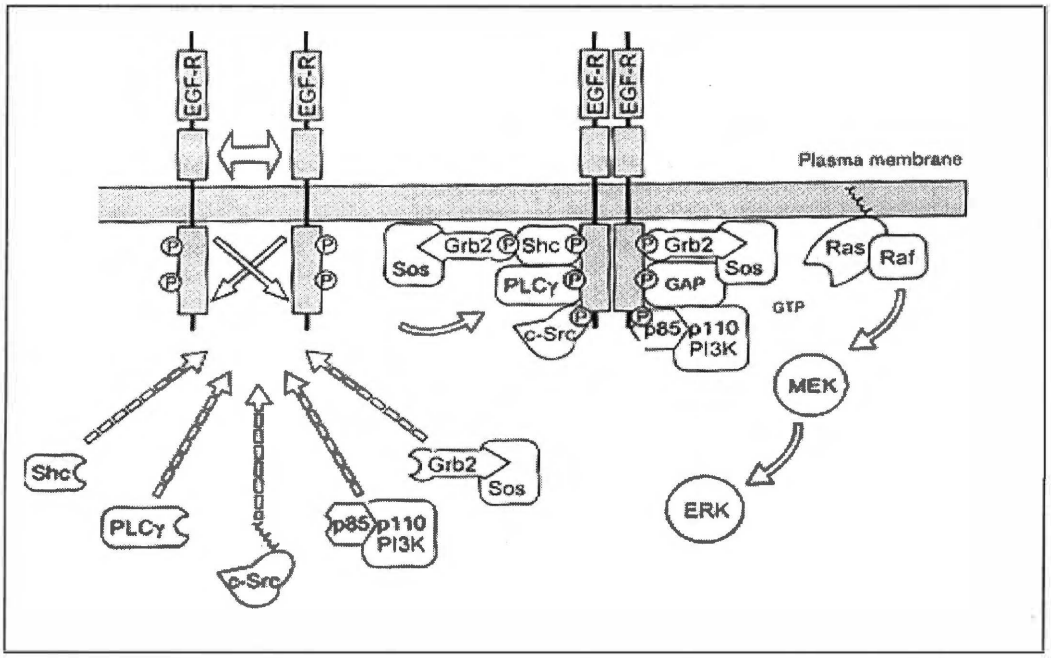


Figure 1. Epidermal growth factor receptor-mediated growth signaling. Classical RTKs such as the EGF receptor are single transmembrane domain proteins. The cytosolic domain, which has an intrinsic kinase activity, dimerizes and transphosphorylate (indicated as cross arrows) upon ligand binding. Phosphorylated tyrosine residues serve as docking sites for phosphotyrosine binding PTB or SH2 domain-containing adaptor proteins, such as Shc and Grb2, and signaling molecules such as c-Src, phospholipase Cγ (PLCγ), Ras.GTPase activating protein (GAP), and PI3K. The various docking molecules are then phosphorylated and activated to initiate a range of signaling pathways. The exchange of Ras.GDP to the active Ras.GTP is mediated by the Grb2-Sosl that is recruited to the activated EGF receptor complex via Shc domain. Activated Ras activates Raf (MAPK kinase kinase), which in turn phosphorylates and activates MEK (MAPK kinase) to initiate the MAPK/ERK cascade. (Adapted from Luttrell et al. (1999).

Activated EGFR also phosphorylates and activates STAT in a JAK-independent manner (David et al., 1996)

The epidermal growth factor receptor and lung cancer

An autocrine growth loop is present when both the receptor and its ligand are coexpressed in a cell with self-regulated proliferation (Tsao et al., 1996). Various autocrine growth pathways have been associated with the pathogenesis of a variety of cancers for which the EGFR has been implicated as a promoter of proliferation and progression into malignant cells (Wells, 2000). Moreover, an internal autocrine stimulation of the PDGF receptors with no need for growth factors has been found in v-sis transformed cells (Keating, 1988; Bejcek et al., 1989).

Several studies have shown that EGFR plays a major role in the autocrine growth of human non-small cell lung cancer (NSCLC) (Liu and Tsao, 1993; Rusch et al., 1997). The family of NSCLC is comprised of adenocarcinoma, squamous cell carcinoma, large cell carcinoma and carcinoid (Kang et al., 2000). Expression and overexpression of EGFR was found in a greater proportion of squamous and adenocarcinomas than in large cell or small cell carcinoma (Rusch et al., 1993; Nakagawa, 2001). The co-expression of transforming growth factor-α (TGF-α) and the EGFR exists in a range of lung cancer cell lines particularly the NSCLC (Liu and Tsao 1993; Rusch et al., 1997; Hsieh et al., 2000). These investigators reported TGF-α as the main and most important autocrine ligand in NSCLC tumor formation. Moreover, activating point mutations of the Ras gene with

constitutively expressed mitogenic signals was observed in about 30% of NSCLCs (Rodenhuis, 1990).

EGFR overexpression is associated with reduced survival (Volm et al., 1998; Nakagawa, 2001), metastasis (Fontanini et al., 1995), and resistance to chemotherapy (Dickstein et al., 1995) in lung cancer. Various chemotherapeutic approaches have targeted the EGFR signaling pathway with encouraging preclinical results (Baselga, 2001; Slichenmyer and Fry, 2001).

Signaling through the G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) are among the largest family of cell surface receptors. They mediate signaling from a number of stimuli and ligands including peptide and glycoprotein hormones, neurotransmitters, phospholipids, and photons. These receptors consist of an extracellular ligand binding domain, a seven transmembrane domain, and heterotrimeric G-proteins (vanBiesen et al., 1996). The heterotrimeric G-protein consists of α, β, γ subunits, which dissociate into active Gα-GTP and G$\beta\gamma$-subunits after interaction with ligand-bound receptor. Although there are numerous G_α-proteins, the main classes are the $G_{\alpha s}$, which activate adenylyl cyclase; the $G_{\alpha i}$, which inhibit adenylyl cyclase; and $G_{\alpha q}$, which activate phospholipase C. Besides the G_α subunits, the G$\beta\gamma$-subunit is also involved in regulating various effectors, including

adenylyl cyclase, phospholipase C and A_2 isoforms, serine threonine kinases, and protein kinase C (vanBiesen et al., 1996) (Figure 2).

Adrenergic receptors, which are a group of GPCRs, are divided into α-ARs and β-ARs based on functional and pharmacological characteristics. Activated β-adrenergic receptors activate adenylyl cyclase through the stimulatory G-protein subunit ($G_{\alpha s}$), leading to cAMP synthesis (Ma and Huang, 2002). Cyclic AMP is an intracellular second messenger that mediates actions of various physiological ligands and of beta-adrenergic agonists. Elevation of cAMP influences cell physiology including proliferation, survival, and differentiation, either stimulating or inhibiting the response depending on cell type and context. Contradictory effects of cAMP might result from cellular specificities, concentration, types, and localization of the cAMP-dependent protein kinases (PKA) (Lange-Carter and Malkinson, 1991). There are nine isoforms of adenylyl cyclase enzyme and the tissue specific expression of the specific isoforms determines the relative amount of cAMP in response to stimuli (Daniel et al., 1998). Moreover, the large number of possible combinations of different G protein subunits and effector molecules allows cells to respond in diverse ways (Lodish et al., 2000). Phosphodiesterase degrades cAMP to 5'-adenosine monophosphate (5'-AMP) terminating the cAMP-mediated effect of hormone stimulation.

Cyclic AMP activates protein kinase A (PKA), which phosphorylates numerous proteins, with subsequent modulation of various cellular functions including gene transcription. Gene transcription is mediated through the PKA-mediated activation of cAMP-

responsive transcription factors including cAMP-response-element-binding-protein (CREB), cAMP-response-element-modulator (CREM), and activating-transcription-factor-1 (ATF-1) (Daniel et al., 1998).

Most GPCR undergo four sequential steps to respond to stimulation by agonists. These are activation, desensitization, internalization, and resensitization (Lefkowitz, 1998). Desensitization, which is a self-regulatory mechanism to responsiveness to agonists, results when sites in the cytoplasmic domains are phosphorylated. In β-adrenergic receptors, this phosphorylation creates a binding site for the SH2 domain of Src. After Src is recruited, it phosphorylates and activates the G protein-coupled receptor kinase (GRK), which in turn phosphorylates the receptor on serine and threonine residues, creating a docking site for β-arrestin. Beta arrestin mediates the internalization of the receptor through the formation of clathrin-coated pits. This process is necessary for resensitization and redistribution of the receptors (Fan et al., 2001).

Growth inhibitory signaling through the G protein-coupled receptors:In many cell types cAMP, the ultimate effector of $G_{\alpha s}$, inhibits proliferation and serves as a counter regulator of signals generated by growth factors such as the EGF and the platelet derived growth factor (PDGF) and may reverse certain transformed cells to normal phenotypes (Graves and Lawrence, 1996). Cyclic AMP modulates receptor tyrosine kinase-dependent signaling pathways and subsequent biological responses (McCawley et al., 2000). This modulation of the EGFR-MAPK pathway occurs at various levels but is

mainly mediated by the cAMP dependent protein kinase (PKA) (Graves and Lawrence, 1996).

Cyclic AMP-dependent inhibition of EGFR activation is cell type dependent. Inhibition of the EGFR was reported in the human squamous cell carcinoma cells, A431 (Mitsui and Iwashita, 1990; Iwashita et al., 1990). Similar effects on the insulin receptor have been observed in lymphoblasts (Stadtmauer and Rosen, 1986). Barbier et al. (1999) determined that cAMP was an inhibitor of EGF-induced activation of EGFR through a serine phosphorylation of the receptor by PKA. Consequently it was proposed that activation of PKA would attenuate the EGFR Tyrosine kinase activity in a feedback regulatory way. Nair and Patel (1993) and Barbier et al., (1999) showed that activation of the adenylyl cyclase and subsequent increase in cAMP inhibited EGFR kinase activity in the heart. In contrast, in some cell types, growth arrest by cAMP occurs without inhibition of EGFR phosphorylation (Lieberman et al., 1996b). Likewise, the effect of cAMP on MAPK activation is cell type dependent (Graves and Lawrence, 1996).

Protein kinase A phosphorylates RAF-1 on specific serine residue, thereby interfering with the ERK/MAPK cascade (Wu et al., 1993; Cook and McCormick, 1993). However, the role of direct phosphorylation by PKA as the inhibitory mechanism is controversial (Sidovar et al., 2000). Other works have demonstrated the inhibitory role of cAMP on RAF-1 in mouse fibroblast cells (Seger and Krebs, 19945; Piiper et al., 2000) but different mechanisms of inhibition have been proposed. Protein kinase A inhibits phospholipase C isotypes via phosphorylation of serine residue in human T cells (Park et

al., 1992; Liu and Simon 1996). Kim et al. (2001) showed that cAMP inhibits the lipid kinase activity of PI3-K reducing the levels of phosphatidyinositols in mouse fibroblast cells. This interferes with the membrane localization of phosphoinositide-dependent kinase (PDK), which phosphorylates and activates protein kinase B (PKB/Akt). Consequently, cAMP inhibits PKB/AKT-mediated outcomes, which includes prevention of apoptosis (Cardone et al., 1998). Cyclic AMP also inhibits the tyrosine phosphorylation of JAK and STAT in myeloma cells (David et al., 1996).

Beta-adrenergic stimulation induces apoptosis independent of PKA. Gu et al. (2000) demonstrated a novel mechanism where Gαs mediates apoptosis of S49 mouse lymphoma cells through activation of Src family tyrosine kinase LcK, independent of PKA. Similarly, the β-adrenergic receptor agonist, isoproterenol, induces apoptosis of $G_{\alpha s}$-overexpressing cardiac myocytes (Geng et al., 1999). Isoproterenol inhibits MAPK activation through the activation of $G_{\alpha s}$ in simian fibroblast cells (Crespo et al., 1995). In summary, in many cell types, β-adrenergic receptor-mediated signaling antagonizes cell proliferation and cell survival.

Mitogenic signaling through the G protein-coupled receptors:In contrast to the reports summarized in the previous section, activation of the GPCRs also stimulates mitogen activated protein kinase cascade that mediates cell growth or proliferation through transcriptional regulation in many cell types. In cell types expressing this pathway, GPCRs induce the activation of mitogenic signaling cascades associated with growth

factor receptors in addition to the classical GPCR signaling pathway that results from the dissociation of heterotrimeric G proteins and the generation of cAMP (Maudsley et al., 2000). Stimulation of G protein-coupled receptors, including the beta-adrenergic receptors, has been shown to have a mitogenic effect via the transctivation of the RTKs (Luttrell et al., 1999; Maudsley et al., 2000). The activated RTK provides a structural scaffold for assembly of mitogenic signaling complexes which containe many of the same intermediates as those activated RTKs including: GPCR → Tyrosine Kinase → Sch → Grb2.mSOs → Ras → Raf → MEK →MAPK (Daaka et al., 1998; Luttrell et al., 1999). Transactivation of RTK is associated with increased tyrosine autophosphorylation and dimerization of EGFR and association with phosphorylated adapters (Luttrell et al., 1997; Daub et al., 1997). The EGFR tyrosine kinase inhibitor, AG1478, abrogated the mitogenic effect of GPCR stimulation, pointing to the obligatory role of RTK transactivation in GPCR mitogenic pathways (Daub et al., 1996).

The mechanism of transactivation is not well understood although $G_{\beta\gamma}$ subunits, Src kinases, and PKC have been mainly implicated (Luttrell et al., 1999). vanBiesen et al. (1995) showed that $G_{\beta\gamma}$ subunit of Gi mediated the phosphorylation of Shc leading to enhanced complex formation of the Shc, Grb2 and Sos, culminating in the activation of the Ras/MAPK cascade. Other works have also shown the stimulatory role of $G_{\beta\gamma}$ for the MAPK pathway (Faure et al., 1994; Luttrell et al., 1997b). Subsequent research indicated that phosphatidylinositol-3-kinase (PI3-K) is an early intermediate upstream of Sos and Ras in this $G_{\beta\gamma}$-subunit-mediated MAPK activation (Hawes et al., 1996). Daub et al.

(1997) later showed that PI3-K is required for MAPK activation but not for EGFR transactivation. Luttrell et al. (1996, 1997), however, demonstrated that $G_{\beta\gamma}$-subunit mediated the activation of Src (non-receptor tyrosine kinase), which in turn facilitated the activation of Shc. Similarly, Chen et al. (1994) showed activated Src was a propagator of signals from the GPCRs to the RTK/MAPK cascade. In addition, Ma et al. (2000) demonstrated a novel pathway in which $G_{\alpha i}$ directly stimulated the c-Src. In support of these findings, Simonson et al. (1996) inhibited GPCR-mediated transcription of c-fos by a dominant negative c-Src demonstrating the role of c-Src in GPCR-mediated cell growth and development.

Many neurotransmitters and growth factors cause activation of PLC upon binding to GPCR. The $G_{\alpha q/11}$ class of G_{α} subunit directly activates PLC, which hydrolyses membrane associated phosphatidylinositol to yield diacylglycerol (DAG) and inositol phosphates. Inositol phosphates mediate elevated intracellular Ca^{++}, which together with DAG activates PKC (Wu et al., 1992). PKC can directly activate Raf-1, initiating the MAPK cascade in a Ras-independent manner (Kolch et al., 1993).

G-protein coupled receptors (GPCRs) stimulate cell growth signaling in a $G_{\alpha s}$-dependent manner. Maudsley et al. (2000) reported a novel pathway where isoproterenol stimulated the EGFR pathway and the formation of a heterodimeric complex containing the B_2 adrenergic and EGFR receptors in simian fibroblast cells. Recently, a novel-signaling pathway in which $G_{\alpha s}$ stimulated the Src family tyrosine kinases in a cAMP-PKA

independent fashion has been demonstrated in murine embryonic fibroblasts (Ma et al., 2000). In contrast to its inhibitory effect on Raf-1, PKA activates the MAPK signaling in a cell type specific manner (Schmitt and Stork, 2000). Faure et al. (1994) had reported earlier that constitutively activated $G_{\alpha s}$ mutant, forskolin, and cAMP analogue increased MAPK activation in simian fibroblast cells. Cyclic AMP-stimulated MAPK/ERK activation also occurs in PC-12 (pheochromocytoma cells of the adrenal gland) (Frodin et al., 1994; Vaillancourt et al., 1994). This was attributed to the presence of Raf-1 independent and cAMP-insensitive pathway in some type of cells (Vaillancourt et al., 1994). Conversely, a recent finding showed that activation of ERK1/2 by cAMP/PKA is mediated through a distinct pathway involving Rap1 (a Ras-related G-protein) and a cell type specific isoform of Raf called Raf-B in lymphoma cells (Wan and Huang 1998) and PC-12 cells (Vossler et al., 1997). Interestingly, this cAMP-mediated Raf-B activation is accompanied by Raf-1 inhibition (Vossler et al., 1997). The activation of Rap1 may be PKA-dependent or independent in a cell specific manner (Schmitt and Stork 2000). Moreover, forskolin-induced cAMP activates GDP/GTP exchange factors called EPACs (Exchange Protein directly Activated by cAMP) or cAMP-GEFs. These factors may activate Rap1 without any requirement of PKA (de Rooij et al., 1998; Kawasaki et al., 1998). Therefore, the expression of the B-raf determines whether cAMP inhibits or stimulates the MAPK (ERK1/2) (Gao et al., 1999; Seidel et al., 1999). In simian fibroblast cells, Crespo et al. (1995) demonstrated a signaling pathway from β-adrenergic receptor where MAPK is activated and inhibited by $G_{\beta\gamma}$ and $G_{\alpha s}$ subunits, respectively.

They proposed these opposing regulatory pathways as a control mechanism of activation of MAPK by the β-adrenergic receptors.

The transactivation of RTKs by GPCRs is a mitogenic pathway in some cancer cell lines. EGFR transactivation by $G_{\alpha i}$ subunit of GPCR with subsequent MAPK activation and DNA synthesis occurs in astroglioma cells (Castagliuolo et al., 2000). Activating mutations of the $G_{\alpha i}$ subunit occurs in ovarian and adrenal neoplasms (Lyons et al., 1990). Moreover, transfection with constitutively active $G_{\alpha i}$ transforms fibroblasts (Pace et al., 1991). Constitutive inhibition of adenyl cyclase hence relieving cAMP-mediated inhibition of Raf-1 and MAPK may explain the mitogenic effect of this mutant $G_{\alpha i}$ (vanBiesen et al., 1996). Mutation of the $G_{\alpha s}$ with a reduced intrinsic GTPase activity occurs in pituitary and thyroid tumors (Lyons et al., 1990). This creates a constitutively activated GPCR with subsequent elevation of cAMP, which is mitogenic for many cell types (Frodin et al., 1994; Vaillancourt et al., 1994).

In summary, GPCRs utilize multiple mechanisms to regulate cell growth. The pathway that connects the GPCR to the MAPK signaling pathway is cell type-specific and dependent on the type of receptor and associated G-proteins. Moreover, the availability, the concentration, and the cellular localization of key signaling molecules probably also play a key role in this cross interaction (Liebmann, 2001).

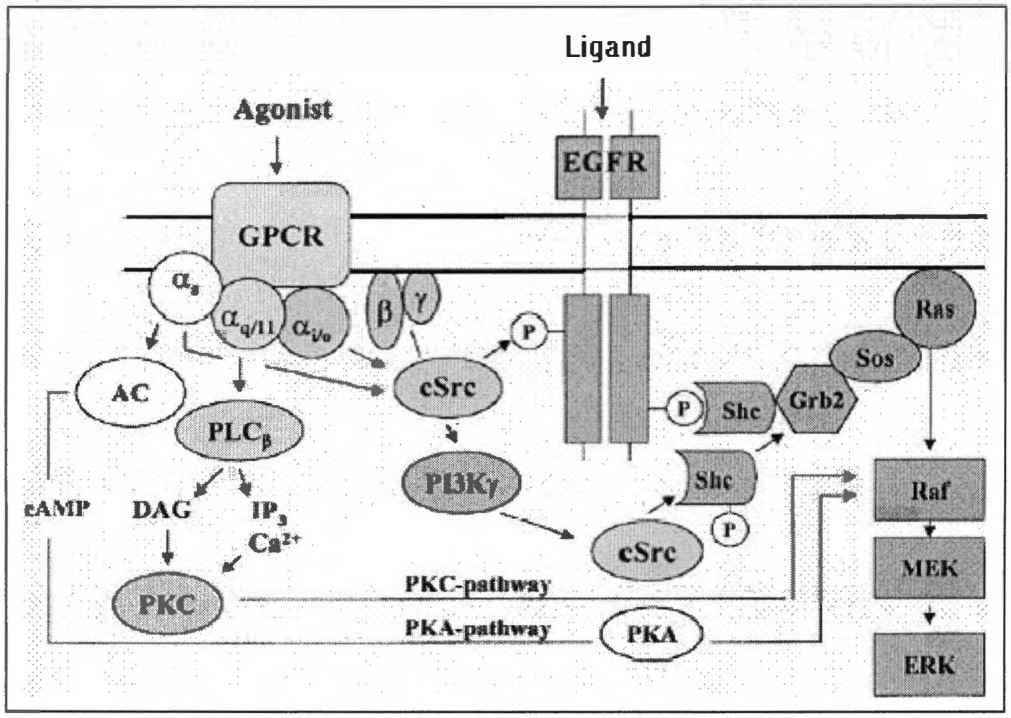


Figure 2. G-protein coupled receptor-mediated growth signaling. The best-characterized MAPK that is activated by G-protein coupled receptors (GPCRs) is the extracellular-regulated kinases (ERK1/ERK2). This activation is mediated by different classes of G proteins 1) $G_{\alpha s}$–mediated PKA-dependent activation of Raf depending on the type of Raf. 2) $G_{\alpha q}$-mediated activation of protein Kinase C (PKC) that activates Raf, hence MAPK and 3) $G_{\beta\gamma}$ and Src-mediated transactivation of receptor tyrosine kinases with subsequent Ras/MAPK signaling 4. $G_{\alpha s}$-mediated activation of Src, which activates PI3k and Shc, with subsequent activation of the MAPK cascade. Adapted from Liebmann (2001).

Beta-adrenergic signaling and cancer

The growth stimulatory and inhibitory effect of β-adrenergic stimulation has been documented in a range of cancer cell lines. Cyclic AMP inhibits growth and promotes apoptosis in many cell types. In macrophages, Kato et al. (1994) showed cAMP as an inducer of p27kip1 that inhibits cyclin D kinase-4 (CDK4), which is required for cell cycle progression by cAMP. Various site-specific cAMP analogues have been identified as cell cycle specific apoptotic agents in human lung carcinoma (Ally et al., 1989) and human neuroblastoma cells (Kim et al., 2001c).

Although limited, data on alveolar type II cells of human or mouse origin indicate that cells of this phenotype are inhibited by cAMP-mediated mechanisms. Banoub et al. (1996) showed the growth inhibitory effect of cAMP on mouse normal and neoplastic alveolar type II cells. Growth inhibitory and differentiation promoting effect of interleukin 1 (IL-1) is associated with up-regulation of β-adrenergic receptors in alveolar type-II cells (Nakane et al., 1990). Interestingly, some tumors of this phenotype are less responsive to β-adrenergic stimulation. Fewer beta-adrenergic receptors and diminished cAMP production upon isoproterenol treatment was found in mouse alveolar type II tumorigenic cells compared to their normal counterparts (Lange-Carter et al., 1992). Furthermore, mouse PACs of alveolar type II cell origin are deficient in cAMP-dependent protein kinase type I isozyme (PKA 1) compared to their non-tumorogenic counterparts (Lange-Carter and Malkinson 1991). However, PKA1 messenger RNA (mRNA) in the neoplastic alveolar cells is more stable, suggesting that the cause for low-

level PKA1 results from transcriptional control (Lange-Carter and Malkinson 1991). This deficiency in PKA might lead to prolonged stimulation by the mitogenic Raf-1, which is inhibited by PKA in small cell lung cancer cells (Schuller, 2001). Consequently, neoplastic alveolar type II cells might have a proliferative advantage by evading negative growth regulation from cAMP/PKA. In addition to these observations, Droms (1996) reported functionally uncoupled $G_{\alpha s}$ but a functional adenylyl cyclase in neoplastic mouse alveolar type II cells. Consequently, tumorigenic and normal alveolar type two cells differed in isoproterenol-mediated, but not in forskolin-mediated, cAMP levels (Droms, 1996). Dent et al. (1998) reported that the total phosphodiesterase enzyme, which is a cAMP catabolizing enzyme, is approximately nine-fold lower in human bronchial epithelial cells than in the human PAC type II cell line A549. As a result, more degradation of cAMP is expected in PAC cells of alveolar type II phenotype. In summary, the β-adrenergic pathway negatively regulates proliferation while promoting non-proliferative processes in alveolar type II cells. Consequently, it may be reasonable to speculate that by down-regulating the number and function of the various β-adrenergic effectors, alveolar type II cells gradually abandon their physiological role and assume an uncontrolled proliferative state during their malignant transformation. This would undoubtedly exacerbate the deteriorating alveolar physiology.

β-adrenergic signaling stimulates the growth of selected types of cancer types. This signaling pathway enhances the growth of adenocarcinoma cell lines derived from the human pancreas (Weddle et al., 2001), lung (Schuller et al., 2000; Park et al., 1995) or breast (Cakir et al., 2002). Park et al. (1995) initially demonstrated the mitogenic role of

β-adrenergic signal transduction in human PAC of the Clara cell phenotype. This work showed the importance of β-adrenergic agonists/cAMP in the proliferation of PAC. Supporting data with an NNK-induced hamster PAC model showed that β-adrenergic agonists promoted tumor development while β-adrenergic antagonists blocked this effect (Schuller et al., 2000). Moreover, the tobacco-specific carcinogen, NNK, is a high affinity agonist for β-adrenergic receptors (Schuller et al., 1999). Exposure of human PAC cell lines of Clara phenotype (NCI-H322 and NCI-H441) to these agonists resulted in production of cAMP, release of AA, and DNA synthesis. These findings identified a novel mechanism for tobacco-induced carcinogenesis. Schuller et al. (1999) also demonstrated the presence of B1 and B2 receptors in PAC cell types. Consequently, chronic treatment of respiratory diseases with β-adrenergic agonists may be a potential risk factor for the increased incidence of PAC (Park et al., 1995).

Finally, current data imply that PAC of Clara cell phenotype and PAC of alveolar type II cell phenotype respond to elevated cAMP levels in an opposite manner. Agents affecting the level of cAMP are expected to have antagonistic effects in the proliferation of PAC in a phenotype-specific manner. The effect of β-adrenergic stimulation on proliferation of EGFR expressing PAC cell lines is expected to be phenotype/cell type specific. This is likely to have important clinical and epidemiological implications since beta-adrenergic stimulators are active ingredients of drug formulations that are widely used in the management of chronic respiratory diseases.

Working hypothesis

- Pulmonary adenocarcinoma of Clara cell phenotype (PACC) and pulmonary adenocarcinoma of alveolar type II cell phenotype (PAC type II) express an EGFR autocrine growth pathway. This pathway is modulated by β-adrenergic pathways in a cell-type specific and antagonistic manner.

Specific aims

1. Assess the presence of the epidermal growth factor receptor (EGFR)-mediated proliferative pathway in human lung cancer cell lines of PACC and PAC-type II phenotypes.
2. Compare the effects of β-adrenergic stimulation and cAMP on proliferation of PACC and PACC-type II cell lines.
3. Compare the basal and post-treatment levels of cAMP in PACC and PAC-type II cell lines.

II. MATERIALS AND METHODS

Cells

Human pulmonary adenocarcinoma cell lines NCI-H322 (Clara cell phenotype) and A549 (alveolar type II cell phenotype) (Giard et al., 1973; Lieber et al., 1976) were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in 75 cm^2 vented cell culture flasks and routinely maintained in RPMI-1640 culture medium (Gibco) supplemented with fetal bovine serum (10%, v/v), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco) at 37°C in an atmosphere of 5% $C0_2$. All experiments were conducted with 1-12 passages.

Chemicals and biologicals

Isoproterenol (broad-spectrum beta-adrenergic agonist) and propanolol (broad-spectrum beta-adrenergic blocker) (Sigma-Aldrich, St. Luis, Mo); Atenolol (selective beta 1 adrenergic blocker) and ICI-118551 (selective beta 2 adrenergic blocker) (Tocris, UK); AG1478 (Selective EGFR Tyrosine kinase inhibitor), forskolin (adenylyl cyclase stimulator), PD98059 ([2-(2'-amino-3'-methylphenyl)-oxanaphtalene-4-one]) (MEK1/MAPK Kinase 1 inhibitor), and SQ22536 (9-(tetrahydro-2-furanyl)-^{9}H-6-amine (adenylyl cyclase inhibitor) (Calbiochem, La Jolla, CA); recombinant human epidermal growth factor (rhEGF); anti beta-actin antibody; and biotynylated antibody against

phosphotyrosine/clone PY-20 (Sigma-Aldrich, St. Louis, MO); human recombinant transforming growth factor-α (rhTGF-α) and antibody against EGF (R & D systems, Minneapolis, MN); antibody against the human EGF receptor; antibody against phosphorylated mitogen activated kinase (anti Phospho-MAPK/ERK1/2); horseradish peroxidase (HRP)-lined anti-rabbit secondary antibody; HRP-linked anti-biotin antibody, biotinylated molecular marker, and chemiluminescence horseradish peroxidase detection kit (Cell Signaling Technology, Beverly, MA, Beverly, MA); [^{3}H]-thymidine (specific activity 82.0 Ci/mMol) (Amersham, UK).

[^{3}H]-thymidine incorporation

General culture conditions for thymidine incorporation: [^{3}H]-thymidine incorporation was conducted to assess DNA synthesis and thus indirectly measure cell proliferation. Cells grown to 60-70% confluency were trypsinized and 6 x 10^3 cells/well were plated into 96 well plates in RPMI medium containing high-serum (10% FBS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were incubated for 24 hours in 37°C in an atmosphere of 5% $C0_2$. In this study, low-serum medium contained RPMI-1640 tissue culture medium with 0.1% fetal bovine serum (FBS) whereas high-serum medium contained RPMI-1640 tissue culture medium with 10% FBS. All incubations for growth assay were done in low-serum containing [^{3}H]-thymidine (0.5 μCi/well) and treatment agents or vehicle in a final volume of 200 μl/well for 24 hours in 37°C in an atmosphere of 5% $C0_2$. The amount of vehicle to dissolve the highest tested concentration of treatment reagent was used for control groups. At the end of the

specified incubation period, thymidine incorporation was assayed as described in the subsequent section.

Modification of culturing conditions was done according to the type of assay. For growth stimulation assays, the medium was changed to low-serum (0.1% FCS) for 24 hours to starve cells, and then replaced by fresh low-serum medium. Agents that were used to stimulate cell growth were recombinant human epidermal growth factor (rhEGF), recombinant human transforming growth factor alpha (TGF-α), isoproterenol (broad spectrum β-adrenergic agonist), and forskolin (adenylyl cyclase stimulator).

For growth inhibition assays, cells were treated in low-serum after the first 24-hr incubation in high-serum. Agents used were antibody against EGF, AG1478 (EGFR-specific Tyrosine kinase inhibitor), PD98059 (MEK inhibitor), and 8-Cl-cAMP (site-specific cAMP analogue). The site-specific cAMP analogue was used to test whether the inhibitory effect of forskolin in A549 cells could be reproduced by cAMP.

Specific culture and treatment conditions for thymidine incorporation assay: Recombinant human growth factor (rhEGF) was tested at 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 20 ng/ml, 100 ng/ml, and 200 ng/ml. Acetic acid (10 mM in 0.1% BSA) was used as a vehicle. Cells in the control group were treated with vehicle only.

Recombinant human transforming growth factor-α (rhTGF-α) was tested at 20 ng/ml, 40 ng/ml, 100 ng/ml, and 200 ng/ml. Acetic acid (10 mM in 0.1% BSA) was used as a vehicle. Cells in the control group were treated with vehicle only.

Antibody against the human epidermal growth factor was tested at 4 μg/ml, 8 μg/ml, and 16 μg/ml. The antibody was dissolved in the low-serum (0.1% FBS) culture medium.

The EGFR-specific tyrosine kinase inhibitor, AG1478, was tested at 1 μM, 10 μM, and 20 μM. Dimethyl sulfoxide (DMSO) was used as a vehicle. Cells in the control group were treated with vehicle only.

The MEK1 (MAPK kinase) inhibitor was tested at 1 μM, 10 μM, and 20 μM. Dimethyl sulfoxide (DMSO) was used as a vehicle. Cells in the control group were treated with vehicle only.

Isoproterenol (Broad-spectrum β-adrenergic agonist) was tested at 100 pM, 1 nM, 10 nM, 100 nM, and 1 μM. Isoproterenol was dissolved in the low-serum culture medium.

Forskolin (adenylyl cyclase stimulator) was tested at 100 pM, 1 nM, 10 nM, 100 nM, and 1 μM. Dimethyl sulfoxide (DMSO) was used as a vehicle. Cells in the control group were treated with vehicle only.

The site-specific cAMP analogue (8-Cl-cAMP) was tested at 0.1 mM, 0.5 mM, and 1 mM. 8-Cl-cAMP was dissolved in the low-serum medium.

Preincubation with the various antagonists was done to verify specificity of the observed isoproterenol-induced effects on DNA synthesis. At the end of the 24-hr starvation period, cells were incubated for 20 minutes in a fresh low-serum medium containing propanolol (broad-spectrum β-adrenergic antagonists, 2 μM), atenolol (selective β1-aderenergic antagonist, 2 μM), ICI-118551 (selective β2-aderenergic antagonist, 2 μM), or SQ22536 (adenylyl cyclase inhibitor, 2 μM). This pre-incubation was followed by the addition of isoproterenol (2 μM) making the final concentration of isoproterenol and antagonists 1 μM. Low-serum medium was used as a vehicle for isoproterenol propanolol, atenolol, ICI-118551 whereas DMSO was used for forskolin and SQ22536. Cells in the control groups were treated with vehicles only. Cells were incubated for an additional 24 hrs, and DNA was harvested at the end of the incubation period. The concentration used for specificity (1 μM) was adopted from earlier work from this laboratory (Park et al., 1995).

Preincubation with SQ22536 (adenylyl cyclase inhibitor) was done to verify the specificity of the observed forskolin-induced effects on DNA synthesis. At the end of the 24-hr starvation period, cells were incubated for 20 minutes in a fresh low-serum medium containing SQ22536 (2 μM). This pre-incubation was followed by the addition of forskolin (2 μM) making the final concentration of forskolin and SQ22536 1 μM. Dimethyl sulfoxide (DMSO) was used as a vehicle for both forskolin and SQ22536. Cells

in the control group were treated with vehicle only. Cells were incubated for an additional 24 hrs, and DNA was harvested at the end of the incubation period.

Preincubation with AG1478 (EGFR-specific Tyrosine kinase inhibitor) was done to assess the involvement of the epidermal growth factor receptor in isoproterenol- and forskolin-mediated effects on DNA synthesis. At the end of the 24-hr starvation period, cells were incubated for 20 minutes in a fresh low-serum medium containing AG1478 (1 μM or 10 μM). This pre-incubation was followed by the addition of forskolin or isoproterenol making the final concentration 1 μM. Low-serum medium was used as a vehicle for isoproterenol whereas DMSO was used for forskolin and AG1478. Cells in the control group were treated with the vehicle only. Cells were incubated for an additional 24 hrs, and DNA was harvested at the end of the incubation period.

In order to examine the effect of serum levels on DNA synthesis, cells were plated in high-serum medium (10% FBS) and incubated for 24 hours. Cells then were rinsed with low-serum medium (0.1% FBS) and fresh high-serum (10% FBS) or low-serum (0.1% FBS) was added and incubated for 4, 8, 12, 18, and 24 hours. DNA was harvested at the end of each incubation period.

DNA harvesting for thymidine incorporation assay: At the end of incubation period, cells were washed 3 times with PBS and lysed by adding 30 μl of 0.1N NaOH. The plate was incubated at room temperature on a horizontal orbital microplate shaker. Incorporated thymidine was selectively harvested from the lysates by vacuum filtration using a

Micromate™ 196 cell harvester (Packard Instrument Company, Meriden, CT) onto a backed glass fiber filter. The fiber was flushed with isopropanol (200µl/well) to fix adsorbed DNA. The harvester cut circular disks corresponding to each well. Each disk was transferred to a scintillation vial containing 3 ml counting cocktail (Bio-Safe-II™, Research Products International Corp., Mount Prospect, IL). A liquid scintillation analyzer (Packard Instruments Company, Meriden, CT) was used to determine radioactivity bound to the filters.

Thymidine incorporation assay for interrupted cultures in 24-well plates: The presence of released growth factors in the low-serum growth condition was indirectly assessed using the modified interrupted-culture-method (Murphy et al. 2001). Cells (20 x 10^3 cells/well) were plated in 24-well plates in high-serum and incubated for 24 hours. Cells were then washed with low-serum and incubated in low-serum medium. In the first treatment group, cells were continuously maintained for 48 hours without any interruption. In the second treatment group, every 12 hours the medium was removed and cells were washed in-situ with low-serum medium. Fresh low-serum medium was added and incubated. In the third treatment group, every 12 hours the medium was removed and saved. Cells were washed in situ with low-serum medium, and the saved primed medium was replaced. The third treatment group served as a control for possible stress from frequent medium manipulation. All treatment groups were maintained as described for 48 hours.

At the end of this period, medium was removed and cells were washed twice with low-serum. This was followed by addition of 0.5 ml of fresh low-serum medium containing

[^{3}H]-thymidine (0.5 μCi/well). After a 3 hours incubation, cells were washed 5X with PBS. This was followed by precipitation with (1:3) (v/v) solution of glacial acetic acid in methanol for 10 minutes, and washed with 80% (v/v) methanol in water. DNA was dissolved by adding of 0.1N NaOH (300 μl). The lysates were thoroughly mixed, and 100 μl of sample was transferred to a scintillation vial containing 3ml of counting cocktail. A liquid scintillation analyzer was used to determine radioactivity.

Western blotting

To examine the effect of serum-starvation on MAPK expression, 5 x 10^5 cells were plated in a 100 mm tissue culture dish and maintained in high-serum medium. At 70-80% confluency, the medium was replaced with low-serum medium. Lysates for western blotting were collected at 0, 15, and 30 minutes and 1, 2, 8 and 12 hours incubation.

To assay the effects of treatment with forskolin (adenylyl cyclase stimulator) on MAPK expression and activation, 5 x 10^5 cells were plated in RPMI-1640 medium supplemented with 10% FBS in a 100 mm tissue culture dish. At 70-80% confluency, the medium was replaced with low-serum medium (0.1% FBS) with or without forskolin. Lysates for western blotting were collected at 0, 15, and 30 minutes and 1, 2, 8 and 12 hours incubation. The same method was used to assess the effect of Ag1478 (EGFR-specific Tyrosine kinase inhibitor).

At the end of each incubation period, cells were washed in situ with ice-cold phosphate buffered saline (PBS). This was followed by lysis with 300 µl of RIPA [cell lysis buffer containing 1XPBS, 1%NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] including protease inhibitors [(10 µl/ml of 10mg/ml phenylmethylsulphonyl fluoride (PMSF); 10 µl/ml of 100mM sodium orthovanadate and 30µl/ml of 1.7 mg/ml aprotinin)]. Cells then were scraped with a rubber policeman, transferred to a microcentrifuge tube, and vortexed. The cell lysate was passed through a 21-gauge needle (2-3x); 10 µl of PMSF (10mg/ml) was added, and the mix was incubated on ice for 30 minutes. This was followed by centrifugation at 14,000 rpm (1600 g's) for 20 minutes at 4°C. The supernate was collected and the protein concentration was determined by bichinchoninic acid assay (BCA™) (Pierce, Rockford, IL). A sample of cell lysate (10 µl) was diluted to a final 1:10 concentration in RIPA buffer. Ten µl of diluted lysate and serial standard albumin preparation (0 to 1200 µg/ml) were added into 96 microplate wells. To this was added 200-µl bichinchoninic acid reagent and incubated at 37°C for 30 minutes. Absorbance was read using a spectrophotometer (BioTeK Instruments Inc., Winooski, VT) at a wavelength of 550nm. Protein concentration (ug/ml) was determined by linear regression (Graph Pad, San Diego, CA). A sample of lysate representing 20-µg protein was used for electrophoresis. Each sample was mixed with an equal volume of 2X SDS (Sodium dodecyl sulfate) loading buffer (4% SDS, 0.1 M Tris-Cl at pH 6.8, 0.2% bromophenol blue, 20% glycerol), centrifuged at high speed for 5 seconds, and boiled for 3 minutes at 95°C. Samples were then centrifuged at high speed for 5 seconds and loaded onto a 10 % Tris-HCl PAGE gel (Bio-Rad Laboratories, Hercules, CA) and subjected to 200 volts for 45 minutes in running buffer containing 10% of 10X Tris SDS

buffer in deionized water. A biotin-labeled molecular weight marker (Cell Signaling Technology, Beverly, MA) was run for each gel. Gels were removed and electrophoretic transfer of proteins to nitrocellulose membrane was conducted at 100 volts for 1 hour in a transfer buffer containing 5.8 g tris-hydroxymethyl-aminomethane, 2.9 g glycin, 3.7 ml 10% SDS, 200 ml methanol, and 800 ml deionized water. Membranes were removed and washed three times (for 5 minutes each) with wash solution containing 1X tris buffered saline (TBS) (200 mM Tris HCl and 1.5 M NaCl per liter) and 0.05% tween-20. Membranes were blocked with 5% non-fat dry milk in 1X TBS and 0.05% tween-20 for 1 hour. This was followed by three five-minute washes with wash solution. Membranes were then incubated overnight at 4°C in primary antibody (rabbit polyclonal IgG for phospho-ERK1/2, 200ug/ml) (Cell Signaling Technology, Beverly, MA) at a 1:1000 dilution in the same blocking solution. The antibody against phospho MAPK (ERK1/2) recognizes the phosphorylated Tyrosine and threonine residues at positions 204 and 202, respectively (Cell Signaling Technology, Beverly, MA). At the end of the incubation period, three five-minute washes were done. Membranes then were incubated for 1 hour at room temperature in blocking solution containing horseradish peroxidase-linked antirabbit IgG and horseradish peroxidase-linked antibiotin antibody at 1:2000 and 1:1000 dilutions, respectively. This was followed by three five-minute washes. Protein bands were detected with a chemiluminescent horseradish peroxidase detection kit (Cell Signaling Technology, Beverly, MA).

Equal loading of protein was ensured by stripping and incubating membranes with anti-actin polyclonal rabbit IgG in blocking solution at a dilution of 1:100. Membranes were

stripped by incubation in a stripping buffer containing 36.5 ml deionized water, 10 ml 10% SDS, 350 µl mercaptoethanol (100mM), 3.125 ml of tris HCl (pH 6.7, 62.5mM) for 45 minutes in a water bath at 50°C with gentle agitation. The same secondary antibodies and procedure were used to detect protein bands.

Immunoprecipitation and western blotting

Immunoprecipitation followed by western blotting was conducted to determine whether the antiproliferative and MAPK inhibitory effect of forskolin, an adenylyl cyclase stimulator, is mediated through inhibition of EGFR phosphorylation. Cells were sampled at 0 (no treatment), 30 minutes (maximal ERK1/2 activation), 8 hours (first highly significant ERK1/2 inhibition), and 24 hours (incubation length for thymidine-incorporation assay and last highly significant ERK1/2 inhibition). These sampling times were selected to show modulation EGFR activation in relation to the ERK1/2 modulation and DNA synthesis by forskolin.

Cells ($5X10^5$) were plated in RPMI-1640 medium supplemented with 10% FBS in 100 mm tissue culture dishes. At 60-70% confluency, the medium was replaced with low-serum (0.1% FBS) with or without forskolin. Harvesting of cell lysates was done at 0, 30 minutes, 8 hr and 24 hr as described for western blotting. At the end of each incubation period, cells were washed with ice-cold PBS and cell lysate preparation and protein determination was done as described for western blotting. A sample of lysate containing 400 µg protein was taken and 1µg or 10 µl of 100 µg/ml rabbit anti-human EGFR

antibody was added (Cell Signaling Technology, Beverly, MA). The mix was incubated overnight at 4 °C with gentle rocking after which 20 uL (50% slurry) of Protein-A beads (Santa Cruz, Santa Cruz, CA) was added and incubated for 4 hours at room temperature with gentle rocking. This was followed by centrifugation at 2500 RPM (1000 X g) for 5 minutes at 4 °C, and the supernate was carefully removed. The pellet was washed five times by repeated centrifugation at 2500 RPM (1000 X g) for 5 minute at 4 °C in 500 µl of RIPA (cell lysis buffer) containing the same protease inhibitors and concentrations indicated previously. Finally, 15 µl of RIPA with the protease inhibitors was added followed by 15 µl of 2X loading/sample buffer to make a final volume of 30µl. Samples then were centrifuged and heated to 95-100 °C for 3 minutes as described for the western blotting. Samples were finally centrifuged at high speed for 5 seconds to homogenize the mix and then divided equally (15 ul/sample) and loaded on separate gels. One of the gels was used to determine the total quantity of EGFR and the other one was used to quantify the total amount of phosphorylated EGFR. A biotin-labeled molecular weight marker was run for each gel. Electrophoresis of proteins and transfer to nitrocellulose membranes were done as described for western blotting. After washing the membrane as described earlier, membranes were blocked in 3% bovine serum albumin (BSA) for 1 hour. One of the membranes was then incubated with anti-rabbit polyclonal antibody and horse radish peroxidase-linked antibiotin antibody at 1:2000 and 1:1000 dilution in 3% BSA, respectively. Incubation was done for 1 hour at room temperature. After washing the membranes as described earlier, protein bands were detected with a chemiluminescent detection kit (Cell Signaling Technology, Beverly, MA). A molecular

marker was used to detect the EGFR protein band. The other gel for detection of phosphorylated EGFR was incubated with biotin labeled monoclonal anti-phosphotyrosine antibody (Clone PY-20) (Sigma-Aldrich, St. Louis MO) at 1:5000 dilution in 3% BSA overnight at 4 °C with gentle rocking. The membrane was washed as described earlier and blocked with 3% BSA. This was followed by the same washing process and incubation with horse radish peroxidase-linked antibiotin antibody at a 1:1000 dilution in 3% BSA for 1 hour at room temperature. Membranes then were washed and protein bands were detected by chemiluminescent detection kit (Cell Signaling Technology, Beverly, MA).

Densitometric analysis of protein bands

Densitometry of protein bands was done with a Scion image analyzer software (NIH, Bethesda, MD). Densitometric values for target protein and beta-actin (internal control protein) was expressed as pixel values. Relative expression of target proteins was computed as ratio of pixel values of target protein/pixel values of beta-actin. Relative quantity of phosphorylated EGFR in the immunoprecipitation assay was computed as a ratio of pixel values of tyrosine-phosphorylated EGFR/pixel values of total EGFR.

Photography

Cells from both cell lines were plated (2 x 10^5/well) in 6-well tissue-culture plates in high-serum medium and incubated at 37 °C for 24 hours. Medium then was replaced with

fresh low-serum medium (0.1% FBS) containing rhEGF at increasing concentrations or vehicle (10 mM of acetic acid in 0.1% bovine serum albumin/BSA). Cells were then incubated for 24 hours. Cells were examined with an inverted microscope and pictures were taken with Nikon FX35A camera (Tokyo, Japan)

Cyclic AMP assay

The level of cAMP in cell lysates was determined with a cyclic AMP immunoassay kit (R&D Systems, Minneapolis, MN).

Principle of the assay: The assay is based on the competitive binding technique in which the cAMP present in a sample competes with a fixed amount of alkaline phosphatase-labeled cAMP for sites on a rabbit polyclonal antibody to cAMP. During the incubation, the polyclonal antibody becomes bound to a goat anti-rabbit antibody coated on the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped after an hour of incubation and the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of cAMP in the sample (R&D systems, Minneapolis, MN)

Cell culture, treatment and sample preparation for cAMP immunoassay: Cells ($4X10^5$/well) were plated in RPMI-1640 medium (10% FBS) in 6-well tissue culture plates. Cells were incubated until they reached 70-80% confluence.

Medium was removed and fresh RPMI-1640 medium (0.1% FBS) containing 1 mM 3-isobutyl 1-methylxanthine (IBMX), a phosphodiesterase inhibitor, was added to all treatment and control groups. Cells were incubated for 30 minutes at 37 °C. To determine control/basal cAMP levels, 4 wells from each cell line were washed three times with distilled water and cells were incubated for 20 minutes in a 400 µl lysis solution containing 0.1N HCl dissolved in 0.1% 100X-Triton. The 0.1% 100X-Triton was made in 1X TBS. Undetached cells were removed by repeated flushing with the cell lysate. Wells were observed under a microscope to ensure complete removal of cells. The lysate was transferred to centrifuge tubes. The wells then were washed with an additional 200 µl of the lysis solution and this was added to the respective lysates. The cell lysates were centrifuged at 600 g for 10 minutes at 4 °C. The supernatants were collected and kept on ice until analyed.

In the stimulation assays, isoproterenol (1 µM) or forskolin (1 µM or 10 µM) was added after the medium containing IBMX was decanted, and cells were washed with low-serum medium. Cells were then incubated for 20 minutes at 37 °C. At the end of incubation, cells were harvested by the method described for the control groups.

In the inhibition assays, the medium containing IBMX was replaced with fresh low-serum medium containing ICI-118-551 (selective β2-adrenergic antagonist) (1 µM) or atenolol (selective β2-adrenergic antagonist) (1 µM). Cells were then incubated for 20 minutes, after which low-serum medium containing isoproterenol were added making the

final concentration of isoproterenol 1 μM. Incubation was done at 37 °C for 20 minutes, and cells were harvested for cAMP assay as described for the control groups.

In all cAMP assays, four additional wells for each cell line were used for determination of protein concentrations/well. Plates for this purpose were harvested at the beginning of the experiment with 200 μL RIPA containing protease inhibitor cocktail. The same procedure of protein determination for lysate preparations described for western and immunoprecipitation was employed. However, the lysates were diluted to 1:2 instead of 1:10 to avoid over-dilution. Mean protein content (mg) of the four wells was used to compute cAMP concentrations in pM/mg of protein.

Sample dilution for cAMP immunoassay: A sample of 125 μL was taken from the each supernate. A 1:2 dilution was made by adding 125 μL of asay buffer. This was mixed well by vortexing and two 100-μL samples were taken for duplicate analysis.

Cyclic AMP standards for cAMP immunoassay: Serially diluted cAMP standards were prepared at 200, 50, 12.5, 3.125, and 0.781 pM/ml by diluting in the assay buffer provided with the kit. Cyclic AMP standards were used within 30 minutes of preparation.

Assay procedure for cAMP immunoassay: Samples and standards were added to assay wells at 100 μL in duplicate. For the zero standard and non-specific binding (NSB) wells, 100 and 150 μL assay buffer was added, respectively. To all wells 50 μL of cAMP conjugate (cAMP conjugated with alkaline phosphatase) was added. This was followed

by the addition of 50 μL of cAMP antibody solution and incubation at room temperature for 2 hours on a horizontal orbital microplate shaker set at 400 rpm. At the end of the incubation period, medium was decanted and wells were washed 4 times with 1X washing buffer in distilled water. The plate was blotted to remove any remaining liquid.

For the measurement of total activity (TA), 5 μL of cAMP conjugate was added in two additional wells. This was followed by the addition of 200 μL of substrate (p-nitrophenyl phosphate) to all wells. The plate was then incubated for 1 hour at room temperature without shaking. At the end of the 1-hour incubation, 50 μL of stop solution (trisodium phosphate) was added. Optical density was determined immediately using a spectrophotometer set at 405 nm primary and 590 nm reference wavelengths (BioTeK Instruments Inc., Winooski, VT).

Calculation of results: The duplicate readings for standards and samples were averaged. The average reading from the non-specific binding wells (NSB) was subtracted from this value. A dose response curve was generated by plotting the logarithm concentrations of standards against respective mean absorbance using a non-linear regression curve fit (R^2 = 0.9895 and 0.9995). The concentration of samples corresponding to the mean absorbance was calculated in pM/ml from the dose-response curve (GraphPad Prism, San Diego, CA). The total cAMP in the 600-μL-sample lysates then was determined. This value was divided by the amount of total protein in the 600 μL to obtain the concentration of cAMP in pM/mg.

Statistical analysis

Graph Pad software was used to analyze data. Mean ± standard error of the mean of three independent experiments with 4-8 replicates/group was generated for count per minute (thymidine incorporation) and cAMP level. Mean values in control groups were set as 100% and values from treatment groups were normalized as percentage of values of control groups. Two-way paired t-test was conducted to compare means between two treatment groups. With more than two treatment groups, one-way analysis of variance (ANOVA) was conducted with Dunnet comparison (comparison of mean of treatment groups versus mean of the respective control group with treatment as the only fixed effect). Basal cAMP content in the two cells was compared with unpaired t-test with Welch's mean comparison for unequal variance. For comparison of the amount of protein expression, computed target/internal-control pixel-ratios from independent experiments were pooled and means were compared by one-way analysis of variance (ANOVA). The level of significance (α-level) was set at 0.05.

III. RESULTS

Effect of serum level on proliferation

The effect of serum level on DNA synthesis was assessed by ^{3}H-thymidine incorporation assay. In both cell lines, DNA synthesis was significantly higher in low-serum (0.1% FBS) than in high-serum condition (10% FBS) ($p<0.001$). The stimulation was consistent for all time intervals (Figure 3 and 4). A 48-hour low-serum incubation with replacement of culture medium every 12 hours was conducted to indirectly assess the presence of released growth stimulating factors in the serum. This caused significant ($p<0.001$) reduction in DNA synthesis in both cell lines with a more pronounced effect in A549 cells (Fig 5 and 6). In order to rule out the effect of possible stress from repeated medium replacement and rinsing, the procedure was conducted in a second control group with some variation. The culture medium for this group was removed temporarily, and the same medium was replaced. DNA synthesis was not significantly different from that of the control group ($p>0.05$) (Figure 5 and 6).

A parallel increase in the level of phosphorylated or activated MAPK (ERK1/2) with serum withdrawal was observed. Phosphorylated ERK1/2 content showed a significant increase as early as 30 minutes after high-serum medium was replaced with low-serum medium ($P<0.05$), a trend that was maintained at all sampling points in the 24-hour-period incubation in low-serum ($P<0.01$) (Figure 7 and 8).

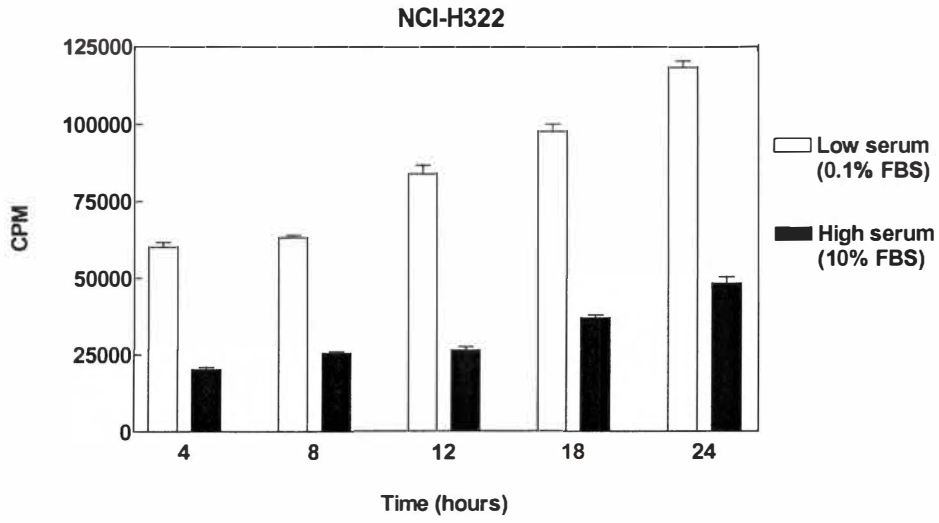


Figure 3. Effect of serum content on DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours, after which fresh medium containing 10% FBS or 0.1% FBS with [^{3}H]-thymidine (0.5 μCi/well) was added. Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments. Means in each paired treatment groups were compared with paired-t test. Serum deprivation significantly stimulated DNA synthesis ($p<0.001$ for all incubation periods)

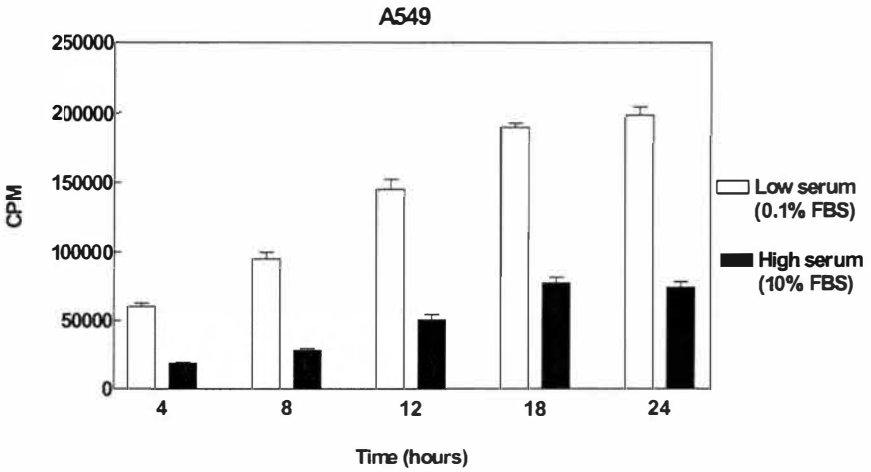


Figure 4. Effect of serum content on DNA synthesis in A549 cells (PAC type II). Cells were plated and incubated in high serum medium for 24 hours, after which fresh medium containing 10% FBS or 0.1% FBS with [^{3}H]-thymidine (0.5 μCi/well) was added. Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments. Means in each paired treatment groups were compared with paired-t test. Serum deprivation significantly stimulated DNA synthesis ($p<0.001$ for all incubation periods)

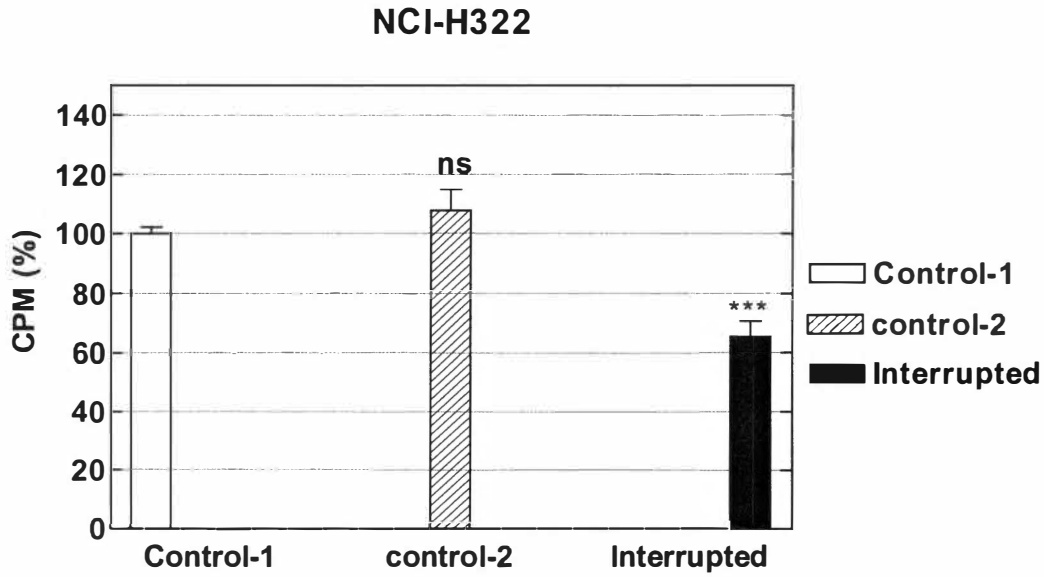


Figure 5. Effect of continuous and interrupted culture on DNA synthesis in NCI-H322 cells (PACC). Cells were plated in 24-well plates in high serum medium and incubated 24 hours. Fresh low serum medium was added to all wells. In control-1, the medium was continuously maintained without replacement. In control-2, every 12 hours, the primed medium was briefly removed, saved, and replaced immediately; In the interrupted group, the primed medium was replaced with fresh low serum medium every 12 hours. This treatment was done for 48 hours. At the end of the last 12-hr incubation, 0.5 μCi [^{3}H]-thymidine/well was added in fresh low serum medium and incubated for 3 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control-1 (continuous culture) group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Periodic replacement of primed medium with fresh medium significantly reduced DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

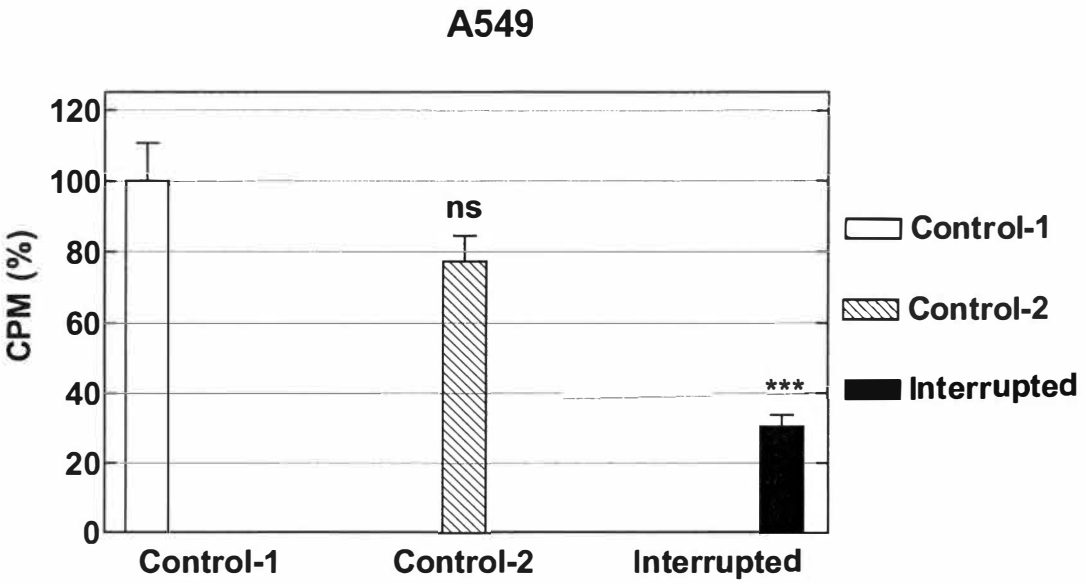
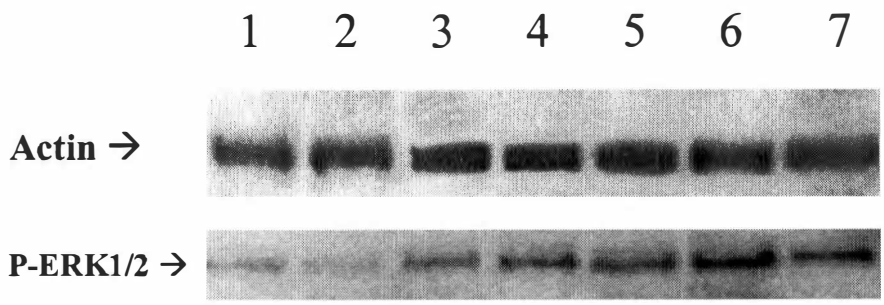


Figure 6. Effect of continuous and interrupted culture on DNA synthesis in A549 cells (PAC type-II). Cells were plated in 24-well plates in high serum medium and incubated 24 hours. Fresh low serum medium was added to all wells. In control-1, the medium was continuously maintained without replacement. In control-2, every 12 hours, the primed medium was briefly removed, saved, and replaced immediately; in the interrupted group, the primed medium was replaced with fresh low serum medium every 12 hours. This treatment was done for 48 hours. At the end of the last 12-hr incubation, 0.5 μCi [^{3}H]-thymidine/well was added in fresh low serum medium and incubated for 3 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control-1 (continuous culture) group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Periodic replacement of primed medium with fresh medium significantly reduced DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)



1 = 0 min.
2 = 15 min.
3 = 30 min.
4 = 1 hr.
5 = 2 hr.
6 = 8 hr.
7 = 24 hr.

Figure 7. Western blot of Phospho-MAPK showing the effect of serum deprivation on activation of ERK1/2 in NCI-A549 cells (PAC-type II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium then was replaced with low serum medium and cell lysates were harvested at the indicated periods for western blotting. ERK1/2 was activated as early as 30 minutes after high serum medium was replaced with low serum. Actin bands serve as an internal control for equal protein loading.

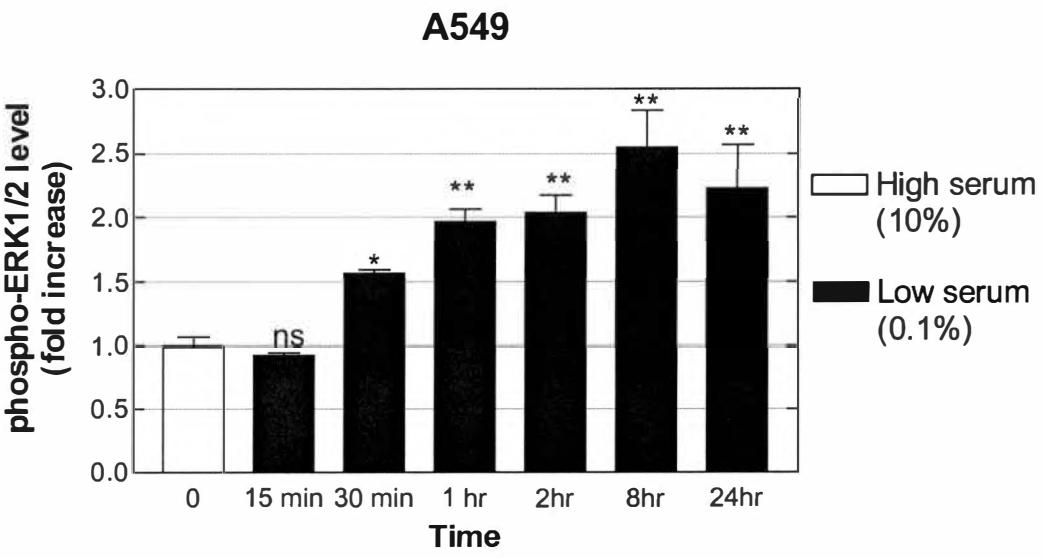


Figure 8. Densitometric values of western blot analysis showing the effect of serum-deprivation on the amount of phospho-ERK1/2 protein in A549 cells (PAC type-II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium then was replaced with low serum medium and cell lysates were harvested at the indicated periods for western blotting. Bars represent the mean ERK/actin pixel ratio as expressed as fold-increase of the mean value of the control group (0 hr) that was normalized at 1. Means in each treatment group were compared with the mean from the control group using ANOVA with Dunnet comparison. ERK1/2 was significantly activated as early as 30 minutes after high serum medium was replaced with low serum. (ns –non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).

Effect of TGFα, EGF and anti-EGF antibody on DNA synthesis

The effect of rhTGF-α (recombinant human transforming growth factor-α), rhEGF (recombinant human epidermal growth factor), and antibody against the human epidermal growth factor (EGF) on DNA synthesis was assessed by ^{3}H-thymidine incorporation assay.

The human recombinant EGF had a differential effect on DNA synthesis in NCI-H322 and A549 cells. In A549 cells, EGF did not have any significant effect on DNA synthesis at all concentrations tested ($p>0.05$) (Figure 10). In contrast, except at lower concentrations (≤ 10 ng/ml), EGF significantly increased DNA synthesis in NCI-H322 cells at 20ng/ml and higher concentrations ($p<0.01$) (Figure 9).

DNA synthesis was significantly inhibited by antibody against EGF in both cell lines. Inhibition in NCI-H322 cells was significant ($p<0.05$) at 4 μg/ml and highly significant ($p<0.001$) at 8 μg/ml and 16 μg/ml (Figure 12). The antibody against EGF also inhibited DNA synthesis in A549 cells (Figure 13). However, the inhibitory effect of EGF neutralization was less pronounced in this cell line as compared to that observed in NCI-H322 cells. Significant ($p<0.05$) inhibition was observed at 8 μg/ml and 16 μg/ml and no significant ($p>0.05$) effect was observed at 4 μg/ml.

The human recombinant TGFα showed no significant effect on DNA synthesis in both cell lines ($p>0.05$) (Figure 14 and 15).

Effect of EGF on cell morphology

In A549 cells, EGF induced morphological changes ranging from spindle to multi-appendaged fibroblastic appearance. This effect was particularly evident at high EGF concentration. This morphological change was not observed in NCI-H322 cells (Figure 11).

Effect of the tyrosine kinase inhibitor, AG1478, on DNA synthesis

The EGFR specific tyrosine kinase inhibitor, AG1478 (Arteaga et al., 2002), invariably inhibited DNA synthesis in both cell lines that was stimulated by serum deprivation. Inhibition was highly significant at all concentrations used in NCI-H322 cells ($p<0.001$) (Figure 16). Highly significant inhibition was also observed in A549 cells with exception of 1μM, which did not significantly affect DNA synthesis ($p>0.05$) (Figure 17). The concomitant MAPK inhibition by AG1478 was shown in A549 cells (Figure 18 and 19)

Effect of the MEK1 inhibitor, PD98059, on DNA synthesis

The MEK1 inhibitor, PD98059 (Dario et al., 1995), inhibited DNA synthesis in both cell lines as monitored by ^{3}H-thymidine incorporation assay (Figures 20 and 16). Inhibition was highly significant at all concentrations ($p<0.001$) with the exception of 1μM, which did not significantly affect DNA synthesis in A549 cells ($p>0.05$) (Figure 21).

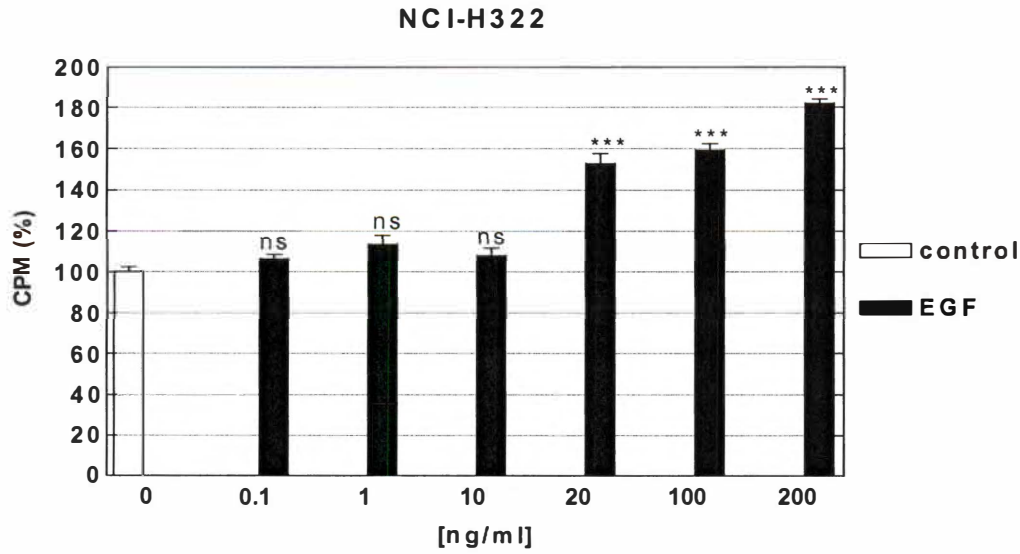


Figure 9. Effect of EGF on DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing rhEGF or vehicle (10 mM acetic acid in 0.1% BSA). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. EGF at ≥ 20ng/ml significantly increased DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

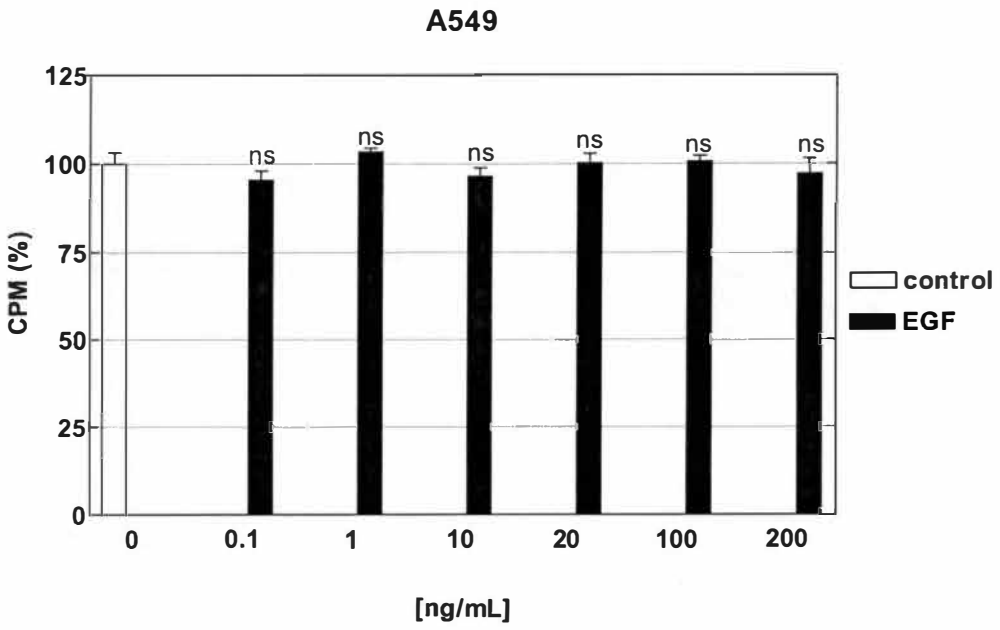


Figure 10. Effect of EGF on DNA synthesis in A549 cells (PAC type II). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing rhEGF or vehicle (10 mM acetic acid in 0.1% BSA). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. EGF showed no significantly effect on DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

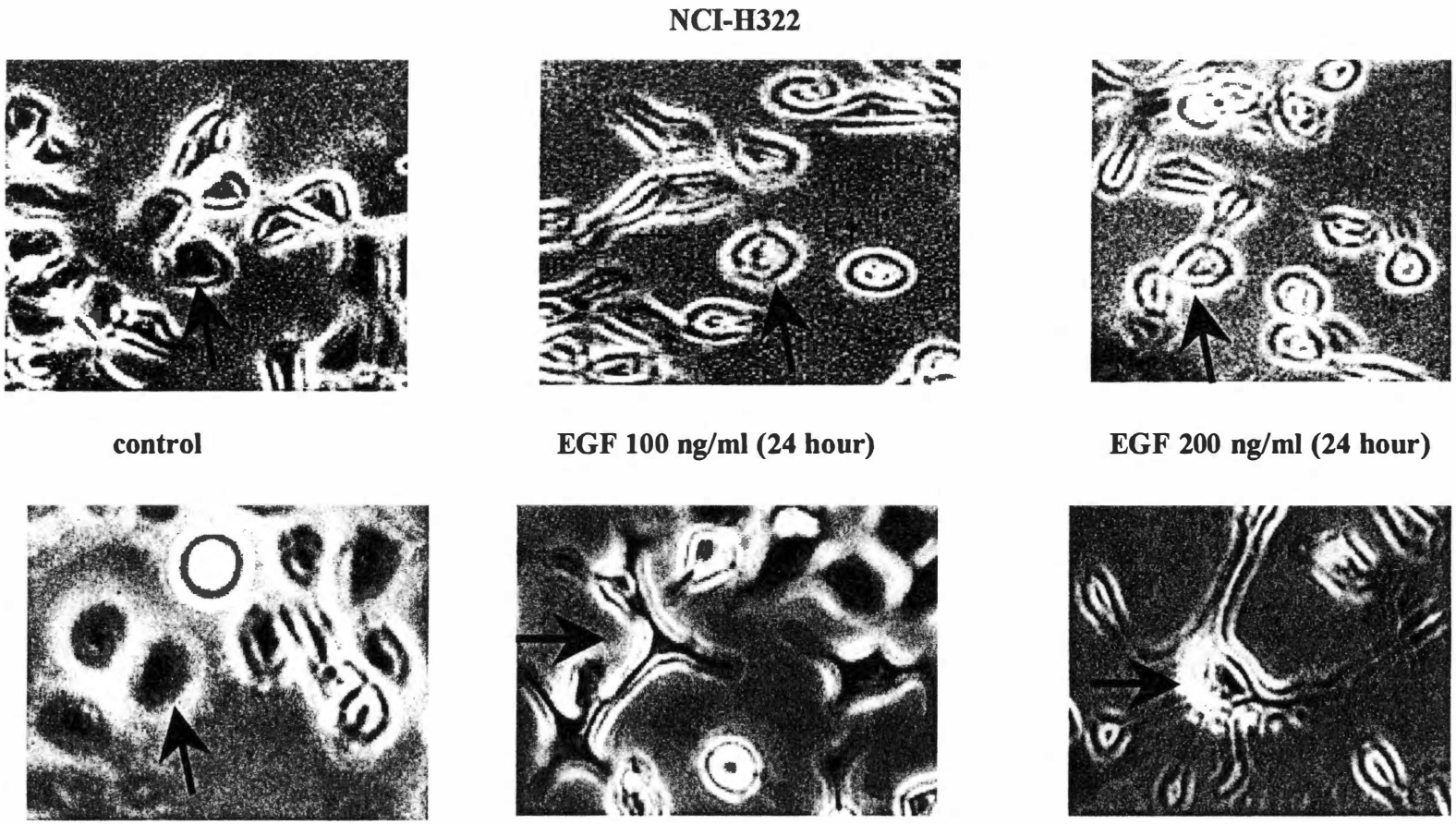


Figure 11. Effect of EGF on cellular morphology. Epidermal growth factor differentially induced fibroblastic morphology in A549 cells (PAC-type II)

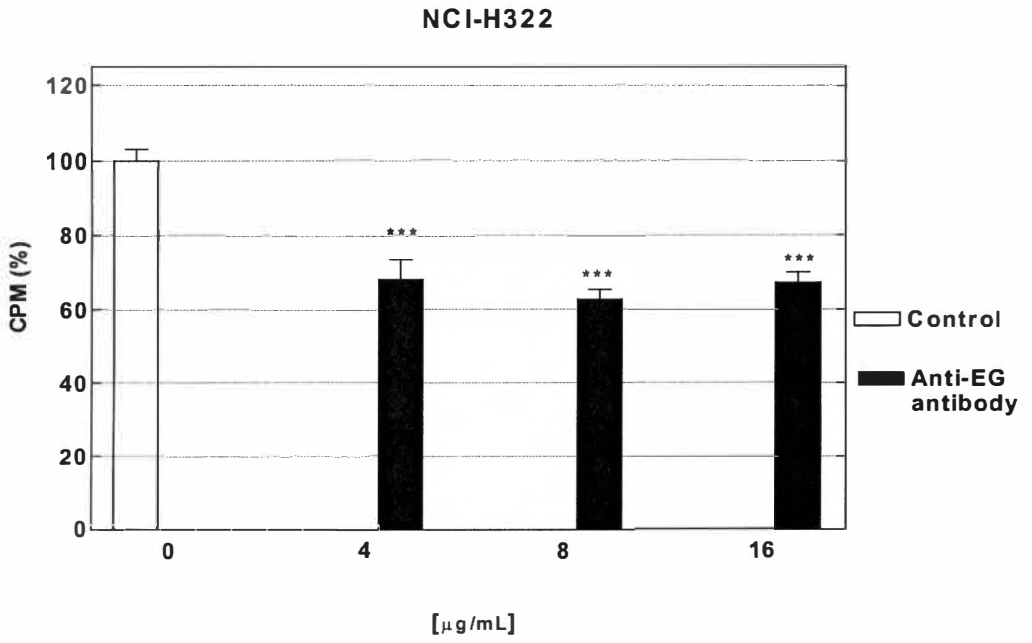


Figure 12. Effect of anti-EGF antibody on DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. This was followed by the addition of low serum medium containing 0.5 µCi [^{3}H]-thymidine/well with or without anti-EGF antibody was incubated 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Antibody against EGF significantly decreased DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

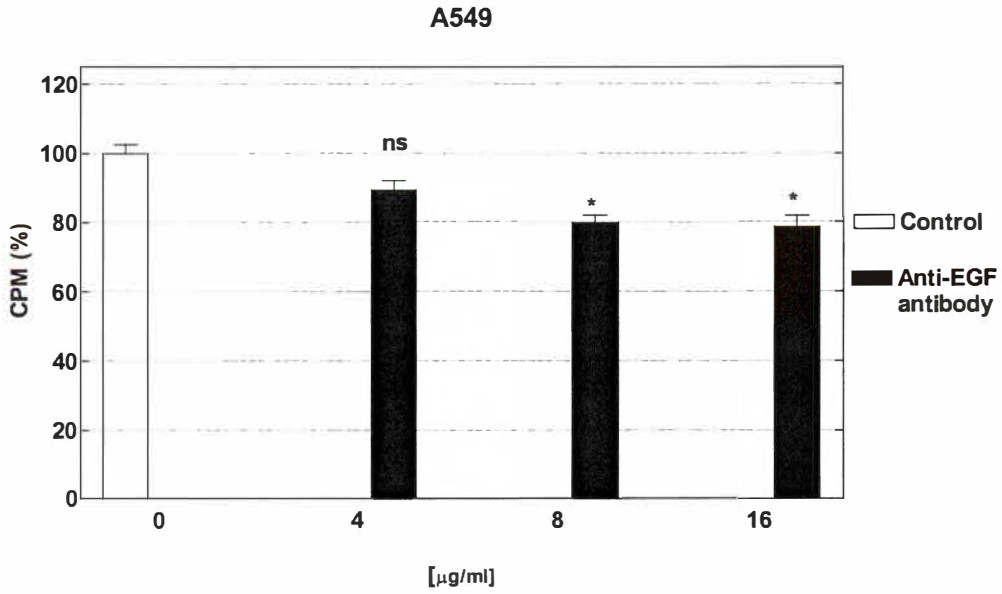


Figure 13. Effect of anti-EGF antibody on DNA synthesis in A549 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. This was followed by the addition of low serum medium containing 0.5 μCi [^{3}H]-thymidine/well with or without anti-EGF antibody was incubated 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Antibody against EGF significantly but moderately decreased DNA synthesis at μ 8 ìg/ml. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

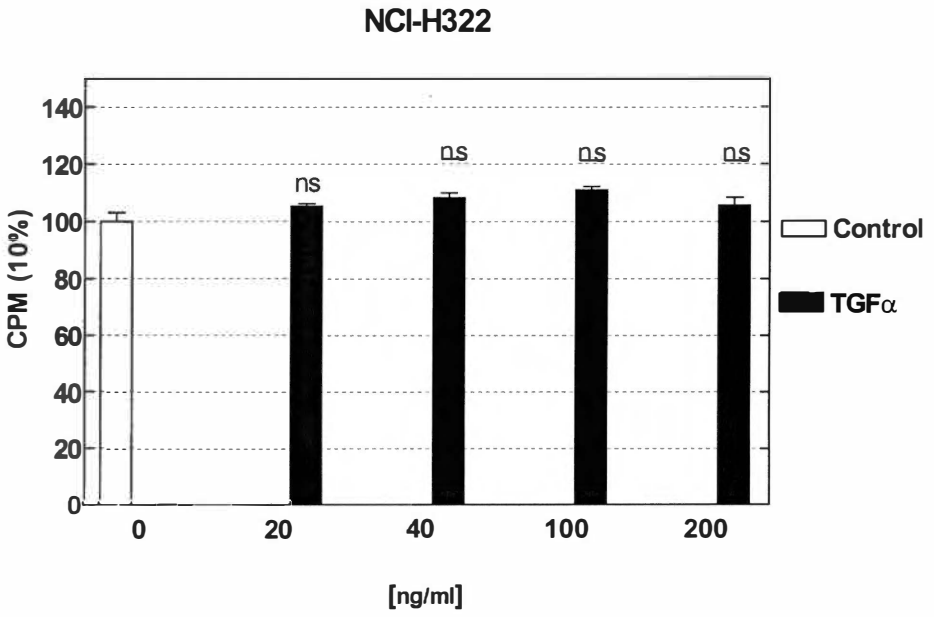


Figure 14. Effect of TGF-α on DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing rhTGF-α or vehicle (10 mM acetic acid in 0.1% BSA). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. TGF-α showed no significantly effect on DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

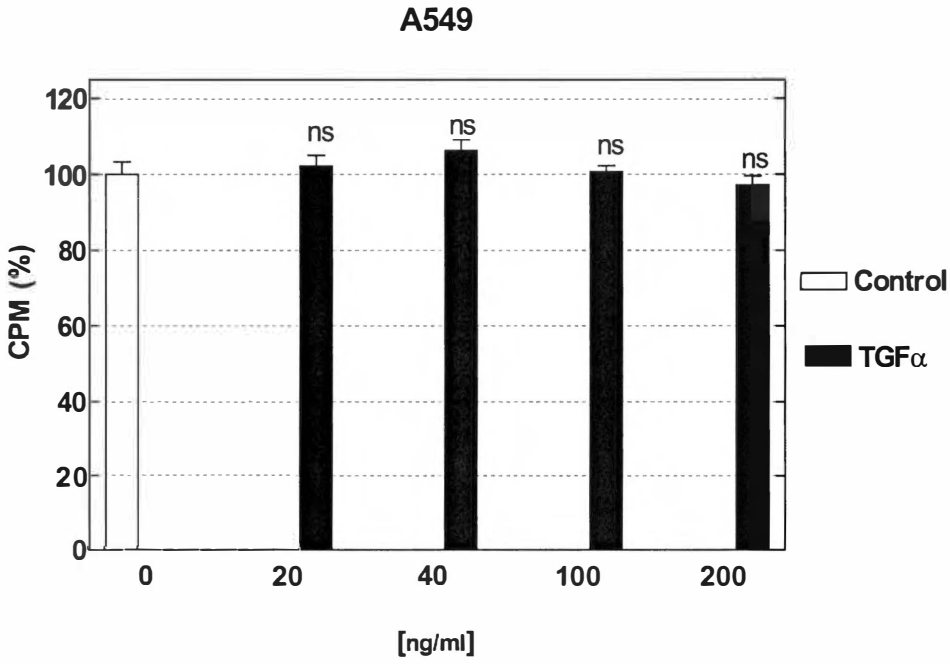


Figure 15. Effect of TGF-α on DNA synthesis in A549 cells (PAC type-II). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing rhTGF-α or vehicle (10 mM acetic acid in 0.1% BSA). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. TGF-α showed no significantly effect on DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

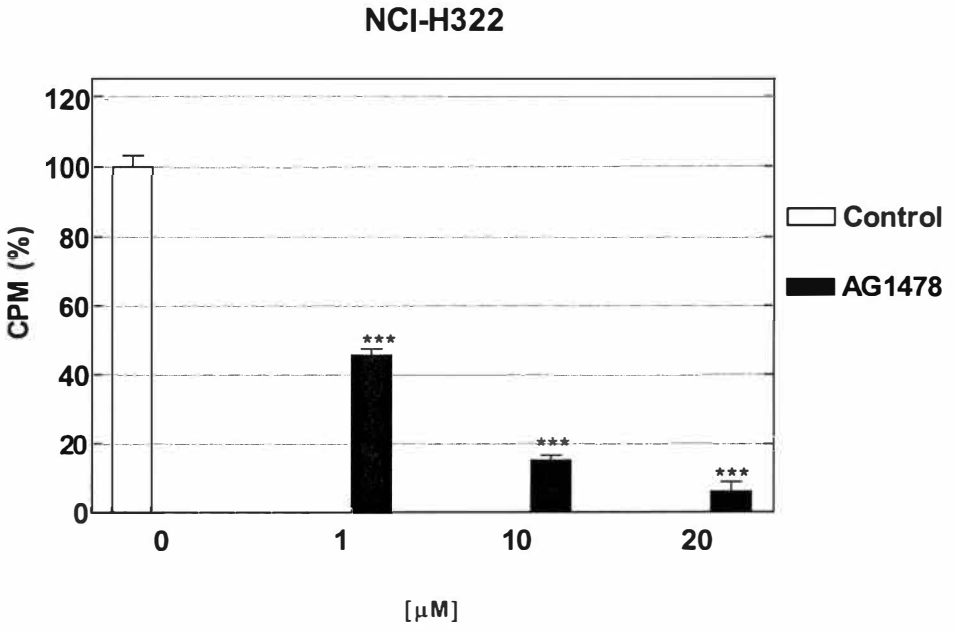


Figure 16. Effect of the EGFR-specific tyrosine kinase inhibitor, AG1478, on DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. This was followed by the addition of low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing AG1478 or vehicle (DMSO). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. AG1478 significantly reduced DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

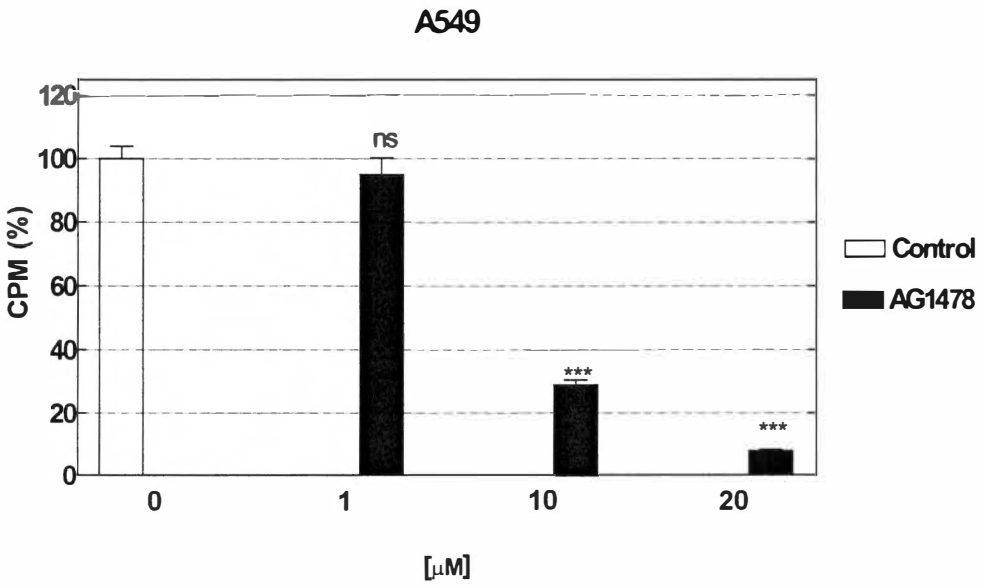
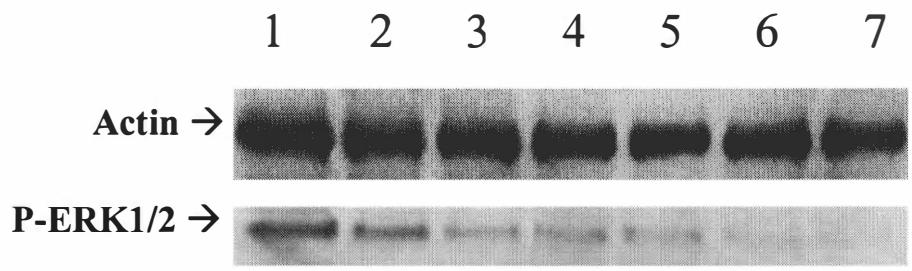


Figure 17. Effect of the EGFR-specific tyrosine kinase inhibitor, AG1478, on DNA synthesis in A549 cells (PAC-type II). Cells were plated and incubated in high serum medium for 24 hours. This was followed by the addition of low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing AG1478 or vehicle (DMSO). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. AG1478, except at 1iM, significantly reduced DNA synthesis that was stimulated by serum deprivation. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)



1 = 0 min.
2 = 15 min.
3 = 30 min.
4 = 1 hr.
5 = 2 hr.
6 = 8 hr.
7 = 24 hr.

Figure 18. Western blot of Phospho-MAPK showing the effect of the EGFR-specific tyrosine kinase inhibitor, AG1478 (10iM) on ERK1/2 activation in NCI-A549 cells (PAC-type II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing AG1478 (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for western blot analysis. Inhibition of ERK1/2 activation was evident as early as 15 minutes with complete inhibition at 24 hours. Actin bands serve as an internal control for equal protein loading.

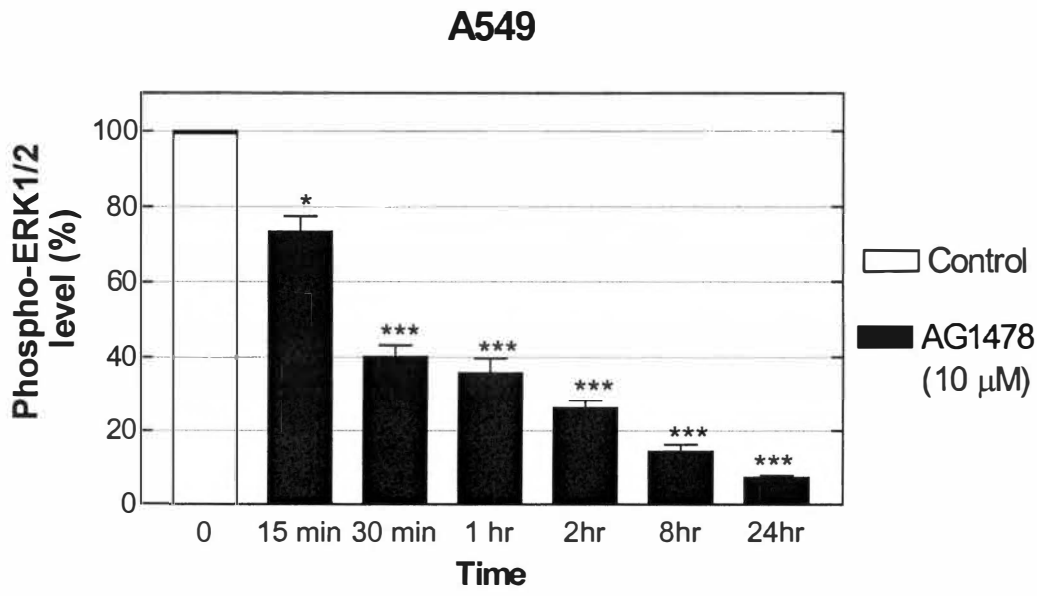


Fig 19. Densitometric values of western blot analysis showing the effect of AG1478 (EGFR-specific tyrosine kinase inhibitor) on the amount of phospho-ERK1/2 protein in A549 cells (PAC type-II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing AG1478 (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for western blot analysis. Bars represent the mean ERK/actin pixel ratio as a percentage of the mean value of the control group (0 hr), which was normalized at 100%. Means in each treatment group were compared with the mean from the control group using ANOVA with Dunnet comparison. AG1478 induced significant inhibition of ERK1/2 activation as early as 15 minutes. (ns –non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).

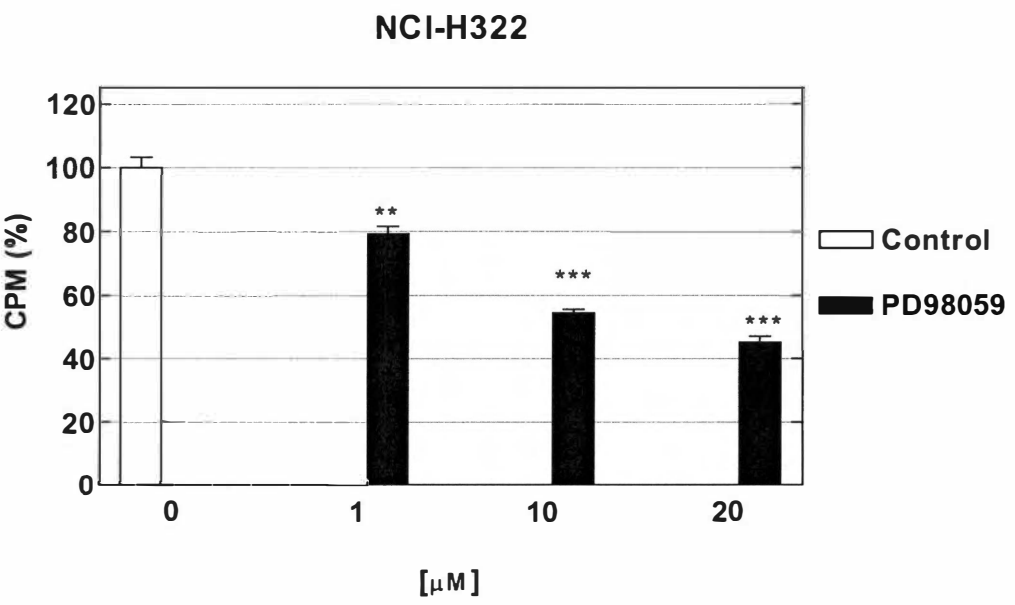


Figure 20. Effect of the MEK (MAPK Kinase) inhibitor, PD98059, on DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. This was followed by the addition of low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing AG1478 or vehicle (DMSO). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. PD98059 significantly reduced DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

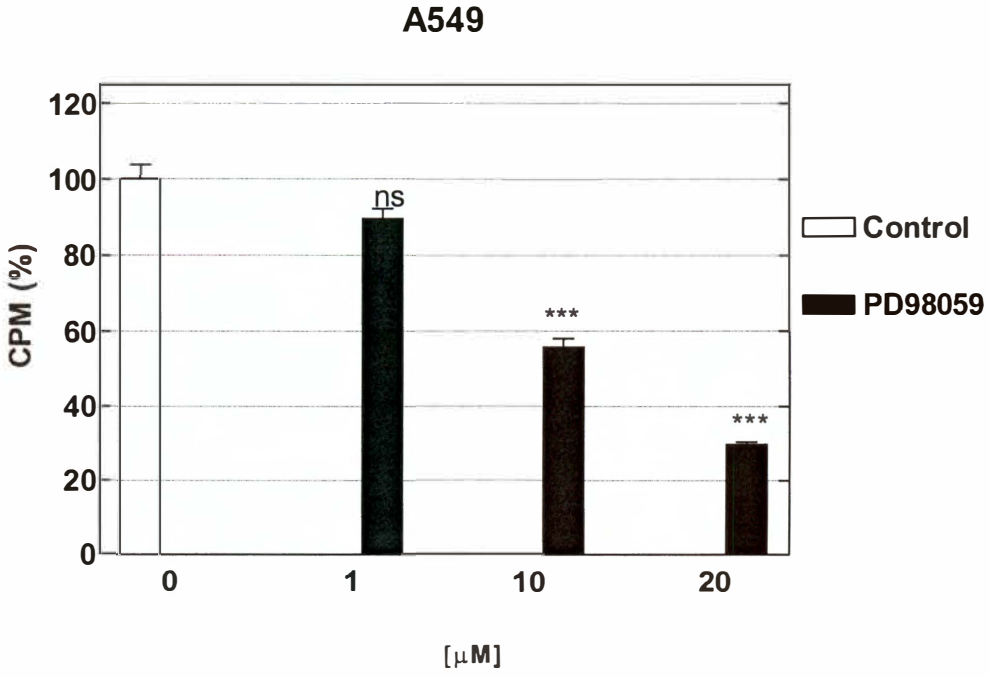


Figure 21. Effect of the MEK (MAPK Kinase) inhibitor, PD98059, on DNA synthesis in A549 cells (PAC type II). Cells were plated and incubated in high serum medium for 24 hours. This was followed by the addition of low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing AG1478 or vehicle (DMSO). Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. PD98059, except at 1 μM, significantly reduced DNA synthesis that was stimulated by serum deprivation. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

Effect of the beta-receptor agonist (Isoproterenol), and the adenylyl cyclase stimulator (Forskolin) on DNA synthesis

^{3}H-thymidine incorporation assays with isoproterenol (β1 and β2 agonist) and forskolin (adenylyl cyclase stimulator) had differential effects on DNA synthesis in NCI-H322 (Figures 22 and 24, respectively) and A549 cells (Figures 23 and 25, respectively). Isoproterenol induced highly significant stimulation of DNA synthesis in NCI-H322 cells at all concentrations used (100 pM to 1uM) ($p<0.01$ – $P<0.001$). Higher concentrations (10 μM and 20 μM) were toxic to both cell lines. The proliferative effect of isoproterenol was significantly blocked ($P<0.001$) by pre-incubation with 1 μM of propanolol (broad spectrum beta-adrenergic antagonist) (Figure 33), atenolol (selective beta-1 adrenergic antagonist) (Figure 36), ICI-118551 (selective beta-2 adrenergic antagonist) (Figure 36), or SQ22536 (the adenylyl cyclase inhibitor) (Figure 33). Similar blockage of isoproterenol-induced proliferation was observed with pre-incubation with 1μM of AG1478 (EGFR-specific tyrosine kinase inhibitor) ($P<0.001$). Pre-incubation with 10 μM of AG1478 blocked both forskolin-induced and basal DNA synthesis in low-serum condition ($P<0.001$) (figure 35).

Similarly, the adenylyl cyclase stimulator, forskolin, caused a highly significant stimulation of DNA synthesis in NCI-H322 cells at 1 μM ($p<0.001$) (Figure 24). Lesser concentrations did not have any significant effect ($P>0.05$). The proliferative effect of forskolin (1 μM) was completely blocked by pre-incubation with the adenylyl cyclase inhibitor, SQ22536 (1 μM) ($P<0.001$) (Figure 33). Forskolin-induced DNA synthesis was

not blocked by 1 μM AG1478 (P>0.05). However, AG1478 at 10 μM blocked both forskolin-induced and basal DNA synthesis (P<0.001) (Fig 35).

By contrast, isoproterenol had no significant effect on DNA synthesis in A549 cells at all tested concentrations (p>0.05) (Figure 23). Contrary to its effect on NCI-H322 cells, forskolin caused significant inhibition of DNA synthesis in A549 cells (p<0.05) (Figure 25). This inhibitory effect was significantly but not completely blocked by pre-incubation of with 1 μM of SQ22536 (P<0.01) (Fig 34).

Effect of the cAMP analogue, 8-chloro-cAMP, on DNA synthesis

The site-specific, cell-permeable cAMP analogue, 8-chloro-cAMP, significantly inhibited DNA synthesis at all concentrations tested (P<0.01) (Figure 26).

Effect of Forskolin on activation of MAPK

Treatment with 10 μM forskolin had a differential effect on the activation of ERK1/2 in A549 and NCI-H322 cells. A highly significant activation of ERK1/2 was evident as early as 15 minutes in NCI-H322 cells, which was maintained throughout the sampling points, with the highest phospho-ERK1/2 quantity observed at 24 hours (P<0.05 – P<0.001) (Figure 27 and 28).

On the other hand, forskolin caused an early activation and subsequent inhibition of MAPK (ERK1/2) in A549 cells (Figure 29 and 30). An early and significant stimulation was observed as early as 15 minutes ($P<0.001$). The highest ERK1/2 activation (based on our sampling time) was observed at 30 minutes. This was followed by a consistent decline with significantly lower quantity (about 75% of control) observed at 8 and 24 hours ($P<0.001$).

Effect of the adenylyl cyclase stimulator, forskolin, on EGFR phosphorylation

The quantity of phosphorylated EGFR was not significantly affected by forskolin (10 μM) at 30-minutes and 8-hours (Figure 31 and 32). The amount of phospho-EGFR in the 0-hr control group was significantly higher than that was found in the 24-hr forskolin treatment group ($P<0.05$). Likewise, the amount of Phospho-EGFR in the 24-hr control group was significantly higher than that was found in the 24-hr forskolin treatment group ($P<0.05$). However, there was also a significant increase in the amount of phospho-EGFR during the 24-hour incubation period in the control group ($P<0.05$).

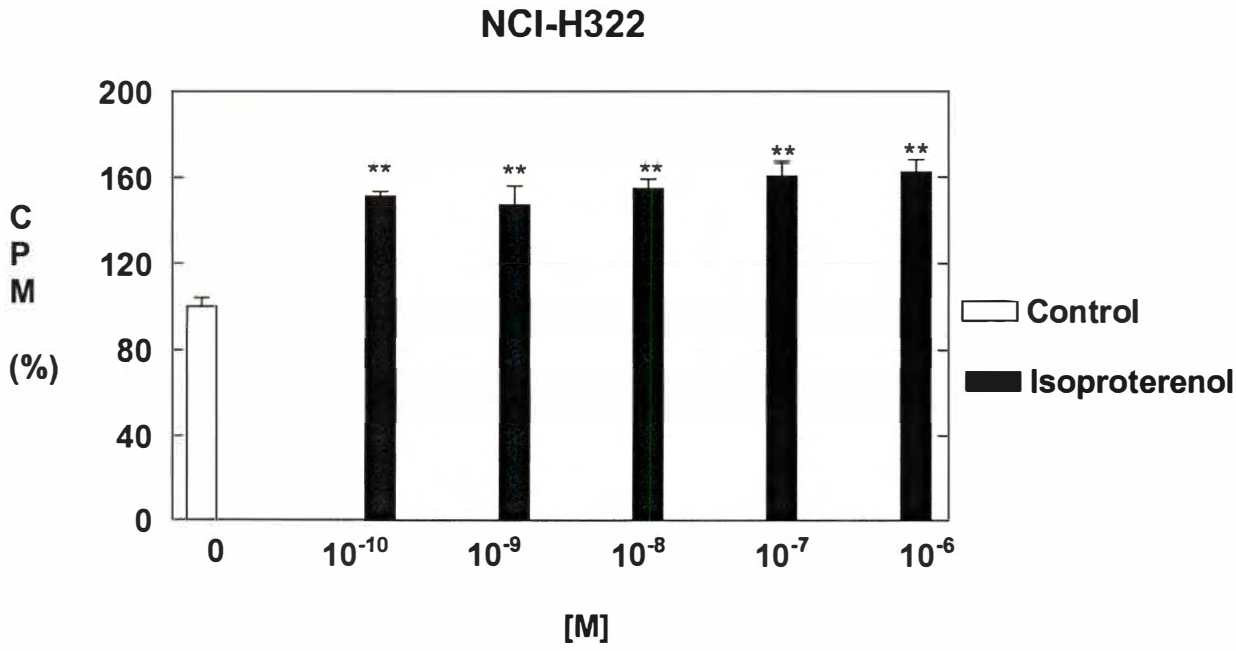


Figure 22. Effect of the broad-spectrum β-adrenergic agonist, isoproterenol, DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium containing 0.5 μCi [^{3}H]-thymidine/well with or without isoproterenol. Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Isoproterenol significantly stimulated DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

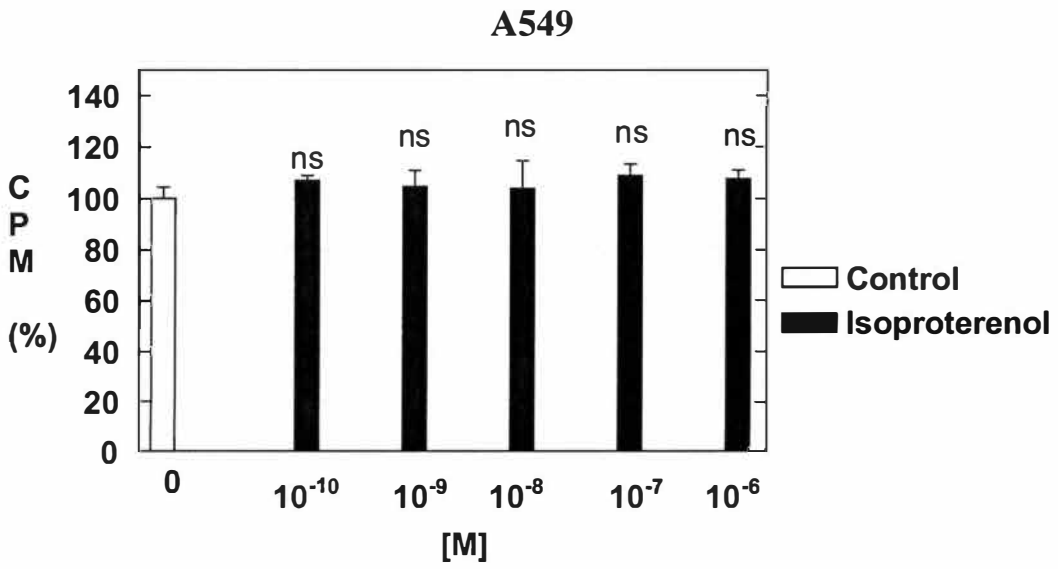


Figure 23. Effect of the broad-spectrum β-adrenergic agonist, isoproterenol, DNA synthesis in A549 cells (PAC type II). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium containing 0.5 μCi [^{3}H]-thymidine/well with or without isoproterenol. Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Isoproterenol showed no significant effect on DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

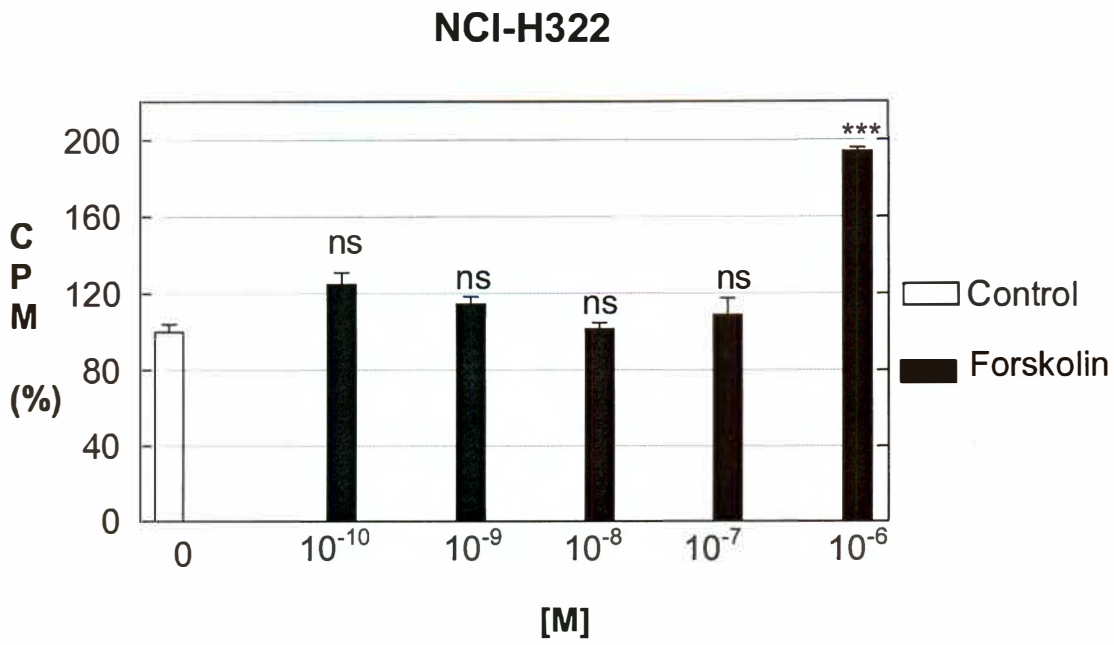


Figure 24. Effect of the adenylyl cyclase stimulator, forskolin, on DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium with 0.5 μCi [^{3}H]-thymidine/well containing Forskolin or vehicle (DMSO) was added. Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Forskolin at 1 μM significantly stimulated DNA synthesis (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

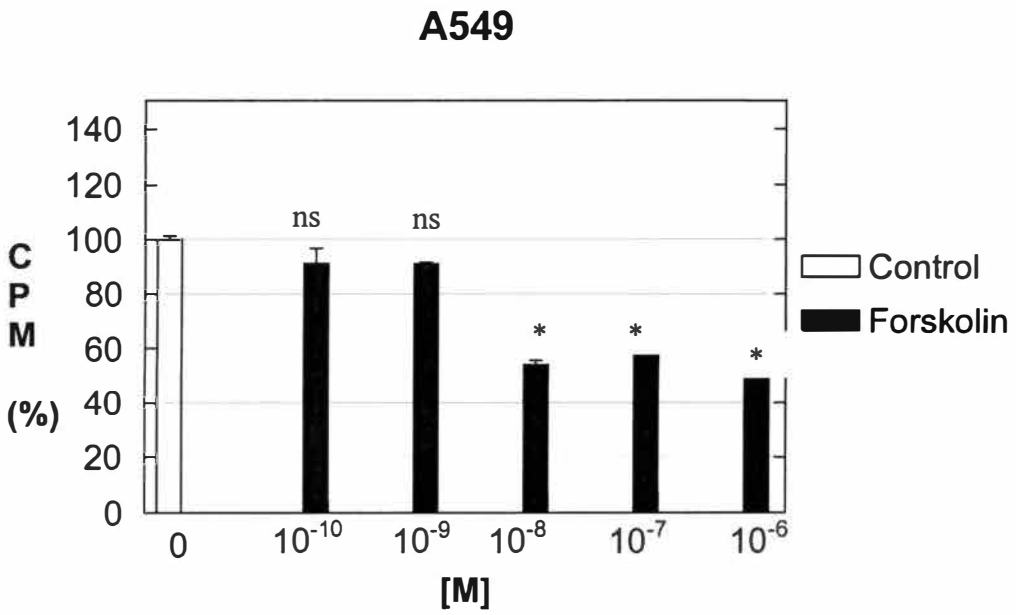


Figure 25. Effect of the adenylyl cyclase stimulator, forskolin, on DNA synthesis in A549 cells (PAC type-II). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium with 0.5 μCi [3H]-thymidine/well containing Forskolin or vehicle (DMSO) was added. Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group normalized as 100%. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. Forskolin at ≥ 10 nM significantly inhibited DNA synthesis. No significant cytotoxicity was observed with trypan blue exclusion assay (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

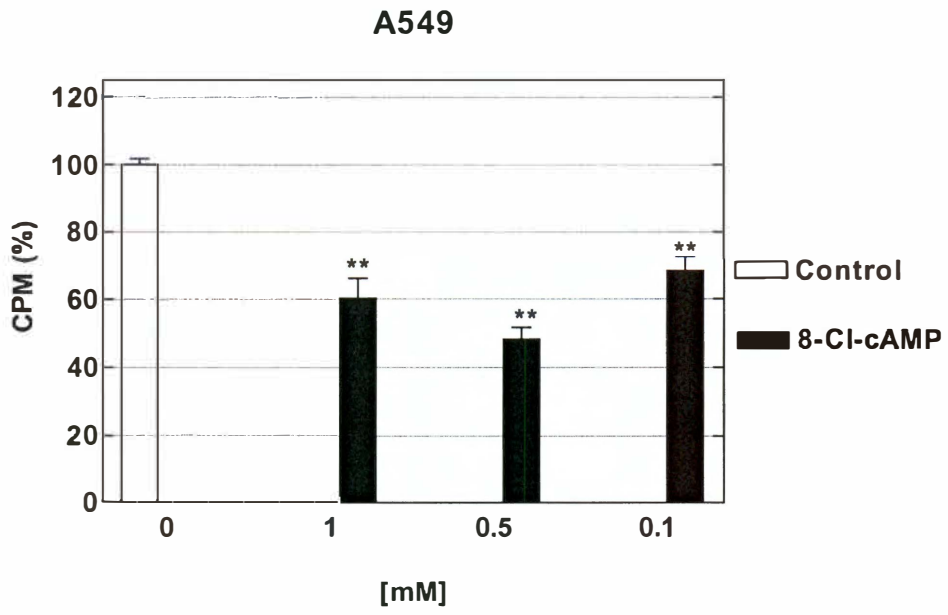
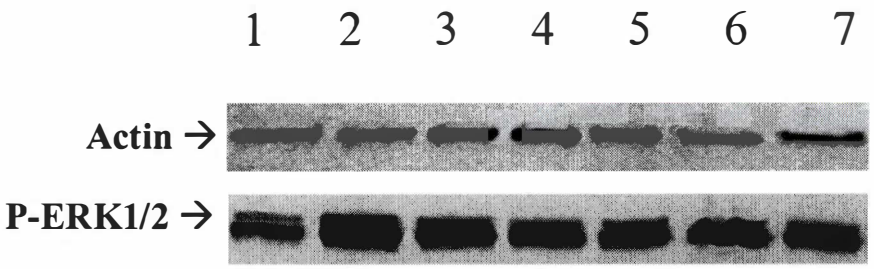


Figure 26. Effect of the site-specific cAMP analogue, 8-Chloro-cAMP, on DNA synthesis in A549 cells (PAC type II). Cells were plated and incubated in high serum medium for 24 hours. This was followed by the addition of low serum medium containing 0.5 μCi [^{3}H]-thymidine/well with or without 8-chloro-cAMP. Cells were incubated for additional 24 hours. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control group (low serum medium) normalized as 100%. Means in each treatment group were compared with the mean in the control group. Means in each treatment group were compared with the mean in the control group using ANOVA with Dunnet comparison. 8-Chloro-cAMP significantly reduced DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)



1 = 0 min. 5 = 2 hr.

2 = 15 min. 6 = 8 hr.

3 = 30 min. 7 = 24 hr.

4 = 1 hr.

Figure 27. Western blot of Phospho-MAPK showing the effect of the adenylyl cyclase stimulator, forskolin on ERK1/2 activation in NCI-H322 cells (PACC). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing forskolin (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for western blot analysis. Activation of ERK1/2 was evident as early as 30 minutes after treatment. Activation was maintained through out the 24-hour incubation period. Actin bands serve as an internal control for equal protein loading.

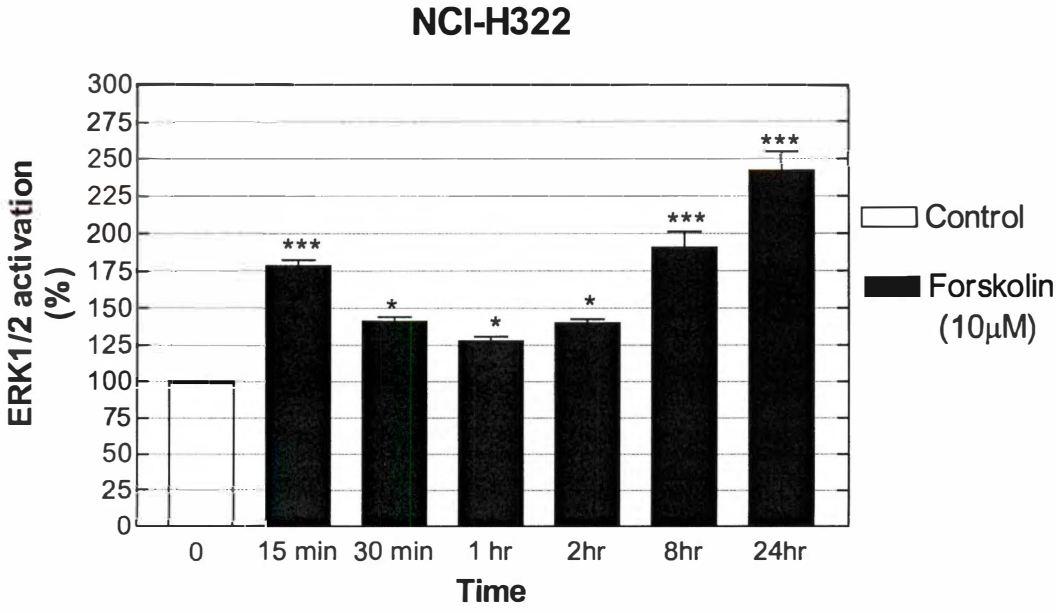
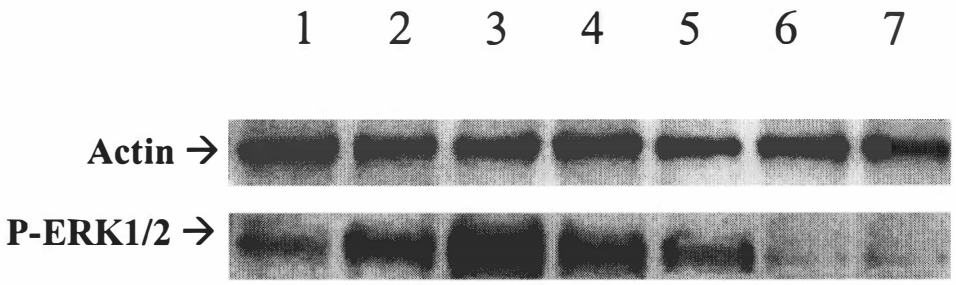


Fig 28. Densitometric values of western blot analysis showing the effect of forskolin (adenylyl cyclase stimulator) on the amount of phospho-ERK1/2 protein in NCI-H322 cells (PACC). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing forskolin (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for western blot analysis. Bars represent the mean ERK/actin pixel ratio as a percentage of the mean value of the control group (0 hr) that was normalized at 100%. Means in each treatment group were compared with the mean from the control group using ANOVA with Dunnet comparison. Forskolin induced significant activation of ERK1/2 as early as 15 minutes. (ns –non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).



1 = 0 min. 5 = 2 hr.

2 = 15 min. 6 = 8 hr.

3 = 30 min. 7 = 24 hr.

4 = 1 hr.

Figure 29. Western blot of Phospho-MAPK showing the effect of the adenylyl cyclase stimulator, forskolin on ERK1/2 activation in NCI-A549 cells (PAC-type II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing forskolin (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for western blot analysis. Forskolin induced early activation of ERK1/2. This was followed by persistent inhibition Actin bands serve as an internal control for equal protein loading.

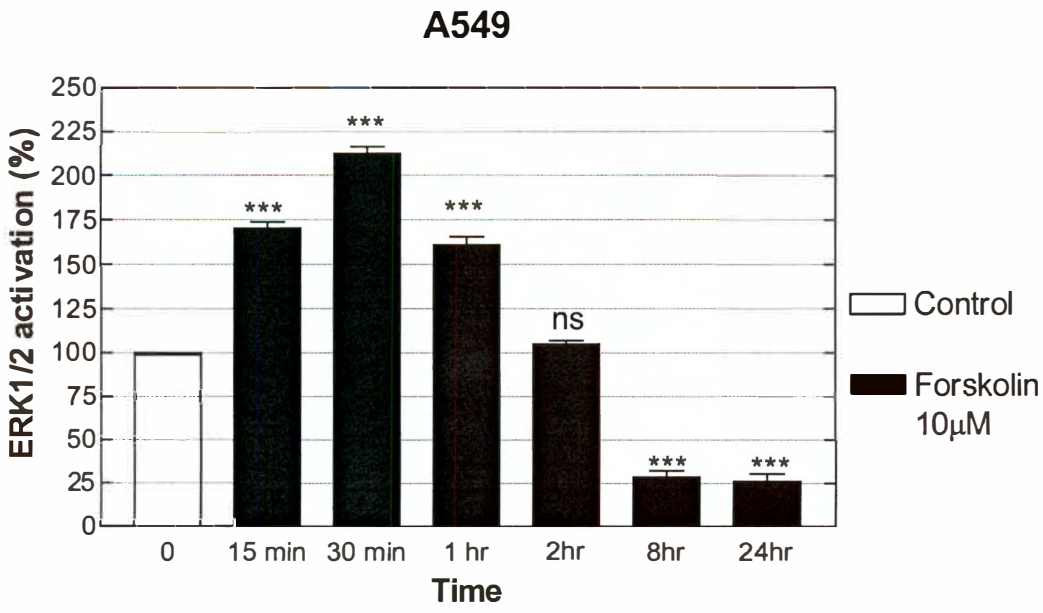
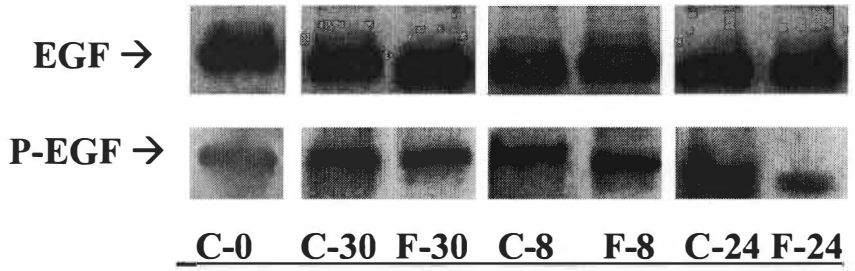


Figure 30. Densitometric values of western blot analysis showing the effect of forskolin (adenylyl cyclase stimulator) on the amount of phospho-ERK1/2 protein in A549 cells (PAC type-II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing forskolin (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for western blot analysis. Bars represent the mean ERK/actin pixel ratio as a percentage of the mean value of the control group (0 hr) that was normalized at 100%. Means in each treatment group were compared with the mean from the control group using ANOVA with Dunnet comparison. Forskolin induced significant early activation of ERK1/2. This was followed by persistent and significant inhibition. (ns –non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).



C-0 = control at 0 min.

C-30 = control at 30 min.

F-30 = control at 30 min.

F-24 = Forskolin at 24 hr.

C-8 = control at 8 hr.

F-8 = Forskolin at 8 hr.

C-24 = control at 24 hr.

Figure 31. Immunoprecipitation and western blot of the epidermal growth factor receptor (EGFR) showing the effect of the adenylyl cyclase stimulator, forskolin (10 μM), on tyrosine-phosphorylation/activation of the EGFR in NCI-A549 cells (PAC-type II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing forskolin (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for analysis. Tyrosine phosphorylation of the EGFR was detected with an antiphosphotyrosine monoclonal antibody. Forskolin did not inhibit EGFR tyrosine phosphorylation except at 24 hour. The EGFR bands serve as an internal control. No significant difference observed in the total EGFR in the immunoprecipitation product.

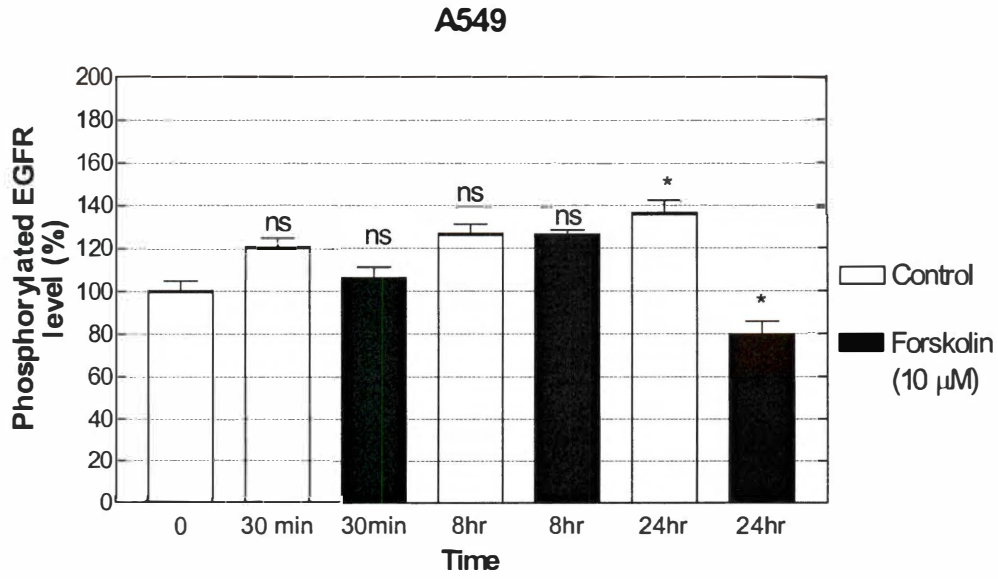


Figure 32. Densitometric values of immunoprecipitaion and western blot analysis showing the effect of forskolin (adenylyl cyclase stimulator) on tyrosine phosphorylation of the EGFR in A549 cells (PAC type-II). Cells were plated and maintained in high serum medium until 70-80% confluency. Medium was then replaced with low serum medium containing forskolin (10 μM) and incubated for the indicated periods. At the end of the respective incubation period, cells were harvested for analysis. Bars represent the mean phospho-EGFR/total EGFR pixel ratios as percentage of the mean value of the control group at 0-hr that was normalized at 100%. Means in each treatment group were compared with the mean from the respective control group with paired t-test. Values with asterisks significantly differ from each other and the 0-hr control in the amount of tyrosine phosphorylated EGFR. (ns –non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).

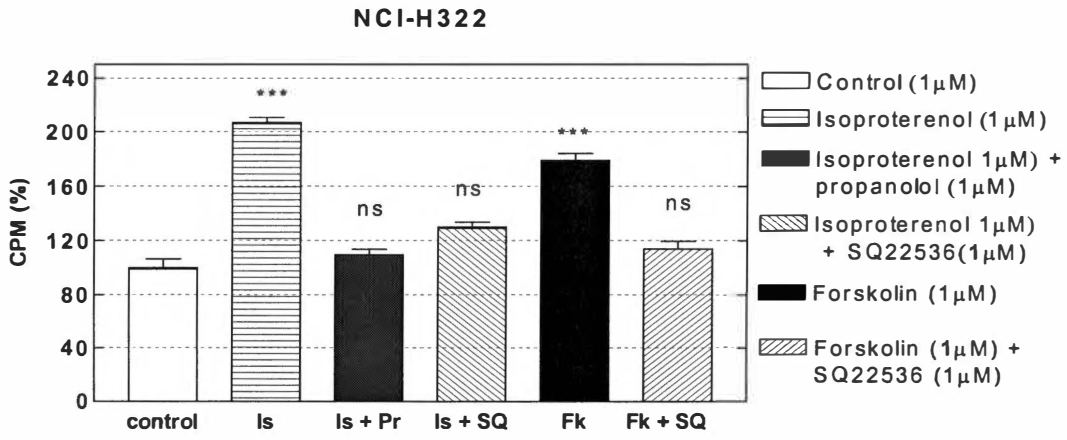


Figure 33. Effect of propanolol (broad-spectrum β-adrenergic antagonist) and SQ22536 (adenylyl cyclase inhibitor) on isoproterenol- and forskolin-induced DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium containing antagonists was added and incubated for 20 minutes. This was followed by the addition of agonists and incubation for 24 hours. All wells contained 0.5 μCi of [^{3}H]-thymidine. Low serum medium and DMSO were used as controls for isoproterenol and forskolin, respectively. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control groups normalized as 100%. Means in each treatment group were compared with the mean from the respective control groups using ANOVA with Dunnet comparison. SQ22536 nullified isoproterenol- and forskolin-induced DNA synthesis. Similarly, isoproterenol-induced DNA synthesis was blocked by propanolol. The vehicle (DMSO) had no significant effect on DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

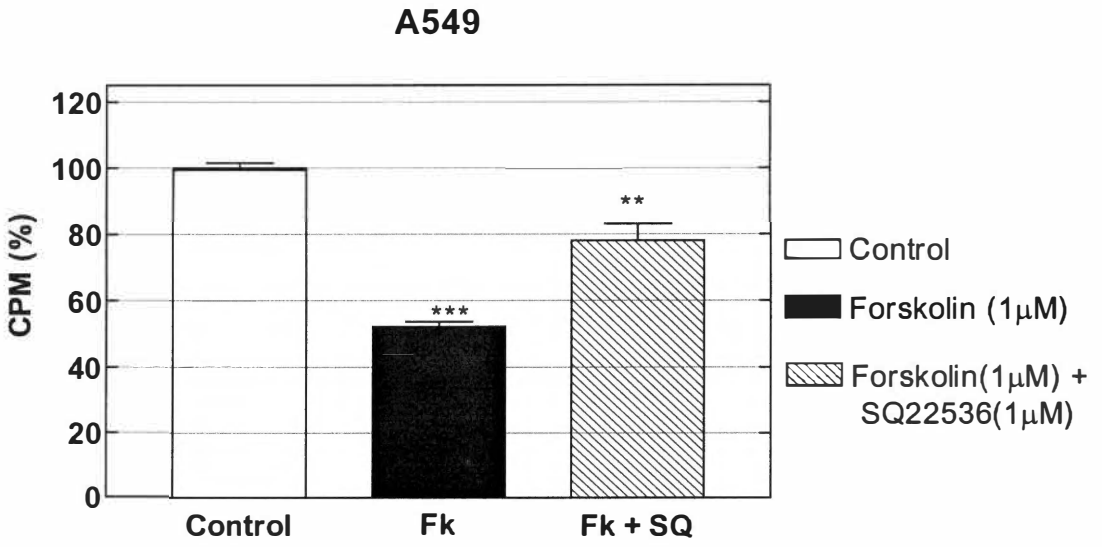


Figure 34. Effect of SQ22536 (adenylyl cyclase inhibitor) on forskolin-induced inhibition of DNA synthesis in A549 cells (PAC-type II). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium containing SQ22536 was added and incubated for 20 minutes. This was followed by the addition of forskolin and incubation for 24 hours. All wells contained 0.5 μCi of [^{3}H]-thymidine. DMSO was used as a vehicle/control for forskolin. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control groups normalized as 100%. Means in each treatment group were compared with the mean from the respective control groups using ANOVA with Dunnet comparison. SQ22536 nullified forskolin-induced inhibition of DNA synthesis. (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

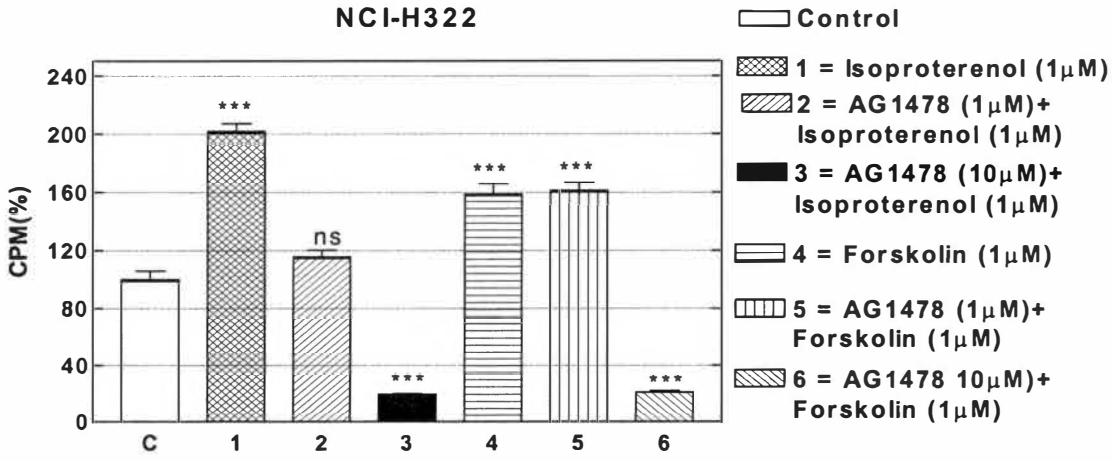


Figure 35. Effect of the EGFR-specific tyrosine kinase inhibitor, AG1478, on isoproterenol- and forskolin-induced DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium containing AG1478 was added and incubated for 20 minutes. This was followed by the addition of isoproterenol or forskolin and incubation for 24 hours. All wells contained 0.5 μCi of [^{3}H]-thymidine. Low serum medium and DMSO were used as vehicle/controls for isoproterenol and forskolin/AG1478, respectively. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control groups normalized as 100%. Means in each treatment group were compared with the mean from the respective control groups using ANOVA with Dunnet comparison. AG1478 (1μM) blocked isoproterenol- but not forskolin-induced DNA synthesis. At 10 μM, AG1478 blocked both isoproterenol/ forskolin-induced and basal DNA synthesis. The vehicle (DMSO) had no significant effect on DNA synthesis (ns – non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

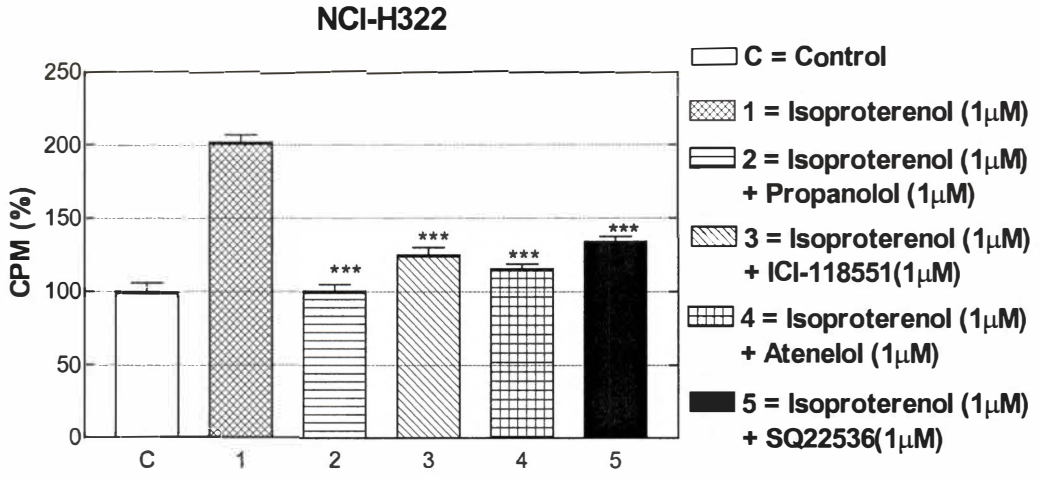


Figure 36. Effect of propanolol (broad-spectrum β-adrenergic antagonist), ICI-118551 (β2 adrenergic antagonist), atenolol (β1 adrenergic antagonist), the SQ22536 (adenylyl cyclase inhibitor) on isoproterenol-induced DNA synthesis in NCI-H322 cells (PACC). Cells were plated and incubated in high serum medium for 24 hours. Medium was replaced to low serum condition to starve cells for 24 hours. After the starvation period, fresh low serum medium containing the various antagonists was added and incubated for 20 minutes. This was followed by the addition of isoproterenol and incubation for 24 hours. All wells contained 0.5 μCi of [^{3}H]-thymidine. DMSO was used as a vehicle for SQ22536. For the rest, low serum medium was used as a vehicle/controls. Bars represent the mean CPM ± SE of each treatment group from three independent experiments as percentage of mean value of the control groups normalized as 100%. Means from each treatment group were compared with the mean from isoproterenol group using ANOVA with Dunnet comparison. All antagonists, showed significant inhibition of isoproterenol-induced DNA synthesis. (ns –non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$)

Basal and isoproterenol/forskolin-induced cAMP level

The basal and isoproterenol- or forskolin-mediated cAMP concentration was assayed with competitive ELISA. Cyclic AMP concentrations were expressed as pM/mg of protein.

The basal cAMP concentration in NCI-H322 cells was significantly higher (3.8 fold) than that of A549 cells ($p<0.001$) (Figure 37).

Isoproterenol (1 μM) significantly increased intracellular cAMP concentration in NCI-H322 cells ($p<0.001$) (Figure 38). This represented a nearly 700% increase as compared to untreated controls. On the other hand, Isoproterenol had no significant effect on intracellular cAMP concentration in A549 cells ($p>0.05$) (Figure 39).

The isoproterenol-induced cAMP accumulation was significantly reduced by ICI-118551 (selective β2-adrenergic antagonist) or atenolol (selective β1-adrenergic antagonist) ($P<0.001$). A more marked reduction (70%) was observed with atenolol than with ICI-118551 (50%) ($P<0.05$) (Figure 40)

Forskolin, at both 1 μM and 10 μM, induced a significant and marked increase in the intracellular cAMP concentration in NCI-H322 cells ($p<0.001$) (Fig 38). There was an approximate 860% and 1100% increase for 1 μM and 10 μM, forskolin, respectively.

On the other hand, a significant, but less marked increase in intracellular cAMP levels was induced in A549 cells by forskolin (1 μM and 10 μM, $p<0.05$) (Figure 39). There was approximate 32% and 44% increase for 1 μM and 10 μM, forskolin, respectively. In contrast to NCI-H322 cells, there was no significant difference between 1 μM and 10 μM forskolin treatments in the concentration of cAMP ($p>0.05$).

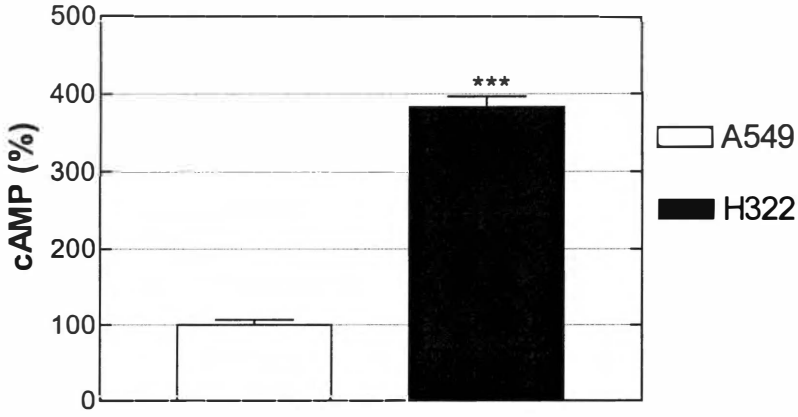


Figure 37. Relative basal cAMP concentration in the human pulmonary adenocarcinoma cell lines, A549 (PAC-type II) and NCI-H322 (PACC). Cells were pre-incubated with 1mM 3-isobutyl 1-methylxanthine (phosphodiesterase inhibitor) for 30 minutes before measurement of cAMP. Bars represent the cAMP concentration (mean± SE) as percentage of the mean concentration in the A549 cells normalized at 100%. Mean cAMP concentration was compared with unpaired t-test. Mean. The basal cAMP concentration in NCI-H322 cells was significantly higher. (ns–non-significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).

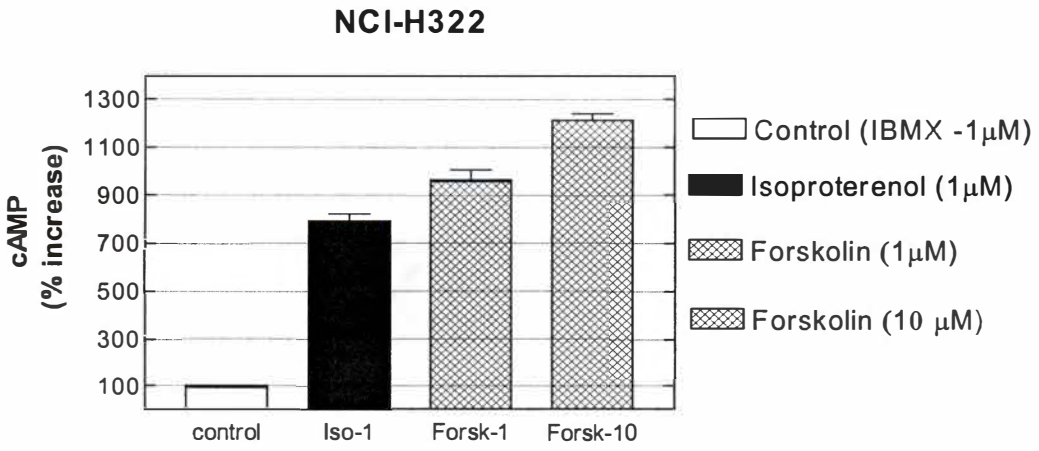


Figure 38. Effect of isoproterenol (broad spectrum β-adrenergic agonist) and forskolin (adenyly cyclase stimulator) on cAMP accumulation in the human pulmonary adenocarcinoma cell NCI-H322 cells (PACC). Cells were pre-incubated with 1mM 3-isobutyl 1-methylxanthine (phosphodiesterase inhibitor) for 30 minutes. Fresh low serum medium containing the indicated agents were added and incubated for 20 minutes. Bars represent the cAMP concentration (mean± SE) as percentage of the mean value in the control group that was normalized at 100%. Means in each treatment group were compared with the mean from the control group using ANOVA with Dunnet comparison. Both isoproterenol and forskolin induced significant cAMP accumulation. (ns– non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).

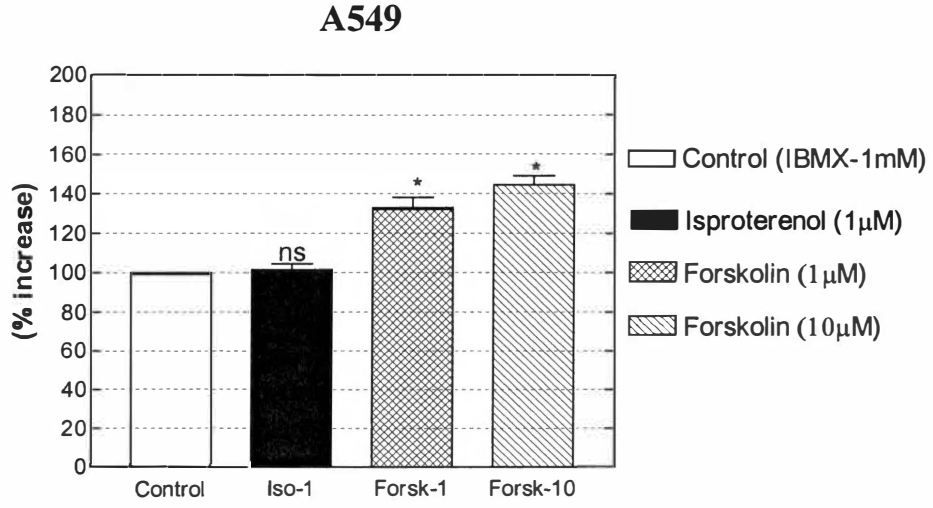


Figure 39. Effect of isoproterenol (broad spectrum β -adrenergic agonist) and forskolin (adenyly cyclase stimulator) on cAMP accumulation in the human pulmonary adenocarcinoma cell A549 (PAC type-II). Cells were pre-incubated with 1mM 3-isobutyl 1-methylxanthine (phosphodiesterase inhibitor) for 30 minutes. Fresh low serum medium containing the indicated agents were added and incubated for 20 minutes. Bars represent the cAMP concentration (mean± SE) as percentage of the mean value in the control group group normalized at 100%. Means in each treatment group were compared with the mean from the control group using ANOVA with Dunnet comparison Forskolin induced significant cAMP accumulation. (ns– non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).

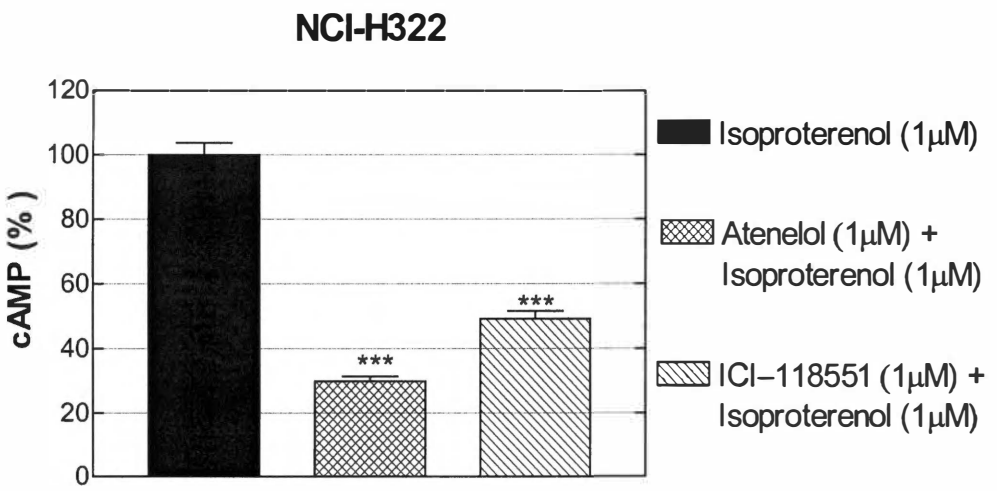


Figure 40. Effect of atenolol (selective β1-adrenergic antagonist) and ICI-118551 (selective β2-adrenergic antagonist) on isoproterenol-induced cAMP accumulation in NCI-H322 cells (PACC). Cells were pre-incubated with 3-isobutyl 1-methylxanthine, phosphodiesterase inhibitor, for 30 minutes. Cells were then incubated with antagonists for 20 minutes. This was followed by addition of isoproterenol and incubation for 20 minutes. Bars represent the cAMP concentration (mean± SE) as percentage of the mean concentration in isoproterenol treatment group that was normalized at 100%. Means in each treatment group were compared with the mean from the isoproterenol group using ANOVA with Dunnet comparison. Both atenolol and ICI-118551 significantly inhibited isoproterenol-induced cAMP accumulation. (ns– non significant/$p>0.05$; ** - $p<0.01$; * - $p<0.05$; *** - $p<0.001$).

IV. DISCUSSSION

The Epidermal growth factor receptor and cell proliferation

Effect of serum on proliferation

In both cell lines, significantly higher DNA synthesis was observed in low-serum condition. This is in contrast with most reports where serum stimulates DNA synthesis and proliferation (Bihl et al., 1998; Thrane and Schwarze, 2001). Comparable findings to ours have been reported recently by Murphy et al. (2001b), who showed increased DNA synthesis and proliferation in pancreatic adenocarcinoma cells weaned from high-serum. In a related study, Murphy et al. (2001a) showed that pancreatic adenocarcinoma cells proliferate indefinitely in serum-free medium by overexpression of the epidermal growth factor receptor and overproduction of TGF-α. Bost et al. (1997) reported a doubling time of 60 hours in low-serum medium for A549 cells. Serum-free medium conditioned for 72 hours by A549 cells stimulate colony formation of normal bronchial epithelial cells, and A549 cells themselves suggesting the presence of released autocrine growth factors (Siegfried, 1987). Proliferation of monolayer cells is significantly reduced by contact inhibition at full confluence. It could then be reasoned that cells in high-serum medium had reached near/at confluence and would transition to a state of reduced DNA synthesis. However, in this study confluence at the end of incubation and DNA harvest varied between 60-70% ruling out contact inhibition as a possible cause of lower DNA synthesis

in high-serum. Croce et al. (1999) showed the lack of anchorage dependency in parental A549 cells. These investigators described that three of the four subpopulations of A549 cells grow as a well-spread monolayer while one of them tends to form cell aggregates that pile up on the monolayer. Consequently, cells assume pavement-like structures as culture approach confluence and start to pile up as a result of anchorage independency (Croce et al., 1999). This growth characteristic was also observed in the A549 cells in the present study. Therefore, contact inhibition might not dramatically prevent these cells from proliferation as observed in our study. However, as stated earlier, the low confluency level at the time of cell harvest rules out this possibility as the source of such a marked variation.

We also found that replacement of the medium with fresh low-serum medium every 12 hours led to a significant inhibition of DNA synthesis in both cell lines. By contrast, DNA synthesis in the second control group where the primed medium was temporarily removed, saved, and replaced showed no significant difference compared to that of the continuous-culture control. The comparable DNA synthesis in the two control groups rules out the physical interference from the repeated medium removal and replacement as a significant factor for the difference in DNA synthesis. Together, these observations point to the presence of secreted endogenous growth stimulatory factors in the culture medium. An internal constitutive autocrine loop without the need for growth factors in v-sis transformed cells has been reported in various cells (Keating 1988; Bejcek et al., 1989). Our observation suggests a less significant role of for an internal growth loop because removal of the conditioned medium caused a marked reduction in DNA

synthesis implying dependence on released growth factors. However, medium removal might remove other extracellular components besides growth factors that might be needed for optimal cell growth and survival. Therefore, our findings do not completely rule out this possibility although they strongly suggest the presence of autocrine growth factors.

In both cell lines, the higher DNA synthesis in low-serum condition was evident as early as the fourth hour of incubation, which implies DNA synthesis was stimulated by low-serum even earlier. In support this interpretation, an early (30 minutes) ERK1/2 activation was observed in A549 cells following medium change from high to low-serum. This activation was maintained throughout the 24-hour incubation in low-serum medium consistent with the higher and significant DNA synthesis observed at all sampling points compared to the high-serum environment. Early response genes such as c-fos and c-jun are induced within minutes of the addition of growth factors to quiescent cells (Lodish et al., 2000). The early activation of MAPK/ERK after medium change is not surprising because these early response genes are induced by MAPK. Murphy et al. (2001b) reported MAPK/ERK, c-jun, and c-fos mRNA content was significantly elevated in pancreatic cancer cells cultured continuously in serum free medium. This activation might suggest a disinhibitory role of low-serum. Serum contains several defined and undefined factors that stimulate or inhibit the growth of cultured cells. Growth inhibitory factors in serum such as TGF-β antagonize EGF- induced colony formation (Tada et al., 2000) and mediate growth arrest (Bennett, 1999) in A549 cells. TGF-β mediates the shift of A549 cells back to a normal senescence via inhibition of telomerase activity. However,

this effect was not accompanied by complete growth arrest (Katakura et al., 1999). In a study by Bennett (1999), growth inhibition by TGF-β was accompanied by a reduction of the expression and secretion of amphiregulin, a member of the EGF/TGF-α family. Transforming growth factor-beta (TGF-β) or other factors in serum might interfere or suppress the release of preformed growth factors in a similar manner. Consequently, low-serum might facilitate the release of growth factors for subsequent self -stimulation.

In normal cells, the supply of preformed stored peptide hormones is sufficient for growth stimulation for about a day (Lodish et al., 2000). We have observed that higher DNA synthesis in low-serum persisted up to 48 hours. This might suggest overexpression/storage of growth factors, which is a characteristic feature of most solid tumors. Furthermore, receptors and ligands for autocrine growth pathways are up regulated when cancer cells are adapted to serum-free culture (Murphy et al., 2001a).

Effect of EGF, TGF-α, and EGF antibody on DNA synthesis

In the present study, both EGF and TGF-α failed to significantly modulate DNA synthesis in A549. This cell line expresses the EGFR, and its proliferation is mediated by this receptor (Al Moustafa et al., 2002). The EGF receptor binds various ligands including EGF, TGF-α, amphiregulin, heparin-binding EGF like growth factor, betacellulin, and epiregulin (Gschwind et al., 2001). There are varied reports as to the type of ligands that cause activation of the receptor to induce a proliferative response in A549 cells (Bost et al., 1999; Thrane and Schwarze, 2001). Imanishi et al. (1988)

detected TGF-α receptors in A549 cells along with TGF-α in medium conditioned by A549 cells. These investigators also stimulated growth of this cell type with exogenous TGF-α. Antibody against TGF-α but not antibody against EGF resulted in reduced proliferation in A549 cells indicating TGF-α as an autocrine growth factor in these cells (Imanishi et al., 1988). Likewise, Thrane and Schwarze (2001) showed that EGF treatment does not stimulate DNA synthesis despite transient ERK activation. TGF-α has been shown to increase the expression of cyclooxygenase-2 (COX-2) in A549 cells, an enzyme frequently localized at invasive edge of early-stage lung adenocarcinomas (Niki et al., 2002). On the other hand, EGF was identified as a potent stimulator of cell proliferation in these cells, and it was shown that the Jun kinase/stress-activated protein kinase (JNK/SAPK) pathway was an essential mediator of this effect (Bost et al., 1997). The proliferative effect of EGF in A549 cells has also been shown by (Al Moustafa et al., 2002). Choudhury et al. (2000) and Croxtall et al. (2000) showed that EGF-activated ERK caused phosphorylation and activation of cytosolic phospholipase A2 (PLA2) leading to the release of arachidonic acid (AA) and cell proliferation in A549 cells. Bost et al. (1997) showed a proliferative effect of EGF by continuous daily addition of 10 nm rhEGF. Continuous EGF treatment induced no significant effect on DNA synthesis in this study. EGF must continuously interact with receptors for many hours before cells enter into the DNA synthesis phase (S-phase) of the cell cycle (Boulougouris and Elder, 2001). Moreover, quiescent cells need 14-16 hours to pass the restriction point and enter the DNA synthesis phase (S-phase) of the cell cycle, which lasts for 6-8 hours (Lodish et al., 2000). It is, therefore, reasonable to assume that the 24-hour incubation time was long

enough to allow cells to advance well into the DNA synthesis in EGF containing medium in our studies. Therefore, our finding suggested that EGF is not a potent/primary EGFR ligand.

Although we did not observe stimulation of DNA synthesis in A549 cells even with high EGF concentration (200 ng/ml), an antibody against EGF caused significant, though minor, inhibition. A possible explanation for this effect is that this cell line might produce enough EGF to maintain maximal proliferation in low-serum in an autocrine or paracrine fashion. In support of this explanation, DNA synthesis was significantly higher in low-serum medium, and it was inhibited when the primed medium was replaced periodically. Therefore, the addition of exogenous EGF might be rendered inconsequential by a saturated self-stimulatory mechanism whereas antibody against EGF would neutralize endogenous EGF and inhibit proliferation. A dual effect of EGF on tumor cell lines that overexpress the EGFR has been documented where low concentration (pM levels) stimulated growth whereas higher concentrations (nM levels) inhibited growth and facilitated differentiation (Boulougouris and Elder, 2001). These investigators proposed that in such cells, low doses of EGF may cause only transient stimulation of the MAPK pathway, resulting in proliferation, whereas higher doses cause sustained stimulation of the MAPK pathway, leading to differentiation. EGF and TGF-α cause motility, expression of cell adhesion molecules, and the appearance of fibroblastic morphology in several epithelial cells (Chen et al., 1993). Similarly, EGF and heparin-binding EGF like growth factor induced epithelial-like to fibroblastic conversion and stimulated motility in NCI-H322 and A549 cells (Al Moustafa et al., 2002). Treatment with antibody against

the EGFR induced upregulation of E-cadherin and suppression of motility (Al Moustafa et al., 2002). In related studies, EGFR overexpression has been associated with invasiveness and metastasis in A549 cells (Niki et al., 2002) and other cancer types (Fontanini et al., 1995; Nguyen et al., 2000). These investigations are in accord with our observation that EGF induced fibroblastic appearance in A549 cells at high concentrations. Treatment with EGF induced characteristic spindle or multi-appendage morphological transformation. Up regulation of matrix-degrading proteases such as matrix metalloproteases (MMPs) via EGFR-mediated signaling has been proposed to explain this phenomenon (Boulougouris and Elder, 2001; Nguyen et al., 2000). In addition, invasive tumor cells exhibit dysregulated motility in response to growth factors. Consequently, growth factor receptor-mediated motility has been suggested as one of the most common aberrations in tumor cells leading to invasiveness. In this regard, the EGF and hepatocyte growth factor (HGF) is particularly associated with tumor invasion (Wells, 2000). Niki et al. (2002) reported that the EGFR might be involved in the invasive mechanisms of A549 cells. In summary, our data suggests that EGF mediates non-proliferative morphological effects in A549 cells, which might play a role in metastasis and invasion.

Earlier reports suggest EGF might not be the primary growth factor in this cell line (Imanishi et al., 1988; Thrane and Schwarze, 2001), A549 cells express amphiregulin (Bennett, 1999) and heparin binding EGF (Al Moustafa et al., 2002), which are potent ligands for the EGFR. Therefore, the minor but significant inhibitory effect of EGF neutralization might suggest a secondary role of EGF in the proliferation of A549 cells.

On the other hand, the presence of an internal constitutively activated receptor might also nullify the effect of exogenous growth factors. However, as reasoned earlier, the highly significant reduction of DNA synthesis in interrupted culture, strongly suggests dependency on released/externally acting growth factors instead of an internally/constitutively-activated receptor.

The EGFR (ERB1) and ERB2 are highly expressed in NCI-H322 cells (Nguyen et al., 2000; Al Moustafa et al., 2002). Moreover, Al Moustafa et al. (1999) demonstrated the presence of a functional EGFR growth pathway that was inhibited by an antibody against EGFR. This growth inhibition was paralleled by differentiation from epithelial-like to epithelial phenotype. Unlike A549 cells, treatment with EGF at a concentration of $\geq$ 20 ng/ml stimulated DNA synthesis of NCI-322 cells in this study. In agreement with these findings, Al Moustafa et al. (2002) also determined that NCI-H322 cells have a significantly higher proliferative response than A549 cells in the presence of EGF. These data are also in agreement with Schuller et al. (1994), who showed the stimulatory effect of EGF in NCI-H322 cells. In this study, TGF-α did not stimulate DNA synthesis in the NCI-H322 cells. TGF-α might not be the primary/active EGFR ligand in this cell line. Nguyen et al. (2000) showed increased secretion of matrix metalloproteases-9 (MMP-9) with EGF and heregulin-a treatment in NCI-H322 cells. Hepatocyte growth factor (HGF) induced EGFR phosphorylation, DNA synthesis, and proliferation in NCI-H441, a related Clara cell line (Chess et al., 1998). Schuller et al. (1994) showed that exogenous EGF stimulated NCI-H322 but not H441 cells. Therefore, variation in the type of primary/active ligand for EGFR also exists within similar phenotypes of pulmonary

adenocarcinoma. On the other hand, EGF-induced morphological change was not remarkable in NCI-H322 cells as was seen in A549 cells. The epidermal growth factor may have a differential effect on these two cell lines, where it stimulated cell proliferation in NCI-H322 cells, while inducing a non-proliferative morphogenic/metastatic changes in A549 cells.

Genetic instability, which is reflected in tumor heterogeneity, might explain some of our results that are not in agreement with some of the earlier literature. Genetic instability, a critical factor in tumor progression, is the hallmark of virtually all-solid tumors (Jallepalli and Lengauer, 2001). Heterogeneity is observed in individual tumors and among tumors of the same type. Such heterogeneity is the basis for histological, karyotypic, molecular, physiological, and biochemical differences that are observed in different parts of the same tumor (Cahill et al., 1999). For instance, heterogeneity in the A549 cell line with subpopulations of varying morphology, growth behavior, and antigenicity has been documented (Croce et al., 1999). Depending on the characteristics of the dominant subpopulation/s, a given tumor cell line might exhibit its own distinct growth and biochemical characteristics.

Effect of EGFR-specific tyrosine kinase inhibition on DNA synthesis

In both cell lines, the EGFR specific tyrosine kinase inhibitor AG1478 caused significant reduction of low-serum-enhanced DNA synthesis. This growth inhibition was accompanied by marked MAPK inhibition in a time-dependent manner. These findings

show that these cells are dependent on the EGFR-MAPK mediated self -stimulated growth in low-serum.

Together with the low-serum proliferation, the observed effect of AG 1478 points to the role of a self-stimulated EGFR-mediated proliferative pathway in both cell lines. A greater proportion of pulmonary adenocarcinomas expresses and overexpresses EGFR (Rusch et al., 1993; Nakagawa 2001). As will be discussed in the next sections, AG1478 completely blocked isoproterenol-mediated proliferation in NCI-H322 cells, suggesting that the EGFR is an essential mediator of the β-adrenergic receptor-mediated growth-signaling pathway. Various EGFR-targeted therapeutic approaches have been developed to treat various types of cancer types that overexpress the EGFR and its ligands. Synthetic EGFR tyrosine kinase inhibitors including the synthetic anilinoquinazoline Iressa/ZD1839 are showing promising results in early clinical trials (Arteaga et al., 2002; de Bono and Rowinsky, 2002). Iressa considerably enhance the efficacy of cytotoxic agents against A549 cells (Sirotnak et al., 2000). Similarly, monoclonal antibodies against the EGFR have been developed with encouraging antitumor activity in early clinical development (Baselga, 2001; Slichenmyer and Fry 2001).

Effect of PD98059 (MEKl inhibitior) on DNA synthesis

The mitogen activated protein kinase (MAPK) induces a wide array of responses, including cell division and differentiation (Seger and Krebs, 1995). This central signaling

molecule is necessary and sufficient for cell differentiation or proliferation depending on the cellular context (vanBiesen et al., 1996; Cowley et al., 1994). Therefore, inhibition of MEK1 (MAPK activator) by PD98059 was employed in this study to assess the role of MAPK in proliferation/DNA synthesis in the two cell lines. In both cell lines, PD98059 significantly inhibited DNA synthesis, demonstrating the importance of MAPK/ERK in the proliferation of these cell lines. On the other hand, as will be described in the coming sections, tyrosine kinase inhibition of EGFR with AG1478 markedly inhibited the activation of MAPK/ERK. Mitogen activated protein kinase (MAPK) was upregulated when cells were grown in low-serum condition. These findings strongly suggest that the EGFR-MEK-ERK pathway is involved in the proliferative signaling of both cell types.

Our finding is in agreement with earlier reports where PD98059 inhibited EGF-induced and basal ERK activation (Chen et al., 2000; Thrane and Schwarze 2001; Bost et al., 1997) and EGF-induced cell migration (Hauck et al. 2001). Activated ERK phosphorylates cytosolic phospholypase-A (2), promoting its translocation to the membrane where it facilitates the release of AA (Croxtall et al., 2000). MEKI inhibition by PD98059 abrogates both IL-l beta and EGF-stimulated arachidonic acid (AA) and prostaglandin E (PGE) release and cell proliferation in A549 cells (Choudhury et al., 2000). Arachidonic acid metabolites enhance cell proliferation in various cancer cell types including pulmonary adenocarcinoma of both phenotypes (Choudhury et al., 2000; Schuller et al., 1999).

The beta-adrenergic/cAMP pathway and cell proliferation

Intracellular cAMP concentration

Data from the cAMP immunoassay showed a significant difference in the basal cAMP concentration in the two cell lines. The basal level of cAMP in NCI-H322 cells was nearly 3.8 times higher than in A549 cells. These data also showed that the intracellular cAMP concentration in A549 cells was not affected by stimulation of the B-adrenergic receptors with isoproterenol. Direct stimulation of the adenylyl cyclase enzyme with forskolin only moderately stimulated cAMP production in this cell line. These responses of A549 cells are in clear contrast with those observed in the NCI-H322 cells, which demonstrated a highly significant accumulation of cAMP in response to isoproterenol and forskolin. Forskolin induced a higher amount of cAMP production than that induced with isoproterenol at equimolar concentrations. This was not surprising since forskolin directly stimulates the enzyme adenylyl cyclase. Previous work in the NCI-H322 cells found a similar result where both forskolin and isoproterenol induced high amount of cAMP production (Park et al., 1995).

The low concentration of cAMP production in the A549 cells corresponded well with previous data generated in alveolar type II cells of mouse and human origins. Mouse

tumorigenic alveolar type II cells have fewer β-adrenergic receptors. Consequently, isoproterenol induced a diminished cAMP production (Lange-Carter and Malkinson, 1991). Other cancer types also show down regulation of these receptors and unresponsiveness to β-adrenergic receptor stimulation that has been correlated with disease progression (Kamp et al., 1997). Droms (1996) showed that neoplastic alveolar type II cells of mouse origin have a defective $G_{\alpha s}$ with subsequent inability to transmit signals from activated G-protein coupled receptors to the effector adenylyl cyclase enzyme. This defect was bypassed by treating cells with forskolin, which led to comparable camp production with that of the non-tumorigenic counterparts (Droms, 1996). Our data are in general agreement with these earlier works. Forskolin induced a significant but a moderate increase in cAMP concentration in A549 cells that was not achieved by isoproterenol in our studies. However, this stimulation was very low (32% and 44% increase) as compared to that observed in NCI-H322 cells (860% and 1100% increase) for 1 and 10 μM forskolin, respectively. The difference in cAMP in basal as well as stimulated c-AMP amount in these two cell lines could result from the differing quantity of phosphodiesterase (cAMP-degrading enzyme). Dent et al. (1998) showed that the total phosphodiesterase enzyme in human bronchial epithelial cells is considerably lower than in A549 cells. This would obviously result in more degradation of cAMP in A549 cells with consequent low concentration of cAMP. However, this possibility could be ruled out in this study because phosphodiesterase activity was inhibited with a phosphodiesterase inhibitor (3-isobutyl 1-methylxanthine). Another explanation for the observed difference in the cAMP concentration may be that various isoforms of adenylyl cyclase enzymes and tissue-specific expression of specific isoforms exist (Daniel et al.,

1998). In summary, our data indicate that NCI-H322 cells have a considerably higher basal and agonist-mediated intracellular cAMP level than A549 cells.

Effect of isoproterenol and forskolin on cell proliferation in A549 cells

Isoproterenol had no significant effect whereas forskolin inhibited DNA synthesis inA549 cells. The inhibitory effect of forskolin was blocked by pre-incubation with SQ22536 (the adenylyl cyclase inhibitor). The site-specific 8-Cl-cAMP (the site-specific cAMP analogue) reproduced the inhibitory effect of forskolin on DNA synthesis. Analysis of cell viability by trypan blue exclusion assay showed no significant reduction in viability of forskolin-treated A549, ruling out cell death as a cause of reduced DNA synthesis. These data provide evidence that the inhibitory effect of forskolin on proliferation is mediated by cAMP-dependent pathway. The adenylyl cyclase inhibitor, SQ22536, did not completely block the forskolin-induced inhibition of DNA synthesis. Consequently, the trypan exclusion assay was relevant to rule out cytotoxicity as a cause of inhibition of DNA synthesis. The difference might be explained by the pharmacodynamic chracterstics of forskolin and SQ22536 vis-à-vis their common target, the adenylyl cyclase, of which little is known.

The growth inhibitory effect of agents that elevate intracellular cAMP has been documented in various cell types including lung cancer cells. Cyclic AMP induces the expression of signaling molecules that inhibit cell cycle progression (Kato et al., 1994). Site-specific agonists, such as 8-chloro-cAMP are cell cycle specific apoptotic agents in

neuroblastoma cells (Kim et al., 2001c). A related cAMP analogue, 8-bromo-cAMP suppresses the growth of ovarian cancer cells (Shaw et al., 2001). Yan et al. (2000) showed that the cAMP/PKA pathway is involved in isoproterenol-induced cell arrest and apoptosis in lymphoma cells. The cAMP-mediated growth inhibitory mechanism is also found in human lung carcinoma (Ally et al., 1989) and in normal and neoplastic alveolar type II cells of human or mouse origin (Banoub et al., 1996).

Beta-adrenergic stimulation is inhibitory to the proliferation of various cancer cell lines. In this regard, norepinephrine, a physiologic agonist for β-adrenergic receptors, induces apoptosis in A549 cells and rat alveolar epithelial cells (Dincer et al., 2001). This effect of norepinephrine was mediated through α- and β-adrenoceptor activation and was inhibited by propranolol, a broad-spectrum β-adrenergic antagonist (Dincer et al., 2001). Cyclic AMP/PKA mediated growth inhibition by isoproterenol also exists in lymphoma cells (Yan et al., 2000). This is in contrast with our finding that isoproterenol had no significant effect on the proliferation of A549 cells. As described earlier, isoproterenol failed to stimulate cAMP production in this cell line.

Up-regulation of β-adrenergic receptors in A549 cells has been associated with growth inhibition and differentiation (Nakane et al., 1990). Consequently, the lack of response to isoproterenol with respect to cAMP production or DNA synthesis could be attributed to down-regulated receptors. As discussed earlier, related tumorigenic alveolar type II cells of mouse origin have defects in the beta-adrenergic/cAMP signaling pathway (Lange-Carter et al., 1992; Droms, 1996). In support of this explanation, forskolin induced significant inhibition of proliferation in A549 cells that was paralleled by significant

increase in the concentration of cAMP. In this study, it was not possible to offset possible insensitivity by the use of isoproterenol at higher concentrations because of cytotoxicity. Reduced number of beta-adrenergic receptors and diminished cAMP production upon β-adrenergic stimulation has been correlated with cancer progression in human chronic lymphocytic leukemia (Kamp et al., 1997).

Effect of Isoproterenol and Forskolin on cell proliferation in NCI-H322 cells

In contrast to our observations in A549 cells, isoproterenol and forskolin significantly stimulated DNA synthesis in NCI-H322 cells. The stimulatory effect of isoproterenol was significantly inhibited by preincubation with propanolol (broad-spectrum β-adrenergic antagonist) and SQ22536 (adenylyl cyclase inhibitor). Similarly, the effect of forskolin was countered by preincubation with SQ22536. These data clearly show that the proliferative effect of isoproterenol involves cAMP down stream of β-adrenergic receptors. These findings are in accord with our earlier observation that Clara cells (NCI-H322 and NCI-H441) are under the β-adrenergic control (Park et al., 1995). In both cell lines, β-adrenergic agonists, including isoproterenol, induced DNA synthesis in a cAMP-dependent fashion (Park et al., 1995). The effect of isoproterenol was concentration-independent probably suggesting functional β-aderenergic receptors in these cells are saturated in the lower concentration range as well.

This study shows that preincubation with 1 uM of AG1478 (the EGFR-specific tyrosine kinase inhibitor) significantly inhibited isoproterenol but not forskolin-induced DNA

synthesis in NCI-H322 cells. These data show that isoproterenol-induced DNA synthesis involves activation of cAMP as well as transactivation of the EGFR pathway. Beta–adrenergic stimulation activates MAPK through EGFR-dependent (Daub et al., 1996; Maudsley et al., 2000) or EGFR-independent mechanisms (Schmitt and Stork 2000; Hoare et al., 1999). G protein coupled receptor-dependent activation MAPK is cell type-specific and follows different mechanisms mediated by different G_s proteins. Some of these mechanisms include $G_{\alpha s}$/cAMP-mediated PKA activation and subsequent activation of B-Raf-MAPK in human embryonic kidney cell (Schmitt and Stork, 2000); $G_{\alpha q}$-mediated activation of protein Kinase C (PKC) that activates Raf-MAPK in Chinese hamster ovary cells (Hoare et al., 1999); and $G_{\beta\gamma}$/Src-mediated transactivation of receptor tyrosine kinases with subsequent Ras/MAPK activation in rat fibroblast cells (Daub et al., 1996; Maudsley et al., 2000). Maudsley et al. (2000) reported a novel pathway, in which isoproterenol transactivated EGFR via formation of a heterodimeric complex containing the B2 adrenergic receptors and the EGFR. Daub et al. (1997) showed that AG1478 blocked GPCR stimulated EGF receptor phosphorylation and ERK activation in rat fibroblast cells. In a similar fashion, Vancorven et al. (1993) showed that the GPCR ligand thrombin-mediated Ras activation in fibroblasts is abrogated by treatment with genistein, a tyrosine kinase inhibitor. In addition, AG1478 (EGFR-specific tyrosine kinase inhibitor) inhibits alpha-adrenergic receptor-mediated MAKP activation, demonstrating the role of transactivation mechanism in wider range of adrenergic receptors (Daniel et al., 1998). Crespo et al. (1995) showed that isoproterenol-induced MAPK activation was inhibited by expression of Ras inhibiting molecules in rat fibroblast cells.

In summary, isoproterenol and other GPCR ligand-induced DNA synthesis is mediated through the transactivation of the various receptor tyrosine kinases in many types of cells. As discussed earlier, a higher concentration (10 μM) of AG1478 inhibited both isoproterenol-induced and basal DNA synthesis. These findings suggest that, in the NCI-H322 cells, the EGFR pathway is not only activated by agonists of the EGFR but also by binding of agonists to the beta-adrenergic receptors.

The NCI-H322 cells were stimulated also by forskolin, an effect that was significantly countered by preincubation with the adenylyl cyclase inhibitor, SQ22536. These findings imply that a cAMP-dependent proliferative pathway is present in NCI-H322 cells in addition to the GPCR-RTK transactivation pathway. This implies also that in contrast to A549 cells, cAMP-dependent PKA may have a stimulatory instead of inhibitory role in NCI-H322 cells. Cyclic AMP and agents like forskolin that cause cAMP accumulation prevent apoptosis induced by DNA topoisomerase II inhibitors in promonocytic leukemia cells (Garcia-Bermejo et al., 1998). Lazou et al. (1994) showed that isoproterenol and norepinephrine stimulated MAPK activation in isolated rat heart. Cyclic AMP-induced MAPK activation has also been reported by Faure et al. (1994), who showed the presence of regulatory differences between cells indicating that G proteins and second messengers regulate MAPK pathway and growth in a cell-type specific manner. A novel signaling pathways where cAMP activates selected Raf isotypes, hence MAPK, in a PKA-dependent and independent manner have been shown by Vossler et al. (1997) and Schmitt and Stork (2000).The type and expression of the Raf isotypes might decide the effect of intracellular accumulation of cAMP (Gao et al., 1999; Seidel et al., 1999).

Therefore, the stimulatory effect forskolin in NCI-H322 cells might be explained by the type and amount of expression of Raf. Therefore, cAMP/PKA could activate or inhibit the MAPK pathway in a cell type-specific manner (Graves and Lawrence, 1996; Schmitt and Stork 2000).

Effect of forskolin on MAPK activation in A549 cells

The role of cAMP as a counter regulator of signals generated by growth factors has been described in various cell lines (Graves and Lawrence, 1996; McCawley et al., 2000). In this study, forskolin inhibited the low-serum-induced DNA synthesis and MAPK (ERK1/ERK2) activation of A549 cells.

This study showed that forskolin caused an early stimulation of MAPK followed by a consistent inhibition in A549 cells. This is in agreement with the thymidine incorporation data of this study, where forskolin induced significant inhibition of DNA synthesis in this cell type. Similar findings were reported by Lieberman et al. (1996a), who showed that forskolin inhibited EGF-induced MAPK activation and cellular proliferation in pancreatic adenocarcinomas. The inhibitory effect of cAMP on MAPK activation may occur at various levels of the EGFR-MAPK pathway. Inhibition of Raf-1 by cAMP dependent PKA has been well documented (Seger and Krebs, 1995; Piiper et al., 2000). Enhanced expression of the regulatory (RI alpha) subunit of cyclic AMP-dependent protein kinase (PKA) has been correlated with cancer cell growth. Moreover, this PKA inhibitory

protein is overexpressed in various cancer cell lines and tumor tissues including human ovarian cancer (McDaid et al., 1999) and Lewis lung carcinoma of mouse origin (Young et al., 1995). Consequently, inhibition/depletion of this regulatory protein by antisense oligoneucleotides suppresses the growth of human breast, colon, and gastric cancer (Yokozaki et al., 1993) and pulmonary adenocarcinoma of A549 cell origin (Wang et al., 1999).

The MAPK pathway is constitutively activated in A549 cells that harbor an activating Ras mutation that is necessary to maintain cell proliferation in these cells (Hoshino et al., 1999). The activation of MEK, which is a critical mediator of this pathway, is inhibited by pertusis toxin, which increases intracellular cAMP by inhibiting the activity of $G_{\alpha i}$ protein (Yano et al., 2002). The inhibition of MEK activation was associated with cAMP-mediated stimulation of PKA activity (Yano et al., 2002). The data from this study together with earlier observations by others, point to the fact that a minor increase in in the concentration of cAMP is inhibitory to the MAPK proliferative pathway in A549 cells. In the present study, the quantity of the catalytically active form of ERKl/2 was considerably reduced after 8 hours of incubation in A549 cells treated with forskolin (10 μM). However, the cAMP assay data showed that the levels of cAMP were elevated only by about 40% above the basal level after 20 minutes of incubation with forskolin at a concentration of 10 μM. It is possible that a significantly higher cAMP accumulation would have been observed with longer incubations similar to those for the MAPK and thymidine incorporation assay. This could be more pronounced in view of the likely low amount of cAMP degrading enzyme in A549 cells, which can further contribute to

enhanced cAMP accumulation (Dent et al., 1998). This suggests a possibility that in A549 cells the concentration of cAMP is more tightly regulated considering its growth inhibitory role. A minor increase in the concentration of cAMP would be detrimental to these cells. In support of this explanation, an approximate 60% increase in cAMP concentration by pertusis toxin caused a significant reduction in the amount of activated MEK that was accompanied by a significant PKA stimulation in A549 cells (Yano et al., 2002). In line with this interpretation, enhancing the activity of the cAMP-dependent PKA has been proposed as a potential cancer therapeutic approach alone or in combination with conventional chemotherapy in malignancies involving A549 cells (Wang et al., 1999).

Effect of forskolin on MAPK activation in NCI-H322 cells

Consistent with its observed mitogenic effect, forskolin (10 μM) induced a persistent MAPK activation in NCI-H322 cells in contrast to its mainly inhibitory effect in A549 cells. The amount of activated MAPK was significantly upregulated as early as 15 minutes post treatment and was maintained throughout the 24-hour period in the NCI-H322 cells. The cAMP concentration following treatment with forskolin (10 μM) showed about an 11-fold increase within 15 minutes. This paralleled the early stimulation of MAPK. There is limited information as to the mechanisms by which cAMP induces MAPK activation. In a recent report, Hanke et al. (2001) showed that the cAMP-PKA pathway is essential for the transactivation of EGFR. These investigators demonstrated

that specific inhibitors of PKA reduced the EGFR transactivation in response to isoproterenol. The mechanism of this transactivation includes stimulation of β-adrenergic receptors which leads to $G_{\alpha s}$-mediated increase in cAMP concentration, after which protein kinase A (PKA) phosphorylates β-adrenergic receptors (heterlogous desensitization), which is followed by switching from $G_{\alpha s}$ to $G_{\alpha i}$ protein. This is followed by MAPK activation by $G_{\alpha i}$-derived βγ-complexes and Ras (Hanke et al., 2001). This is in contrast with other reports in other cell lines, where the cAMP-PKA pathway inhibits EGFR activation (Mitsui and Iwashita, 1990; Iwashita et al., 1990; Stadtmauer and Rosen, 1986).

Recently, a novel signaling pathway in which cAMP activates MAPK in PKA-dependent and independent manner has been demonstrated (Vossler et al., 1997; Schmitt and Stork 2000). Protein kinase A (PKA) activates Rap1, which in turn activates B-Raf. On the other hand, cAMP can directly activates the EPAC (cAMP-GEFs/cAMP-Guanylyl Exchange Factor), which activates B-Raf directly and independent of PKA (de Rooij et al., 1998; Kawasaki et al., 1998). The type of Raf isotypes is an important precondition to determine inhibition or stimulation of MAPK by cAMP (Gao et al., 1999; Seidel et al., 1999). Consequently, the marked differential effect of forskolin on cell proliferation and MAPK activation in the A549 and NCI-H322 cell lines might be attributed to a difference in the type and amount of expression of Raf-B or Raf-1. Therefore, cAMP/PKA could activate or inhibit the MAPK pathway in a cell type-specific manner (Graves and Lawrence, 1996; Schmitt and Stork 2000). Contradictory effects of cAMP on mitogenic signaling might also result from cellular specificities in concentration, types, and

localization of the cAMP-dependent protein kinases (PKA) (Lange-Carter and Malkinson 1991).

In both cell types, the level of activated MAPK (p-ERK1/2) moderately declined after the initial surge as a result of forskolin treatment. However, contrary to the persistent and marked inhibition observed in A549 cells, the moderate decline in the level of MAPK in NCI-H322 cells was reversible with maximal activation at 24 hours treatment. This may suggest the presence of a mechanism to regulate MAPK activation within a certain optimal level. Barbier et al. (1999) proposed serine-phosphorylation of the EGFR by the cAMP/PKA as a regulatory mechanism of the amount of MAPK activation. On the other hand, PKA might have a dual effect on the MAPK regulation in that it activates MAPK upstream via $G_{\alpha i}$ (Hanke et al., 2001) and inhibits its activation by acting down-stream at the level of Raf-1 (Seger and Krebs, 1995; Piiper et al., 2000). The balance between the two pathways might determine the net response to signals mediated through the cAMP-PKA pathway in a cell-type specific manner. This might also attribute to the more or less cyclic trend of activated MAPK observed in the NCI-H322 cells.

Effect of forskolin on tyrosine phosphorylation of the EGFR

The inhibition of MAPK by forskolin observed in this study could result from the inhibition of tyrosine phosphorylation of the EGFR by cAMP dependent PKA (Barbier et al., 1999; Nair and Patel 1993). Cyclic AMP-dependent inhibition of EGFR activation may be cell-type dependent. Inhibition of the EGFR and related receptors occurs in

various cancer cell types (Mitsui and Iwashita, 1990; Iwashita et al., 1990; Stadtmauer and Rosen, 1986). We wanted to determine if forskolin-mediated MAPK in A549 cells occurs at the EGFR level. This is particularly important since the EGFR is commonly expressed in a greater proportion of the NSCLC (Rusch et al., 1993; Nakagawa 2001). Immunoprecipitation of the EGFR receptor followed by immunoblotting with an antibody against phosphotyrosine was done after treating cells with forskolin in an attempt to address this question.

Forskolin did not significantly modulate EGFR phosphorylation/activation at 30-minutes and 8-hours of incubation. Significant inhibition was observed after 24 hours of incubation. Highest amount of activated MAPK was observed at 30-minutes wherease lowest amount was obsereved at 8 and 24 hours of incubation. Significantly reduced DNA synthesis was found after 24-hour treatment with forskolin. Consequently, these time intervals were expected to show cAMP-mediated modulation of EGFR tyrosine-phosphorylation, if it existed. However, no such effect was observed after 8 hours, which had similar degrees of MAPK inhibition as 24 hours. The 24-hour treatment with forskolin showed significant cell cycle arrest as measured by inhibition of DNA synthesis. By contrast, a greater proportion of cells in an active cell cycle are expected in the 24-hour control/untreated group. Hence the 24-hours control group would likely have greater number of cells with activated/phosphorylated EGFR receptors. Therefore, the observed down-regulated EGFR phosphorylation after 24-hour incubation in forskolin could be confounded by a possible lower proportion of newly divided active cells compared to the control group. In support of this explanation, the amount of

phosphorylated EGFR in the 24-hour control group was significantly higher than the 0 hr control. As reasoned earlier, a comparable down-regulation of EGFR activation at the 8-hour time interval was expected since there was a similar down regulation of MAPK as that of 24-hour forskolin treatment. In summary, our data did not enable us to conclude if the cAMP-mediated down regulation of ERK1/2 (MAPK) activity observed in A549 cells acts at the receptor level. Cyclic AMP-dependent inhibition of EGFR activation appears to be cell type dependent. In some cancer types, growth arrest and MAPK inhibition by cAMP occurs independent of inhibition of EGFR phosphorylation (Lieberman et al., 1996b).

In summary, the differential effect of isoproterenol and forskolin in the two cell types suggests the presence of a cell-type dependent growth-regulatory pathway within pulmonary adenocarcinomas. The unresponsiveness to the β-adrenergic stimulation in A549 cells might have arisen from down regulated β-adrenergic receptors, functionally uncoupled G-stimulatory alpha protein, or functional state of the adenylyl cyclase. However, the differential response may have been due to cell specific signaling peculiarities, where cAMP is stimulatory or inhibitory in a cell-type dependent manner.

V. CONCLUSION

In the developed world, lung cancer became a major epidemic of the 20th century from a rare disease at the beginning of the century (Janssen-Heijnen and Coebergh 2001). Pulmonary adenocarcinoma (PAC), which originates from bronchiolar epithelial/Clara cells or the alveolar type II cells, constitutes one of the most rapidly increasing lung cancer types. Depending on the patient population examined, PACC (Clara cell origin) and PAC-type II (alveolar type II cell origin) occur in a comparable proportion. Unlike the non-small cell lung carcinoma, which exists almost exclusively in smokers, PAC develops in smoking and non-smoking individuals. Consequently, other factors besides smoking may contribute to the development and progression of PAC.

This study showed that isoproterenol (a broad-spectrum beta-adrenergic agonist) and forskolin (a adenylyl cyclase stimulator) induced significant proliferative response in cell line NCI-H322 (PACC). The selective blocking of the beta 1 and beta 2 adrenergic receptors and the adenylyl cyclase activity nullified this response, indicating the involvement of both receptors and cAMP in this proliferative pathway. Furthermore, the isoproterenol-induced proliferation was abrogated by pretreatment of cells with AG1478, an EGFR-specific tyrosine kinase inhibitor. This finding suggested that beta-adrenergic receptor stimulation transactivates the EGFR pathway. A persistent activation of MAPK (ERK1/2) accompanied the forskolin-mediated growth stimulation in the NCI-H322 cells.

Recently, a novel signaling pathway where cAMP activates MAPK in a PKA-dependent and independent manner has been proposed (Vossler et al., 1997; Schmitt and Stork, 2000). These studies showed that the involvement of Rap1-mediated activation of B-raf is an essential step in this cAMP/PKA-mediated MAPK activation. Consequently, the type and expression of the Raf isotype may be an important determinant as to whether cAMP is inhibitory or stimulatory to MAPK activation in a specific cell type (Gao et al., 1999; Seidel et al., 1999). Consequently, analysis of the dominant Raf isotype and activity might provide a clue to the contradictory effect forskolin had on cell proliferation and MAPK activation in the A549 and NCI-H322 cells.

The results from this study are in agreement with previous studies with these and related cell lines. A potential tumor-promoting mechanism involving the beta-adrenergic/cAMP signaling pathway has been described in PAC of Clara phenotype (Park et al., 1995; Schuller et al., 1999). The tobacco-specific carcinogen, NNK, is a high affinity beta-adrenergic agonist enhancing this novel growth pathway in PAC (Park et al., 1995; Schuller et al., 1999). According to these studies, beta-adrenergic receptor agonists such as isoproterenol, epinephrine and agents that cause intracellular accumulation of cAMP such as theophyilline would promote the development of pulmonary adenocarcinoma. As a result, the chronic management of respiratory diseases such as asthma, chronic bronchitis, and chronic obstructive lung disease (COPD) by these agents could pose a potential risk for the development of PAC (Park et al., 1995; Schuller et al., 1999). In addition, there is considerable epidemiological data showing the association of chronic respiratory diseases such as bronchitis and COPD and the development of lung cancer

(Osann, 1991; Weiss 1991). A recent study by Brownson and Alavanja (2000) showed a significant risk of lung cancer in the presence of chronic bronchitis, emphysema, and pneumonia. Consequently, the possible role of the extensive use of these agents might in part explain the spatial and temporal co-distribution of chronic respiratory diseases and lung cancer.

On the other hand, DNA synthesis in A549 cells (PAC-type II) was inhibited by forskolin and 8-Cl-cAMP. These cells were also unresponsive to beta-adrenergic stimulation with isoproterenol. No significant effect in DNA synthesis was observed following treatment with isoproterenol. Cyclic AMP is growth inhibitory in various cell types including lung cancer. It has been associated with cell cycle arrest and apoptosis in various cancer cell lines (Kato et al., 1994; Kim et al., 2001c; Yan et al., 2000) including human lung carcinoma (Ally et al., 1989). Although limited, data from earlier studies suggested that the growth of PAC type II of mouse and human origin are inhibited by beta-adrenergic/cAMP pathway (Dincer et al., 2001; Banoub et al., 1996; Nakane et al., 1990). Studies in mouse tumorigenic alveolar type II cells indicated down regulation or deregulation of the various beta-adrenergic signaling mediators from receptors to the enzyme effectors (Lange-Carter and Malkinson, 1991; Lange-Carter et al., 1992; Droms, 1996). Reduced number of beta-adrenergic receptors and diminished cAMP production upon beta-adrenergic stimulation have also been correlated with progression of chronic lymphocytic leukemia (Kamp et al., 1997). Consequently, the lack of responsiveness to isoproterenol might have arisen from either of the aforementioned defects in the beta-adrenergic/cAMP pathway. In support of this rationalization, data on basal and post-

stimulation cAMP concentration showed a marked difference between the two cell lines. A significantly lower cAMP concentration was found in the A549 cells compared to NCI-H322 cells. Also, beta-adrenergic stimulation with isoproterenol showed no significant increase in the concentration of cAMP in A549 cells. This is in complete contrast with the NCI-H322 cells where isoproterenol induced a marked increase in cAMP concentration. Contrary to the response to isoproterenol, A549 cells showed a moderate but significant increase in cAMP concentration following the direct stimulation of adenylyl cyclase enzyme. However, the increase in forskolin-mediated cAMP amount was distinctly lower than that observed in NCI-H322 cells. Our data demonstrated a remarkable difference between the two cell lines. The β-adrenergic signaling pathway appears to have a dual regulatory function in the normal alveolar type II cells, where it facilitates a range of physiological processes (Dumasius et al., 2001; Isohama et al., 2001) while discouraging cell proliferation (Nakane et al., 1990). Consequently, it might be reasoned that alveolar type II cells down-regulate the beta-adrenergic/cAMP pathway during their tumorigenic progression, which would, otherwise, preclude their uncontrolled proliferation. This transformation to a physiologically incompetent and proliferative phenotype would be expected to greatly exacerbate the ailing alveolar physiology. Compromised re-epithelialization of the alveolar surface, which is done by alveolar type II cells, may result in pulmonary fibrosis (Witschi, 1990). Consequently, it could be hypothesized that treatment of chronic respiratory diseases with agents that stimulate the beta-adrenergic/cAMP pathway, might selectively promote PACC tumorigenesis. On the other hand, in the presence of PAC originating from alveolar type II cells, such agents might have a net beneficiary effect by suppressing the proliferation

of tumorigenic cells. However, a concurrent inhibition of healthy alveolar type II cell population could be the untoward consequence of such agents in both cases. This would be expected to affect the normal re-epithelialization/alveolar repair and other physiological contributions of alveolar type II cells to the proper functioning of the lungs. Phosphodiesterase inhibitors, such as theophylline, are the primary components in the management of chronic pulmonary diseases such as asthma. These agents induce the intracellular accumulation of cAMP, which is known to exert a widespread anti-inflammatory activity (Giembycz, 2000). This effect alleviates inflammation and the subsequent airway dysfunction. However, in the presence of concomitant diagnosed/undiagnosed pulmonary tumors, this effect might be detrimental by suppressing cellular immunity against malignant cells. Since PAC diagnosis is generally not characterized at the cellular level, the treatment of concomitant chronic respiratory diseases should be exercised with caution. In this regard, epidemiological studies of the association of chronic respiratory diseases and the development of lung cancer vis-a-vis the use of beta-adrenergic medications is essential.

Contrary to the pattern observed in NCI-H322 cells, forskolin induced down regulation of MAPK (ERK1/2) activation in A549 cells. The cAMP/PKA-mediated inhibition of Raf, which is an upstream activator of the MAPK mitogenic pathway, has been extensively documented (Seger and Krebs, 1995; Piiper et al., 2000). Another mechanism of MAPK inhibition by cAMP is via cAMP/PKA–mediated inhibition of EGFR tyrosine phosphorylation (Mitsui and Iwashita, 1990). Immunoprecipitation and western blotting of the EGFR following forskolin treatment showed no significant effect on receptor

tyrosine phosphorylation. Therefore, the cAMP-mediated MAPK inhibition in A549 may be mediated by a mechanism other than inhibition of EGFR tyrosine phosphorylation.

The epidermal growth factor receptor (EGFR) plays a major role in the progression of various types of solid tumors. Overexpression of EGFR has been found in a greater proportion of adenocarcinomas (Rusch et al., 1993; Nakagawa 2001). This study showed that both PAC cell types showed a low-serum induced proliferation manifested by a marked DNA synthesis in low-serum. Western blot analysis showed that this proliferative response was accompanied by a significant induction of MAPK activation. These findings suggested the production of autocrine growth factor(s) in response to serum deprivation. Pancreatic adenocarcinoma cells have been shown to proliferate indefinitely in serum-free conditions by overexpressing the epidermal growth factor receptor and respective ligands (Murphy et al., 2001a). Our results with interrupted cultures strongly support this interpretation. An involvement of EGFR as a mediator of the observed low-serum-induced proliferation was supported by a significant inhibition of low-serum-induced DNA synthesis by the tyrophostin AG1478 (the EGFR-specific tyrosine kinase inhibitor). This effect on proliferation corresponded with a significant MAPK (ERK1/2) inhibition in a time-dependent manner.

In both cell lines, treatment with transforming growth factor alpha (TGF-α) failed to stimulate DNA synthesis. However, the epidermal growth factor (EGF) differentially stimulated DNA synthesis in NCI-H322. However, an antibody against EGF inhibited

DNA synthesis in both cell lines. A possible reason for this apparent inconsistency is that the use of exogenous EGF might not stimulate cells that already express EGF. However, neutralizing antibodies would be effective against endogenous growth factors released by cells in an autocrine and/or paracrine fashion. A varying range of primary or active ligand/s for the EGFR has been reported for the same cell line, A549 (Imanishi et al., 1988; Bennett, 1999; Thrane and Schwarze, 2001; Al Moustafa et al., 2002). However, despite the diversity in the type of primary ligand/s, the EGFR appears to be the common denominator in the proliferation of PACs. The beta-adrenergic receptor mediated proliferation in NCI-H322 cells was completely abrogated by pretreatment with AG1478. These findings indicate that β-adrenergic receptor stimulation transactivates the EGFR pathway in these cells. G-protein coupled-receptor (GPCR) mediated cell proliferation via the transactivation of the receptor tyrosine kinases has recently been shown in fibroblasts (Maudsley et al., 2000; Luttrell et al., 1999; Faure et al., 1994). Therapeutic targeting of the EGFR-mediated proliferative pathway showed promising results in early clinical trials of non-small cell lung cancer (Arteaga et al., 2002; de Bono and Rowinsky 2002; BASELGA 2001; Slichenmyer and Fry 2001). However, for the subpopulation of PACC, beta blockers which inhibit EGFR transactivation as well as cAMP-activation, may be more effective.

In summary, the two cell lines derived from phenotypically different PAC cells showed differential growth responses to beta-adrenergic agonists and elevated cAMP. However, a similar study with a larger number of cell lines might be necessary to arrive at a more

meaningful comparison between the two phenotypes. Nonetheless, based on earlier findings in both cell lines and related mouse cell lines, the current study points to an essential difference in growth signaling regulation of these PAC phenotypes. Equally important, it showed that PAC constitutes cell lines that are different not only in morphological and histochemical characteristics, but also in their growth regulatory pathways. Such differences preclude a blanket-type chemoprevention approach. A complete understanding of mitogenic signaling mechanisms is vital to the study of pathophysiology of tumorigenesis, which is a critical step in rational chemopreventive approaches. Care should be exercised when cancer chemoprevention is based on invitro or laboratory animal cancer models that represent a specific signal trasduction pathway. The unsuccessful and even tragic outcomes of carotene-retinol chemoprevention trials (Omenn et al., 1996) underscore the importance of characterizing the regulatory pathways involved in the various cancer cell types. Carotene and retinols, which increase intracellular cAMP (Prasad et al., 1994) have significant chemoprevention activity in hamster tracheobronchial squamous cell carcinoma (Newton et al., 1980; Furukawa et al., 1999). Interestingly, epidemiological data showed that dietary beta-carotene is associated with reduced lung cancer risk in non-smoking individuals (Mayne et al., 1994). Such agents also have been shown, in this and earlier studies, to function as promoters of tumorigenesis in PAC of human and hamster models (Park et al., 1995; Schuller et al., 2000). Recently, Massaro and Massaro (2000) and Mao et al. (2002) reported a potential mechanism and role of retinoids in the treatment of pulmonary emphysema, a condition highly associated with smoking (Petty, 2002; Seagrave, 2000). Consequently, the use of agents, such as retinols, for the treatment of smoking-induced emphysema might

potentially contribute to the development of PACC by promoting tumor progression that might have been initiated by the cigarette specific carcinogen, NNK. In fact, a significant association between previous lung diseases, particularly emphysema, and lung cancer has been reported recently (Brownson and Alavanja 2000).

Amidst the apparently complicated growth-signaling network, common and key elements such as the EGFR, Raf and MAPK represent convergence points that may provide effective pharmacological targets for a broader family of cancer types and phenotypes. Therefore, signaling complexity could paradoxically offer practical simplicity, hence unique opportunities for safer and more effective interventions.

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