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13. ABSTRACT *(Maximum 200)*

Epidermal growth factor (EGF) and transforming growth factor α (TGF) exert their effects through membrane-associated EGF receptors (EGFR). Although their mechanisms of action are not completely understood, these growth factors and their receptor are physiological regulators of normal mammary gland development, and are often overexpressed in estrogen receptor negative breast cancers. Initially, we examined the effects of EGF and TGF on the *in vitro* development of normal rat mammary epithelial cells (MEC). PD158780, a potent and selective inhibitor of the tyrosine kinase domain of the EGFR, was then used to demonstrate that EGFR signaling was required, at least in part, for normal MEC proliferation, survival, and functional differentiation. In addition, normal rat mammary fibroblasts (MFC) were used to examine rapid signal transduction events including tyrosine phosphorylation protein profiles and MAPK phosphorylation in response to EGFR activation by EGF or TGF. PD158780 was then employed to confirm that the signal transduction responses were EGFR-dependent. We will continue to examine the roles that EGFR play in the development of normal MEC cultured alone as well as MEC co-cultured with mammary fibroblasts, pre-adipocytes, or mature adipocytes, and then to examine their role during rat mammary tumor progression and metastasis. Ultimately, we hope that this data will help to identify new therapeutic targets and develop effective therapies to treat patients with estrogen receptor negative breast cancer.

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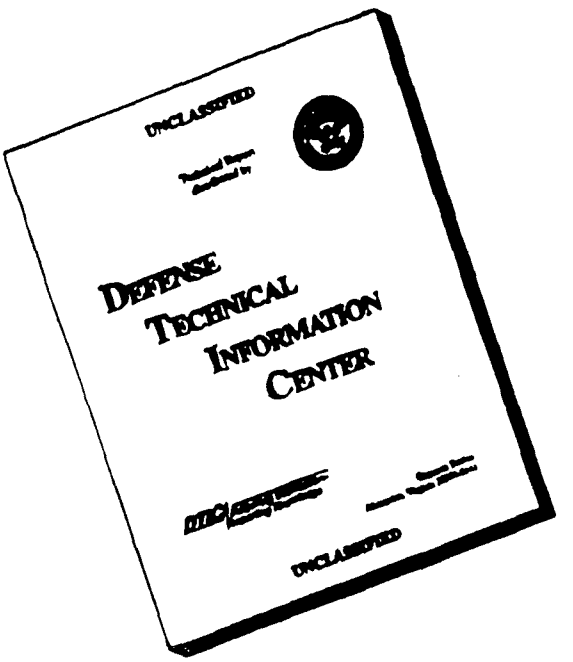

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Kathleen M Darcy 7/25/96

PI - Signature Date

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INTRODUCTION

A. NATURE OF THE PROBLEM

For women in the United States, breast cancer incidence and mortality rates are terrifying. Incidence rates are among the highest in the world and continue to rise with time, while mortality rates have remained unchanged. It seems essential to identify the factors that stimulate or inhibit breast cancer progression, and to clarify the present understanding of the factors that regulate the proliferation, differentiation, adhesion, invasion and death of breast cancer cells and the normal cells from which this cancer is derived. Breast cancer development appears to involve a progressive deregulation of the developmental pathways operative in normal mammary epithelial cells (MEC). Steroids, polypeptides and extracellular matrix (ECM) components appear to coordinately regulate the development of the normal MEC as well as many breast cancer cells. Unfortunately, the mechanisms of action of these regulators, and their interactions are only partially understood. Breast cancer progression is classically characterized by a loss in responsiveness to ovarian steroids, growth inhibitors, and/or inducers of apoptosis, an upregulation in the expression of autocrine and paracrine growth factors, growth factor receptors and matrix remodeling enzymes, as well as an alteration in the adhesive properties of cells to other cells and to the different ECM components.

Epidermal growth factor (EGF) and transforming growth factor-α (TGF) have been shown to stimulate the development and malignant progression of breast cancer. These growth factors act by binding to the EGF receptor (EGFR), and activating its tyrosine kinase domain. A number of proteins involved in signal transduction have been shown to be tyrosine phosphorylated in response to EGFR activation by EGF. The functions and intracellular mechanisms of action of EGF and TGF in normal MEC and breast cancer cells are not fully defined. Changes in the expression of EGFR and its ligands appear to be important in breast cancer progression. EGFR transcripts are overexpressed in ~50-60% of primary human breast tumors, and these carcinomas usually have an estrogen receptor and progesterone receptor negative phenotype, high proliferation rates, poor response to endocrine therapy, and reduced patient survival rates (1). Overexpression of EGFR confers a conditional ligand-dependent growth advantage to the tumor cells.

EGF and TGF, natural ligands of the EGFR, have been shown to stimulate the proliferation of breast cancer cells, and the proliferation and morphogenesis of the normal MEC from which this cancer is derived. Surgical removal of the salivary gland which eliminates the main source of circulating EGF, or treatment with a neutralizing anti-EGF antibody was shown to inhibit the development of spontaneous mammary tumors, the growth of established mammary tumors, and the implantation of transplantable mammary tumors in mice (2). Administration of EGF was able to reverse these effects (2). TGF has been shown to mediate the mitogenic effects of estrogen, progesterone and prolactin in breast cancer cell lines (3,4), and part of the growth promoting effects of an activated ras gene in MEC (5). TGF can also act as a dominant transforming gene product in MEC expressing normal levels of EGFR (6). Moreover, TGF mRNA and protein can be detected in ~50-70% of primary human breast tumors (7).

An important perspective in understanding the mechanisms of growth control in cancer is the knowledge of growth regulation in the normal cells from which this cancer is derived. A detailed understanding of the mechanisms of action, and the factors that control the type and/or magnitude of the response(s) induced by EGF and TGF could be used to design effective forms of treatment that inhibit breast cancer progression. The successful design and implementation of curative therapies also requires an understanding of (1) the role that mammary gland stromal cells play in regulating the progression and metastatic spread of the breast cancer cells and (2) the mechanism of action and resistance to the proposed therapy. Thus far investigations in normal MEC have lagged behind studies examining the influence of growth factors in breast cancer because of the difficulties of supporting physiologically relevant development of normal MEC in culture. The main advantage of culture experiments is that experimental conditions can be controlled. Controlled experiments allow for more definitive interpretation of the results. A unique and powerful primary culture system was developed in our laboratory which permits non functional MEC, isolated from pubescent female rats (at a time of development when these cells are highly sensitive to carcinogen-induced transformation), to undergo extensive physiologically relevant proliferation, functional differentiation, and branching alveolar morphogenesis. This model system is uniquely suited to examine the mechanism(s) by which EGF and TGF, acting through their common cellular receptor, the EGFR, regulate the *in vitro* growth, differentiation, morphogenesis, and invasion of (1) primary MEC derived from normal female rats and then cultured in the absence and presence of a phorbal ester tumor promoter known to synergize with certain activities of EGF and TGF in MEC, as well as (2) primary MEC derived from rats exposed *in vivo* to the carcinogen N-methyl-nitrosourea (MNU) and then cultured in the absence and presence of a phorbol ester tumor promoter known to synergize with certain activities of EGF and TGF in MEC. Our model system was recently upgraded such that MEC can be cultured alone or in combination with different populations of mammary stromal cells. This upgrade allows for the examination of direct epithelial-specific effects when the MEC are cultured without mammary stromal cells, and of direct and indirect effects when the MEC are co-cultured with different types of mammary stromal cells. Development of a defined serum-free primary mammary co-culture system represents a significant advancement for research aimed at examining stromal-epithelial interaction during normal MEC development as well as breast cancer progression and metastasis.

B. BACKGROUND

The normal mammary gland undergoes its most extensive development during puberty and again during pregnancy and lactation. This type of development, referred to as branching morphogenesis, involves the precise regulation of cell proliferation, differentiation, apoptosis, as well as invasion, and leads to the formation of morphologically and functional distinct organs including the salivary gland, liver, lung, and kidney as well as the mammary gland. Mammary epithelial cells (MEC) undergo branching morphogenesis in response to the coordinate presence of distinct extracellular matrix (ECM) components including laminin, type IV collagen, entactin, fibronectin, and glycosaminoglycans (8), and hormones-derived from the pituitary, adrenal, ovarian, and salivary glands (2,9-13). Induction and maintenance of this process is dependent on the differential ability of the epithelial cells to produce, degrade, and/or activate mammary gland parenchymal and mesenchymal cell regulators, and to adjust their polypeptide, steroid and ECM receptor status (14-18). Currently, the MEC-, and the mesenchymal cell-specific mechanisms of action of the systemically- and

locally-derived regulators of MEC branching morphogenesis, and their interactions are incompletely understood.

The most basic phenotypic difference between breast cancer cells, and the normal epithelial cells from which this cancer is derived appears to be the atypical morphology of the cancer cells compared to the highly specialized and well polarized appearance of the normal cells. Breast cancer cells generally develop within the highly proliferative and invasive end buds, and along the ducts of the mammary gland (19-24). The distinct histologic types of breast cancer (25,26), and the late stages at which this disease is often diagnosed have made it difficult to identify and characterize all of the factors that initiate and/or promote either the conversion of a normal cell to a precancerous cell, or the progression of a precancerous lesion to a carcinoma *in situ*, and finally to an invasive carcinoma. The appearance of morphologic atypia indicates a deregulation of normal branching morphogenesis. The factors which stimulate proliferation and invasion of normal MEC seem to also stimulate breast cancer progression and metastasis. These growth factors and their receptors are, therefore, viable therapeutic targets. A detailed understanding of the regulators of normal branching morphogenesis is required if these therapeutic strategies are to effectively inhibit breast cancer cell growth and metastasis.

1. EPIDERMAL GROWTH FACTOR RECEPTORS (EGFR)

The EGFR is a 170 kDa transmembrane glycoprotein with an extracellular domain for binding of epidermal growth factor (EGF)-like ligands of which EGF and transforming growth factor-α (TGF) are the most well characterized members. The intracellular domain of the EGFR has a tyrosine kinase domain with an ATP-binding site, as well as binding sites for proteins that contain SRC homology 2 (SH2) or phosphotyrosine-binding domains (27-30). Upon binding EGF, the EGFR undergoes conformational changes in the extracellular domain which result in rapid oligomerization and intermolecular phosphorylation of occupied EGFR, followed by the association with and phosphorylation of signal transduction proteins such as: phospholipase C-gamma-1, $p21^{ras}$ GTPase-activating protein (GAP), phosphatidylinositol 3'-kinase, src and src-like tyrosine kinases, raf and MAP kinase, lipocortin I, c-erbB-2, c-erbB-3, and c-erbB-4 (28,29,31-40). EGFR activation has been shown to induce membrane hyperpolarization, probably by activation of K+ channels, alkalinization of the cell cytosol by increasing membrane Na+/H+ antiport activity, accumulation of intracellular calcium by indirect activation of protein kinase C, and amino acid and glucose transport (28,41). EGF has also been shown to stimulate the synthesis of type IV collagen (42), fibronectin (43), a 95 kDa type IV collagenase and interstitial fibroblast-type collagenase (44), to induce the expression of the EGF receptor (45), TGF-α (45), and the cellular protooncogenes c-fos and c-myc (46), and to activate casein kinase II (47,48) which has been shown to phosphorylate DNA topoisomerase II (48), and the transcription factors myc (49), E7 (50), large T antigen (51), serum response factor (52), and c-erb A (53). Recently, EGFR activation by EGF was shown to result in a decrease in the binding of ZPR1, a zinc finger binding protein, to the intracellular tyrosine kinase domain of this receptor, and dissociated ZPR1 protein was then observed to translocate and accumulate in the nucleus (54). The biological function of ZPR1 in the nucleus has yet to be defined.

The signal transduction pathways induced by TGF-mediated activation of the EGFR have not been studied as extensively as the intracellular consequence of EGF-mediated

activation of the EGFR. It is interesting to speculate that each ligand initiates the induction of distinct signal transduction cascades within the different histologic types of normal MEC and/or breast cancer cells, and that these transduction pathways ultimately lead to the induction or suppression of distinct cellular responses.

Both high (Kd of 0.1 nM) and low affinity (Kd of 3.6 nM) EGFR are present within cultured mouse MEC (55), and mammary glands from young and mature virgin, pregnant and lactating mice (56,57). Competitive *in situ* binding assays and autoradiography in the mammary glands of pubescent female mice were used to demonstrate that EGFR were concentrated in the cap cells of the terminal end buds, in the myoepithelial cells of the mammary gland ducts, and the stromal cells adjacent to the end bud flank and the subtending ducts (58). Immunohistochemical analysis of normal human breast and benign mammary tumors indicated that EGFR were usually expressed at a low level (59).

Changes in the expression and distribution of EGFR could play an important role in the pathogenesis of breast cancer. Analysis of EGFR in breast carcinomas using binding assays demonstrated an increased level of EGFR in 35-45% of the cases (60,61), an inverse relationship with estrogen and progesterone receptors, a poor response to endocrine therapy, shorter disease-free period, and reduced overall survival. Such tumors also have a higher proliferation rate (62). The increased levels of EGFR are generally due an increased level of EGFR mRNA expression (63,64). Gene amplification has only been observed in ~3% of the primary carcinomas studied (64,65). *In situ* hybridization for EGFR mRNA shows that there generally is a good correlation with immunohistochemically detectable EGFR protein, but there are tumors in which EGFR mRNA can be detected in the absence of EGFR protein (66). In fact, EGFR transcripts are overexpressed in ~50-60% of primary human breast tumors (1). Data from breast cancer cell lines suggests that increased expression alone is not sufficient to produce hormone or growth factor independence (67). EGFR-mediated induction of cell proliferation and invasion requires its interaction with an EGF-like peptide. Therefore, EGFR overexpression in the absence of an EGF-like mitogenic peptide cannot be expected to promote cancer progression. Transfection studies in rodent fibroblasts demonstrated that overexpression of the EGFR can predispose cells to expression of a transformed phenotype upon stimulation by EGF (68-70).

2. EGF AND TGF

EGF has been shown to stimulate the proliferation and occasionally the differentiation of cells within the kidneys, lung, bone, brain, and skin, to affect hormone production in the hypothalamus, pituitary, placenta, ovaries, adrenals, and thyroid gland, as well as to modulate the immune system (41). Our laboratory and others have demonstrated that EGF is a critical physiologically relevant regulator of the growth, differentiation, and morphogenesis of normal MEC in culture as well as *in vivo*. Specifically, EGF appears to stimulate cell proliferation, and to support MEC branching morphogenesis. EGF has also been shown to inhibit, enhance, or differentially regulate milk protein production and/or expression of distinct milk components. The exact role that MEC-derived and/or salivary gland-derived EGF plays in inducing and/or maintaining the cytological differentiation of these cells, and the mechanism(s) of action of EGF in mammary cells remains to be determined. TGF, another member of the growing family of EGF-like peptides, has also been shown to be a physiologically relevant regulator of mammary gland morphogenesis. The biological activities

of TGF have not been studied as extensively as that of EGF, and its mechanism(s) of action in mammary cells are largely unexplored. Administration of exogenous EGF or TGF in Elvax pellets *in vivo* can stimulate end bud and ductal growth (end bud branching morphogenesis) in the mammary glands of virgin ovariectomized mice (71). EGF and TGF also enhanced lobuloalveolar development in hormonally primed virgin mice (72). TGF has been shown to be secreted by rat mammary myoepithelial cells and epithelial cell lines in culture, as well as in the rat mammary gland were it can act as an autocrine and/or paracrine growth factor (73).

Primer-directed enzyme amplification was used to demonstrate that mammary glands from virgin (pubescent and adult), and mid-pregnant mice express both EGF and TGF transcripts, whereas only EGF-transcripts are detected in the mammary glands of mid-lactating mice (71). Using conventional Northern blot analysis, TGF mRNA was detected within mammary glands of pregnant rats, but not in mammary glands of virgin or lactating female rats (73). Interestingly the concentration of TGF in mammary gland extracts from virgin and pregnant rats was 0.2 ng/g of tissue, but in the lactating mammary gland the concentration was 1.2 ng/g of tissue (73). Immunohistochemical localization studies in prepubescent mouse mammary glands demonstrated that EGF was localized in the inner layers of the terminal end bud, and in the ductal cells of the mammary gland (71). These cells tend to be cytologically differentiated. In contrast, TGF was localized in the epithelial cap-cell layer of the advancing terminal end bud, and the stromal fibroblasts at the base on the highly proliferative and invasive terminal end bud. The peripheral cap cell layer is considered to be a population of proliferative stem cells. TGF is also expressed in the rat and human mammary glands in 10-15% of the surrounding stromal cells, as well as in both alveolar and ductal epithelial cells (74). TGF expression is enhanced in human mammary glands during pregnancy and lactation which may account for the relatively high level of this growth factor in human milk (74). Immunohistochemical studies in the mammary glands of lactating rats localized EGF to the luminal surface of the secretory cells (75). Taken together, endogenous TGF may play a key role in the local regulation of stem cell proliferation during end bud branching morphogenesis by autocrine and paracrine mechanisms, whereas EGF may be responsible for stimulating cell growth, but also for inducing or maintaining differentiation of MEC. EGF appears to be derived primarily from the salivary gland as well as from the MEC, whereas TGF is derived from both mammary gland parenchymal and mesenchymal cell types.

It seems interesting to speculate that TGF is responsible for inducing cell growth and invasion during end bud branching morphogenesis, while EGF may be responsible for inducing or maintaining the cytological differentiation of MEC. Comparative analysis of the biological activities of EGF and TGF is required to determine whether each polypeptide plays a distinct role in normal mammary gland morphogenesis. If TGF is a preferential inducer of normal end bud proliferation and invasion, it may also play a similar role in breast cancer by promoting tumor cell growth and metastasis. Additional immunohistological localization studies of EGFR and its ligands during the different stages of mammary gland morphogenesis should provide in-depth insight into the distinct roles that EGFR, EGF and TGF play in the different phases of MEC branching end bud morphogenesis during puberty, MEC branching alveolar morphogenesis during pregnancy and lactation, and neoplastic transformation. It would also be interesting to directly compare the type of signal transduction pathways that are induced in response to EGF or TGF within mammary gland

ducts, end buds, and alveoli, as well as precancerous lesions, carcinoma *in situ*, and invasive carcinoma.

EGF and TGF have both been implicated as factors which can stimulate the proliferation of various types of tumors. Both EGF and TGF have been shown to be potent mitogens for breast cancer cells (1,76-82). Since the salivary gland is the dominant source of biologically active EGF in the body, Oka and co-workers surgical removed the salivary glands of certain mice to examine the role that salivary gland-derived EGF plays in mouse mammary tumorigenesis (2). The incidence of spontaneous mammary tumors in control virgin mice was ~63%. Sialoadenectomy reduced the tumor incidence to ~13% and increased the latency period of tumor development. These effects were partially reversed by administration of EGF. Sialoadenectomy of mammary tumor-bearing mice caused a rapid and sustained inhibition of tumor growth, whereas EGF stimulated tumor cell growth. Implantation of mammary tumor transplants was completely inhibited by sialoadenectomy. In addition to sialoadenectomy, the administration of anti-EGF antiserum inhibited the growth and implantation of mammary tumors (2). EGF treatment was able to reverse or block these effects.

TGF mRNA can be detected in both benign and malignant breast tumors at a similar frequency and level of expression (83). Immunohistochemical studies revealed minimal levels of TGF in normal human breast tissue, and increased levels in ductal hyperplasia, atypical ductal hyperplasia and ductal carcinoma *in situ* (84). TGF mRNA expression and immunoreactive TGF have been found in 40-70% of primary and metastatic human breast tumors (7). It is predominantly expressed in the tumor cells, not in the surrounding stromal cells or in the infiltrating lymphoid cells, and is generally expressed at a level that is significantly higher than in benign breast lesions, or in adjacent, non involved breast epithelium. Transformation of several spontaneously immortalized non transformed mouse mammary epithelial cell lines, NOG-8 and HC-11, and a human mammary epithelial cell line, MCF-10A by an activated c-Has-ras protooncogene increased the level of TGF mRNA and protein expression by 5-10 fold (7). Addition of an anti-TGF neutralizing monoclonal antibody to the ras-transformed MEC inhibited anchorage-independent growth of these cells by 50-80% which indicated that TGF mediates at least part of the mitogenic effects of an activated ras gene in MEC. Overexpression of TGF can act as a dominant transforming gene in NOG-8 mouse cells, and human MCF-10A cells (6,7,85). These cells have previously been shown to express EGFR. Introduction of rat or human TGF into the germ line of transgenic mice induces abnormal morphogenesis and proliferation of the mammary glands (86).

3. BASIC DESCRIPTION OF THE PRIMARY CULTURE MODEL SYSTEM

The unique and powerful model system utilized for our studies was developed in our laboratory (87,88). This system was chosen because it can be used to simultaneously monitor effects on the proliferation, cytological and functional differentiation, morphogenesis as well as invasion of normal and transformed MEC in primary culture under defined, serum-free culture conditions. In addition, this model system was upgraded last year to allow MEC to be cultured alone or co-cultured with different types of primary rat mammary stromal cells such as fibroblasts, pre-adipocytes, and/or adipocytes. This exciting upgrade of our model system allows for the examination of epithelial cell-specific effects when the MEC are

cultured without distinct types of mammary stromal cells, and of actual, physiologically-relevant effects that arise when the MEC are co-cultured with their natural counterparts, the stromal cells. The different co-culture systems attempt to more faithfully reconstitute the complex microenvironment of the mammary gland in a defined culture setting.

MEC were disaggregated from excised mammary glands of 7 week old pubescent female rats, and dissociated from their surrounding mammary gland adipocytes and fibroblasts. The MEC were isolated as organized cell aggregates termed mammary colonies. Most of the isolated mammary colonies were spheroidal in shape, pale in appearance (89), and composed of cells arranged in a terminal end bud or a blunt end bud type of cellular organizational pattern (89). Electron microscopic examination revealed that most of the isolated mammary colonies were composed primarily of cytologically differentiated that were non functional (non milk producing) (89).

Newly isolated mammary epithelial organoids (MEO) were cultured for up to 21 days within the Engelbreth-Holm-Swarm (EHS) sarcoma-derived reconstituted basement membrane (RBM) in the presence of ALV Media (phenol red-free F12/DMEM, bovine insulin [10 µg/ml], ovine prolactin [1 µg/ml], hydrocortisone [1 µg/ml], progesterone [1 µg/ml], human apotransferrin [5 µg/ml], ascorbic acid [880 ng/ml], fatty acid-free bovine serum albumin [1 mg/ml], and gentamycin [50 µg/ml]) supplemented with *mouse EGF* [10 ng/ml]. With time in culture, many of the MEO underwent external changes in size, appearance, and shape, and internal changes in cell number, composition, organizational arrangement and functional capacity. Using tritiated thymidine incorporation to analyze effects on DNA synthesis and an MTT assay to quantitate viable cell number, Hahm and Ip demonstrated that ALV Media with *mouse EGF* [10 ng/ml] induced extensive cell proliferation over a 21 day experiment (87). In general, cell number per culture well usually drops during the first few days of culture development under optimal growth conditions, but thereafter rises rather sharply through day 14, and then more gradually during the last 7 days of the culture period. In addition, cell number increased between 15 and 30 fold over the coarse of a 21-day culture period (87,90,91). In the presence of ALV Media with *mouse EGF* [10 ng/ml], tissue-specific differentiation was rapidly induced, and appeared to be continuous over a 21 day experiment (88,89). Specifically, casein production was detected within the cultured mammary organoids as early as day 2, and the amount of casein within the individual culture wells rapidly accumulated to µg quantities (89). The increase in casein production correlated with a rise in the expression of total ß-casein mRNA by the cultured organoids (88). Milk protein secretion, indicated by the appearance of casein micelles within the luminal compartment of cultured organoids, was first observed within mammary organoids cultured for as little as 4 days, and appeared to continue over the three week culture period (89). In addition, the three-dimensional luminal organoids accumulated extensive intracellular lipid, and occasionally vectorally secreted lipid globules into their luminal compartment (88,89). Oil red O stain, specific for lipid, was used to verify that the cultured MEC were actually accumulating extensive intracellular lipid (88).

C. PURPOSE OF THE PRESENT WORK

A number of factors have been identified that stimulate the development and/or progression of breast cancer. EGF and TGF are among those factors that stimulate breast cancer cell proliferation and invasion. Studies to directly compare the biological activities of

EGF and TGF in normal MEC are in their infancy. In addition, the factors that control the type or the magnitude of response induced by EGF or TGF in normal MEC, or breast cancer cells have not been fully explored. Preliminary data from other laboratories suggests that these growth factors play distinct roles during mammary gland development. TGF may be a more dominant stem cell mitogen, whereas EGF may be responsible for supporting epithelial cell differentiation. If this hypothesis is true then TGF should stimulate proliferation, end bud morphogenesis, invasion of the organoids through the ECM, and the elaboration of matrix-degrading metalloproteinases. In contrast, EGF should induce more differentiated functions. Our preliminary data indicates that EGF stimulates cell proliferation [0.1-100 ng/ml], branching alveolar morphogenesis [1-100 ng/ml], and functional differentiation [1-100 ng/ml]. The effect of EGF on organoid invasiveness and metalloproteinase secretion have not yet been examined. Our data indicates that at low concentrations [0.1 ng/ml] mouse EGF (mEGF) appears to be a selective mitogen. Localized accumulation of these growth factors could play a significant role in influencing the type of response induced by either EGF or TGF. The promotional activities of EGF and TGF may also be different. Do these growth factors play different roles in stimulating breast cancer progression? It is interesting to speculate that there are differences in the magnitude of the responses induced by these growth factors in normal MEC compared to carcinogen-exposed and/or tumor-promoted MEC. Both of these growth factors have been shown to stimulate metalloproteinase secretion in rat mucosal keratinocytes (44). It is, therefore, possible that these growth factors elicit similar effect in rat MEC and/or breast cancer cells. Overexpression of TGF has already been described in 40-70% of primary and metastatic human breast cancers suggesting the importance of understanding the functions of the EGF-like peptides in normal and transformed MEC. Mammary gland stromal cells that naturally surround developing nomal MEC as well as breast cancer cells have been shown to express EGFR as well as TGF The roles that EGFR positive and/or TGF expressing mammary gland stromal cells play in the normal development of MEC, and the progression and/or metastasis of breast cancer cells are presently unclear. A comprehensive understanding of *all* of the regulators of MEC and breast cancer development is essential for the development of effective prevention of and curative therapies for breast cancer.

Since both of these growth factors induce their cellular responses through the EGFR, changes in EGFR expression, the number and affinity of binding sites for EGF and TGF, the regional localization of cells expressing EGFR mRNA and protein and/or the functional activity of the EGFR during branching end bud or alveolar morphogenesis could influence the type and/or magnitude of responses observed when the normal MEC are exposed to EGF or TGF. It is important to determine whether the response to EGF or TGF in rat MEC is determined by the location, and number of the EGFR, or by the binding affinity of the receptor. The binding activity, kinase activity, and internalization of the EGFR appear to be regulated by autophosphorylation on tyrosine residues, other protein kinases that phosphorylate the receptor on specific threonine/serine residues, and by tyrosine-specific phosphoprotein phosphates (92). Such tight regulation seems to suggest that EGFR expression, the number and affinity of EGF and TGF binding sites, the regional localization of cells expressing EGFR, and EGFR tyrosine kinase activity are hormonally and developmentally regulated. Characterization of such regulatory mechanisms would expand the present understanding of the role that growth factor receptor tyrosine kinases play in normal development, and might explain why these receptors appear to be important in the pathogenesis of many forms of cancer including breast cancer. Deregulation or upregulation

of EGFR levels, affinity and/or activity would be expected to provide the tumor cells with a ligand-dependent growth advantage. Overexpression of EGFR has been described in 50-60% of human breast cancers, and is usually a poor prognostic indicator.

D. METHODS OF APPROACH

Aim 1. To compare the biological activity of exogenous EGF and TGF on the *in vitro* development of normal MEC cultured within a complex RBM in the presence of defined serum-free medium. This aim will focus on evaluating the ability of both of these growth factors to modulate cell proliferation, differentiation, morphogenesis, and invasion with the goal of determining whether these two growth factors play distinct or similar roles in normal MEC morphogenesis.

Methodologies utilized thus far:

a. Isolation of the Mammary Epithelial Organoids (MEO) for the Primary Culture and Co-Culture Studies
b. Preparation of the Reconstituted Basement Membrane for Culturing the MEO
c. Primary Culture System for Culturing the Isolated MEO
d. 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) Assay for Quantifying Viable Cell Number
e. ^{3}H-Thymidine Incorporation Assay for Evaluating the Rate of DNA Synthesis
f. Enzyme Linked Immunosorbant Assay for Quantifying Casein Accumulation
g. Morphologic Classification of the Culture Mammary Colonies for Evaluating Colony Morphogenesis as well as MEO Branching Morphogenesis
h. Gelatin Zymogram Analysis
i. Isolation of Different Mammary Stromal Cells for the Co-Culture Studies
j. Co-Culture System for the Isolated MEO and Distinct Types of Mammary Stromal Cells (fibroblasts, pre-adipocytes and mature adipocyte)
k. Immunocytochemistry for the Characteristization of the Different Mammary Stromal Cells
l. Preparation of Whole Cell Lysates
m. SDS-Polyacrylamide Gel Electrophoresis
n. Electrophoretic Transfer of Proteins from an SDS-Polyacrylamide Gel to a Nitrocellulose or an Immobilon Membrane
o. Western Blot Analysis

Aim 2. To compare the biological activity of exogenous EGF and TGF in normal MEC undergoing either end bud or alveolar branching morphogenesis with that observed in NMU- and/or PDBu-treated MEC. The goal of this aim is to determine whether the type and/or the magnitude of the effects induced in normal cells are different than observed in transformed cells, and to evaluate whether either or both of these polypeptides enhances tumor progression in the absence as well as in the presence of the tumor promoter, PDBu.

Methodologies utilized thus far:

a. Intraperitoneal Injection of Carcinogen into Female Rats
b. Palpation and Measurements of Rat Mammary Tumors
c. Mammary Gland Whole Mount Preparation
d. Mammary Gland Whole Mount Staining and Photography
e. Process Mammary Gland Whole Mounts for Histological Analysis

Aim 3. To examine the temporal changes in the expression of EGFR mRNA, the number and affinity of EGF and TGF binding sites, and the regional localization of EGFR transcripts and

proteins within normal MEC undergoing either end bud or alveolar branching morphogenesis in primary culture in response to EGF or TGF with the goal of determining the relationship between the expression, number, affinity and/or regional localization of EGFR, and MEC proliferation, differentiation, morphogenesis, and/or invasion.

Methodologies utilized thus far:

a. Isolation of the Mammary Epithelial Organoids (MEO) for the Primary Culture and Co-Culture Studies
b. Preparation of the Reconstituted Basement Membrane for Culturing the MEO
c. Primary Culture System for Culturing the Isolated MEO
d. Formalin-Fixation and Paraffin-Embedding of the Cultured MEO, Excised Mammary Glands, mammary fibroblasts, and MDA-MB-468 human breast cancer cells
e. Immunocytochemsitry

Aim 4. To examine the temporal changes in the expression, number, affinity, and regional localization of the EGFR within NMU-treated, PDBu-treated, and NMU- plus PDBu-treated, and then compare those profiles with that observed within normal MEC undergoing either end bud or alveolar branching morphogenesis in response to EGF or TGF with the goal of determining the relationship between EGFR and breast cancer development and/or progression, and the difference(s) in the EGFR within normal compared to transformed MEC.

Studies not yet initiated.

Aim 5. To compare the functional consequence of EGF- and TGF activation of the EGFR within normal MEC undergoing end bud or alveolar branching morphogenesis, or within NMU- and/or PDBu-treated MEC with the objective of determining whether these two growth factors activate distinct signal transduction pathways during end bud morphogenesis, and alveolar morphogenesis, and whether EGFR signaling in normal MEC differs from that of transformed MEC.

Methodologies utilized:

a. Isolation of the Mammary Epithelial Organoids (MEO) for the Primary Culture and Co-Culture Studies
b. Preparation of the Reconstituted Basement Membrane for Culturing the MEO
c. Primary Culture System for Culturing the Isolated MEO
d. Isolation of Different Mammary Fibroblasts from Normal Mammary Glands and Carcinogen-Transformed Mammary Tumors for Cell Culture and Co-Culture Studies
e. Cell culture of Isolated Primary Mammary Fibroblasts from Normal And DMBA-
f. Co-Culture System for the Isolated MEO and mammary fibroblasts
g. Preparation of Whole Cell Lysates
h. SDS-Polyacrylamide Gel Electrophoresis
i. Electrophoretic Transfer of Proteins from an SDS-Polyacrylamide Gel to a Nitrocellulose or an Immobilon Membrane
j. Western Blot Analysis

BODY and CONCLUSIONS

A. AIM 1. COMPARATIVE ANALYSIS OF THE BIOLOGICAL EFFECTS OF EXOGENOUS EGF AND TGF ON THE *IN VITRO* DEVELOPMENT OF NORMAL MEC IN PRIMARY CULTURE.

1. OVERVIEW

A variety of experiments have been undertaken to examine and compare the biological effects of EGF and TGF on the proliferation, differentiation, morphogenesis, and invasion of normal rat MEC. It is hoped that this information will provide a deeper understanding of the roles that each of these growth factors play in regulating the normal developmental processes of branching end bud and alveolar morphogenesis. We also hope that this information will provide a solid baseline for comparative studies between normal MEC and breast cancer cells (that is carcinogen-transformed and/or tumor-promoted MEC). Thus far, EGF and TGF have been found to exert a wide variety of biological effects in the normal rat MEC and appear to regulate such distinct cellular processes as proliferation, morphological as well as functional differentiation, morphogenesis, invasion as well as apoptosis. Surprisingly, however, the type and magnitude of the effects induced by EGF and TGF have in large part been identical despite the preliminary results reported by other laboratories that suggest that the biological activities of these two growth factors are distinct. It should be emphasized that our studies are unique in that they are being carried out in normal pubescent rat MEC that are cultured under defined serum-free conditions that retain *in vivo* relevant cellular responsiveness to steroids, as well as polypeptide hormones, growth factors, cytokines and extracellular matrix components.

During the last year, our laboratory obtained a newly developed, potent and selective inhibitor of the tyrosine kinase domain of the EGFR from Parke-Davis. This inhibitor, referred to as PD158780, was used in several different types of primary culture and cell culture studies. This drug provided us with a powerful and effective way of specifically turning off EGFR *in vitro*, and as such represents a dramatic advancement for those studying EGFR biology and for those trying to regain control of cells that overexpress EGFR and/or its ligands. Thus far, PD158780 has allowed us to more accurately (1) identify the role(s) that endogeneous EGFR ligands play in regulating the *in vitro* development of MEC cultured in the absence of exogenously supplied EGF or TGF, and (2) define the specific role(s) that functional EGFR play during the first, second and third week of MEC development when these normal epithelial cells were cultured under conditions that promoted either branching alveolar morphogenesis or branching end bud morphogenesis. A majority of the results presented for this aim will focus on the specific role that EGFR and its ligands play in regulating the proliferation and functional differentiation of MEC. The effect of EGFR regulation on the morphogenesis, invasion and apoptosis of cultured MEC requires additional experimentation and will not be presented in this report.

It should also be noted that I worked with several other members of Dr. Margot Ip's laboratory during the last 18 months (1) to establish protocols to isolate either primary mammary fibroblasts or pre-adipocytes from the mammary glands of normal pubescent female rats, (2) to develop culture conditions that support the proliferation and survival of mammary fibroblasts, and then to induce mammary pre-adipocytes to proliferate and

differentiate into functionally mature mammary adipocytes *in vitro*, and (3) to carry out several experiments designed to evaluate the interaction(s) and biological consequence of co-culturing primary rat MEC with either primary rat mammary fibroblasts or primary rat mammary pre-adipocytes. It should be emphasized that these co-culture experiments were extremely large and cumbersome, but they yielded a tremendous amount of experimental data. In fact, we are still analyzing the results from our first few co-culture experiments and, therefore, will not present this data in the present report. During the next year, we will complete the analysis of the preliminary co-culture experiments and then carry out additional co-culture studies designed specifically to (1) further expand our understanding of the physiological role that functional EGFR play in regulating the *in vitro* development of mammary epithelial cells, fibroblasts, pre-adipocytes and mature adipocytes, and (2) characterize the effects as well as the mechanism of action of EGF and TGF in a reconstituted and defined microenvironment where normal MEC are either cultured alone or co-cultured with the distinct types of normal mammary stromal cells.

2. EXPERIMENTAL METHODS

a. BASIC EXPERIMENTAL DESIGN

(Ann Wohlhueter assisted in carrying out one of the primary culture experiments.)

Non functional rat MEC, isolated from pubescent female rats, were cultured for up to 21 days within the EHS-derived RBM in the presence of defined serum-free medium which supports either branching alveolar or end bud morphogenesis. Alveolar morphogenesis was studied when the MEC were cultured in the presence of alveolar media (ALV Medium) which is F12/DMEM medium supplemented with bovine insulin [10 µg/ml], ovine prolactin [1 µg/ml], progesterone [1 µg/ml], hydrocortisone [1 µg/ml], human apo-transferrin [5 µg/ml], ascorbic acid [880 ng/ml], fatty acid-free BSA [1 mg/ml], and gentamycin [50 µg/ml]. End bud morphogenesis was studied when the MEC were cultured in the presence of end bud media (EB Medium) which is ALV Medium without hydrocortisone.

Several types of primary culture studies were carried out to further expand our understanding of the role that EGFR and its ligands play in regulating the *in vitro* development of MEC. First, to determine whether EGF and TGF play similar or different roles in regulating the proliferation and functional differentiation of MEC *in vitro*, newly isolated normal primary rat MEC were cultured for up to 21 days in ALV Medium or in EB Medium either without human recombinant (hr) EGF or hrTGF (No GF), with 10 ng/ml hrEGF alone, or with 10 ng/ml hrTGF alone. Second, to identify the role(s) that endogenous EGFR ligands play in regulating the *in vitro* development of MEC cultured in the absence of exogenously supplied EGF or TGF, newly isolated MEC were cultured in the presence of ALV Medium without either hrEGF or hrTGF, but with 0, 0.5, 0.5 or 5 µM of the EGFR inhibitor PD158780 from day 0 through day 21 of the study. Third, to define the specific role(s) that functional EGFR play during the first, second and third week of MEC development, newly isolated MEC cultured in the presence of either ALV Medium or EB Medium were exposed to 0 or 0.5 µM PD158780 from day 3.5 through day 7, from day 10.5 through day 14, or from day 17.5 through day 21, respectively. In each of these experiments, viable cell number was monitored using a tetrazolium dye assay, whereas MEC-functional differentiation was assessed using a Western blot procedure to monitor

casein isoform protein expression or using an enzyme linked immunoabsorbant assay to quantitate total casein protein accumulation.

b. TETRAZOLIUM DYE ASSAY TO QUANTIFY VIABLE CELL NUMBER

Specific changes in the number of viable cells was evaluated using a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay (87,93). Briefly, cultures were incubated with MTT at a final concentration of 1 mg/ml for 16 hr at 37°C. The RBM was digested away from the MEC using 5 units/ml grade II dispase in F12/DMEM medium. The aqueous-insoluble formazan crystals were collected, washed and solubilized in 2-propanol. Sample absorbance was read with a Bio-tek EL-311 automatic plate reader at 570 nm. Production of fromazan crystals and the absorbance of the solubilized crystals was directly proportional to viable cell number. A standard curve was set up with the newly isolated MEC for each experiment.

c. WESTERN BLOT PROCEDURE TO MONITOR CASEIN PROTEIN EXPRESSION

(Ann Wohlhueter assisted in carrying out the casein Westerns.)

Casein protein expression was examined in whole cell lysate samples harvested from triplicate culture wells per treatment type for each of the different time points. Each whole cell lysate sample was prepared after the culture medium within each well was aspirated away and the MEC embedded within the RBM solubilized within a 1% triton X-100 tyrosine phosphorylation lysis buffer [0.1 % (w/v) sodium dodecylsulfate (SDS), 0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 50 mM Tris pH 8.0 at 4°C, 2 mM ethylenedinitrilotetraacetic acid, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM sodium orthovandate, 0.1 mM phenylmethyl-sulfonyl fluoride [PMSF],100 ng/ml soybean trypsin inhibitor, and 20 ng/ml leupeptin]. Ice-cold lysates were then sonicated using 3x10 sec bursts of a Tekmar sonic distruptor set at 80% output, and then centrifugated for 15 minutes at ~12,000 x *g*. Lysate supernatants were then mixed with a reducing and denaturing sample buffer and then samples representing an appropriate number of cells separated on a 4-20% SDS-polyacrylamide gradient gel. Proteins were then electrophoretically transferred for 2 hours to an Immobilon P membrane. Membranes were then (1) blocked overnight at 4°C in BLOTTO, (2) washed several times with phosphate buffered saline [PBS] at pH 7.3 and containing 0.1% (v/v) Tween 20, (3) incubated for 90 minutes at room temperature with a 1:1 mixture of the rabbit anti-rat casein polyclonal 877 and 878 antibodies diluted 1:2000 in PBS with 0.05% (v/v) Tween 20 and 3% (w/v) bovine serum albumin, (4) washed several time with PBS containing 0.1% (v/v) Tween 20, (5) incubated for 60 minutes at room temperature with a 1:5000 dilution of an affinity purified donkey anti-rabbit IgG antibody that was preabsorbed against rat, human, and mouse serum proteins and conjugated to horseradish peroxidase [Jackson Immunoresearch Laboratories Inc.], and (6) washed several time with PBS containing 0.1% (v/v) Tween 20. Immunoreactive proteins were visualized using an enhanced chemoluminescent reagent [Amersham], and X-ray films exposed in a dark room were developed using an automatic developer.

d. ENZYME LINKED IMMUNOSORBANT ASSAY TO QUANTIFY CASEIN ACCUMULATION, A MARKER OF MEC FUNCTIONAL DIFFERENTIATION

(Casein ELISA were run by Suzanne Shoemaker)

Casein accumulation, used as an indicator of MEC functional differentiation, was monitored using a previously described non-competitive enzyme linked immunosorbant assay (ELISA) with a rabbit anti-rat casein polyclonal antibody (88,93). Casein levels were quantified in triplicate culture wells per treatment type for each of the different time points. For each culture well, conditioned medium was dirscarded and then organoid plus RBM samples harvested with 600 µl or 1 ml of a 1% (v/v) triton X-100 lysis buffer [150 mM sodium chloride, 50 mM Tris, 2 mM ethylenedinitrilotetraacetic acid, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 0.1% (w/v) sodium dodecylsulfate, 0.5% (w/v) sodium deoxycholate, 1% (v/v) nonidate P-40, 0.1 mM PMSF, 100 ng/ml soybean trypsin inhibitor and 20 ng/ml leupeptin]. The harvested organoids plus RBM samples were sonicated on ice for 15 seconds using a Tekmar Sonic Disruptor (Cincinnati, OH). The samples were then centrifuged at 12,000 x *g* for 15 min at 4°C. The resulting supernatants were stored at -20°C. The total level of casein within each culture sample was measured using the casein ELISA with a phosphate-buffered saline (pH 7.4) buffer system. Each individual culture sample was assayed in duplicate or triplicate at four to six dilutions. Nunc immunosorbant plates were processed using a Bio-tek automatic plate washer, and sample absorbance analyzed at 405 nm using a Bio-tek EL-311 automatic plate reader.

3. ***RESULTS and CONCLUSIONS***

a. EFFECTS OF EGFR REGULATION ON THE PROLIFERATION OF MEC

To compare and characterize the mitogenic response of hrEGF and hrTGF in MEC, newly isolated MEC were cultured within the RBM in the presence of either ALV Medium or EB Medium [EB Medium = ALV Medium without hydrocortisone] alone, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF. Modest cell proliferation was observed when MEC were cultured in the continuous presence of either ALV Medium or EB Medium without either growth factor [Figure 1]. Both hrTGF and hrEGF were able to induce extensive proliferation throughout the 21 day culture period in the presence of ALV Medium or EB Medium [Figure 1]. The magnitude of the mitogenic response to each of these EGF receptor ligands was, however, equivalent [Figure 1]. Interestingly, the mitogenic response induced by both of these growth factors was 2-fold higher in ALV Medium compared to EB Medium. It is worth noting that colony survival in this experiment was not different between the distinct culture groups. The significance of this 2-fold difference will have to be re-evaluated in another study.

To evaluate the role that endogenous EGF receptor ligands play in regulating the proliferation of primary rat MEC in vitro, newly isolated MEC were cultured for up to 21 days in the presence of ALV Medium with 0, 0.05 0.5 or 5 µM PD158780. MEC cultured in the presence ALV Medium supplemented with either 10 ng/ml hrEGF or 10 ng/ml hrTGF represent the positive controls for this experiment. Figure 2 illustrates the extensive proliferation induced in the presence of an exogenous supply of either of these growth factors. In this experiment, moderate cell proliferation was observed during the second and the third week of this study when MEC were cultured in the presence of ALV Medium alone [Figure 2]. PD158780 at all three concentrations tested was able to inhibit cellular proliferation when provided continuously from day 0 through day 21 of the experiment. The 0.5 and the 5 µM concentrations were, however, slightly more effective than the 0.05 µM

concentration [Figure 2]. Taken together, these data suggest that endogenous EGFR ligands are responsible, at least in part, for mediating the moderate cell growth observed when the MEC were cultured in the absence of an exogenous supply of EGF or TGF. It should be noted that at 5 μM, PD158780 was able to cause a ~ 40% reduction in colony survival compared to that observed in the absence of any PD158780 or in the presence of PD158780 at 0.05 or 0.5 μM. As such, PD158780 at 0.5 μM appears to be an optimal, non cytotoxic concentration for inhibiting the growth of MEC in our primary MEC culture system.

To evaluate the role that EGF receptors play in mediating the mitogenic responses observed during the first, second and third week of culture development, MEC (cultured in the continuous presence of either ALV Medium or EB Medium alone, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF) were exposed to 0.5 μM of the EGFR inhibitor PD158780 from day 3.5-7, day 10.5-14 or day 17.5-21 of culture development, respectively. Cell growth by day 7 of the study was inhibited by 0.5 μM PD158780 in the presence of hrEGF or hrTGF [Figure 3]. Surprisingly, 0.5 μM PD158780 did not exert any significant effect on cell growth by day 14 of the study when the MEC were cultured in any one of the six types of medium [Figure 4]. Intriguingly, 0.5 μM PD158780 decreased viable cell number by day 21 of culture development when MEC were cultured in the presence of ALV Medium with 10 ng/ml hrTGF, but not in ALV Medium with 10 ng/ml hrEGF [Figure 5]. In contrast, PD158780 did not exert any effect on cell growth by day 21 of the experiment when MEC were cultured in the presence of EB Medium.

This study revealed that EGF receptor activity was required for the growth of MEC from days 3.5 to day 7 [Figure 3], but not from days 10.5 to 14 of culture development [Figure 4], and suggested that by day 21 that EGFR activition may provide a survival signal for MEC cultured in the presence of ALV Medium with 10 ng/ml hrTGF [Figure 5]. Additional follow up studies are required to characterize the mechanism by which EGFR activation from day 3.5 through day 7 induces the proliferation of MEC cultured in the presence of ALV Medium or EB Medium, and to determine whether EGFR activation from day 17.5 through day 21 provides a survival signal for MEC cultured in the presence of ALV Medium with 10 ng/ml hrTGF, but not with 10 ng/ml hrEGF. It will also be of interest to determine whether MEC cultured from day 3.5 through day 7, as well as from day 17.5 through day 21, but not from day 10.5 through day 14 express functional EGFR on their cell surface. If EGFR are down regulated or functionally inhibited from day 10.5 through day 14, then it will be important to define the factors and the mechanisms by which these regulators down regulate and/or functionally inhibit the EGFR during the second week of culture development and conversely upregulate and/or relieve the repression of the EGFR during the third week of culture development.

2. EFFECTS OF EGFR REGULATION ON THE FUNCTIONAL DIFFERENTIATION OF MEC

To examine the role that EGFR play in inducing the functional differentiation of MEC derived from pubescent virgin female rats, primary MEC were cultured for up to 21 days within the RBM in the continuous presence of ALV Medium alone, ALV Medium with 10 ng/ml hrEGF or ALV Medium with 10 ng/ml hrTGF. Total casein levels in day 7, 14 and 21 lysates were quantitated using a casein ELISA. Figures 6-8 demonstrate that MEC cultured in the presence of ALV Medium with an exogenous supply of either 10 ng/ml hrEGF or 10 ng/ml

hrTGF for 7, 14 or 21 days accumulated significantly more casein than observed when the MEC were cultured in ALV Medium alone.

Primary MEC were also cultured for up to 21 days in the ALV Medium lacking EGF or TGF, but with the continuous presence of 0, 0.05, 0.5 or 5 μM PD158780 to evaluate the role that *endogenous* EGFR ligands play in regulating the functional differentiation of MEC. Again total casein levels were quantitated using a casein ELISA procedure. MEC cultured with PD158780 at all three concentrations accumulated significantly less casein by day 7 of culture development when compared to the levels observed when MEC were cultured in ALV Medium alone [Figure 6]. In fact, PD158780 at 0.5 and 5 μM were maximally effective and appeared to completely inhibit casein expression and/or accumulation by day 7 of the study. By day 14 of the study, however, only MEC cultured in the presence of ALV Medium with 5 μM PD158780 accumulated less casein than the controls cultured in the presence of ALV alone [Figure 7]. Interestingly, MEC cultured in ALV Medium alone for 21 days accumulated rather high levels of casein by day 21 of the experiment, whereas MEC cultured with 0.5 and 5 μM PD158780 produced and/or accumulated moderate levels of casein by day 21 of culture development [Figure 8].

Taken together, these data suggest that EGFR activation from day 0 through day 7 of culture development is required for the expression and/or accumulation of casein milk proteins by day 7 of culture development. More frequent time points using shorter exposure periods will help define the exact requirement of EGFR activation for the induction of MEC functional differentiation as indicated by the production and accumulation of casein. In addition, with increasing time in the continuous presence of PD158780, MEC appear to compensate for the shutdown of the EGFR by as yet undefined factor(s) that allow the accumulation of casein by day 14 of culture development. This latter observation indirectly suggests that EGFR activation during the second week in culture is not absolutely required for casein expression and/or accumulation. Furthermore, EGFR activation may again play a lactogenic (and/or lactogenic competence) type of role during the third week of culture development, since MEC cultured in the continuous presence of ALV Medium with either hrEGF or hrTGF produced and/or accumulated more casein on a per cell basis by day 21 of culture development than by day 14 whereas casein levels in these two culture groups did not change very much between the day 7 and day 14 time points. Moreover, MEC cultured in the continuous presence of ALV Medium with 0.5 or 5 μM PD158780 accumulated less casein by day 21 than observed for MEC cultured in ALV Medium alone. This latter observation suggests that endogenous EGFR ligands may play a role in inducing casein accumulation during the third week of culture development. Additional experiments are required to determine whether endogenous EGFR ligands are exerting a direct or indirect effect on casein accumulation. A direct mechanism may involve the activation a STAT transcription factor known to specifically turn on beta-casein gene expression, or the stimulation of extracellular matrix components known to activate transcription factors that upregulate beta-casein gene expression. Alternatively, an indirect mechanism may involve the inhibition of proteases either by down regulation of protease expression, secretion or activation, or by upregulation of a protease inhibitor. Changes in EGFR expression and/or function in the cultured MEC may also explain why EGFR activation does not appear to be required during the second week of culture development.

To examine the specific role that EGFR and its ligands play in regulating the functional differentiation of MEC and specifically the expression of the distinct casein milk protein isoforms, primary MEC were cultured in the presence of ALV Medium or EB Medium either alone, with 10 ng/ml hrEGF or with 10 ng/ml hrTGF for either 7, 14 or 21 days. The cultured MEC were then exposed to 0 or 0.5 μM PD158780 from day 3.5 through day 7, from day 10.5 through day 14, or from day 17.5 through day 21 of the study. A casein Western blot procedure was used to monitor casein protein isoform expression in day 7, 14 or 21 lysates. Casein Western analysis of day 7 [Figure 9], 14 [Figure 10] and 21 [Figure 11] lysates revealed that hrEGF or hrTGF was required for MEC cultured in the presence of ALV Medium to produce and/or accumulate all forms of the casein milk protein family [alpha 1, alpha 2, kappa, beta and gamma caseins]. In contrast, MEC cultured in EB Medium accumulated very low levels of casein [Figure 12 and casein ELISA data not shown]. Furthermore, MEC cultured in EB Medium with either hrEGF or hrTGF expressed low levels of alpha 1, alpha 2 and kappa caseins [Figure 12]. Surprisingly, however, MEC cultured in the presence of EB Medium without either growth factor for 14 days [Figure 12] or 21 days [data not shown] expressed significant levels of alpha 1, alpha 2, kappa and gamma caseins, and accumulated significantly more casein than observed when MEC were cultured in the presence of EB Medium supplemented with either hrEGF or hrTGF. This observation was completely unexpected and suggests that when MEC were cultured in the absence of hydrocortisone that EGF or TGF inhibited casein expression and/or accumulation. Additional studies will be required to understand how EGF and TGF can act as a lactogen (or at least a competence lactogen) in the presence of hydrocortisone and an anti-lactogen in the absence of hydrocortisone.

PD158780 exposure at 0.5 μM from day 3.5 through day 7 of culture development inhibited the EGF receptor-mediated expression of casein by day 7 of the experiment when the MEC were cultured in ALV Medium with or without EGF or TGF [Figure 9]. By day 14 of culture development, PD158780 had no effect on casein expression when the MEC were cultured in ALV Medium either alone, with 10 ng/ml hrEGF or with 10 ng/ml hrTGF [Figure 10]. The effect of EGF receptor regulation on casein accumulation by day 21 of culture development was rather complex, and will no doubt require more detailed analysis of these samples. Briefly, PD158780 at 0.5 μM PD158780 exposure from day 17.5 through day 21 did not affect the casein expression profile when MEC were cultured in ALV Medium without either growth factor. In contrast, PD158780 at 0.5 μM PD158780 exposure from day 17.5 through day 21 did reduce the expression and/or accumulation of alpha 1, kappa and beta caseins and increase the expression of alpha 2 and gamma casein when MEC were cultured in ALV Medium with either 10 ng/ml hrEGF or 10 ng/ml hrTGF [Figure 11]. Taken together these data suggest that EGFR activation from day 3.5 through day 7 and also from day 17.5 through day 21, but not from day 10.5 through day 14 play significant and rather complex roles in regulating casein milk protein expression and/or accumulation in cultured primary rat MEC. Additional experiments are required to define then mechanisms by which EGFR ligands stimulate or inhibit casein expression and/or accumulation, depending on the composition of the culture medium and the development state of the cells. It should be emphasized that thus far, hrEGF and hrTGF exhibit equivalent effects on MEC functional differentiation.

B. AIM 2. COMPARATIVE ANALYSIS OF THE TYPE AND THE MAGNITUDE OF THE EFFECTS INDUCED BY EXOGENOUS EGF AND TGF IN NORMAL MEC, AND IN DIFFERENT TYPES OF CARCINOGEN-EXPOSED MEC IN PRIMARY CULTURE

(Margot Ip, Suzanne Shoemaker, Nannette Stangle and Mary Vaughn assisted in carrying out this experiment.)

1. OVERVIEW

During the last 6 months, considerable effort has been exerted by myself and three other members of Dr. Margot Ip's laboratory to establish a carcinogen-induced rat mammary tumor model recently developed in the laboratory of Dr. Henry Thompson (94). This model system is unique in that it allows rapid induction (5-10 weeks post-carcinogen exposure, rather than 6-9 months) of mammary intraductal hyperplasia, ductal and lobular carcinoma *in situ*, as well as adenocarcinomas following the injection of pre-pubescent female rats with 1-methyl-1-nitrosourea. Thus far my efforts have been exerted on learning how (1) to inject the carcinogen intraperitoneally, (2) to palpate and caliper mammary tumors, (3) to excise, process and stain mammary gland whole mounts, and (4) to prepare and embed mammary gland whole mounts for histological characterization and immunohistological analysis. Dr. Thompson and different members of his laboratory were extremely helpful in providing us with detailed protocols and technical advice, and as such, we were extremely successful in setting up Dr. Thompson's model system. We are now ready to set up primary culture studies with mammary epithelial organoids isolated from mammary glands excised from 50-60 day old female rats injected with a single intraperitonealy injection of 50 mg/kg 1-methyl-1-nitrosourea when the rats were 21-23 days of age.

2. EXPERIMENTAL METHODS

Female Sprague-Dawley rats [Charles River Laboratories], 23 days of age, were given a single intraperitoneal injection of 50 mg/kg 1-methyl-1-nitrosourea prepared in a 0.9% (w/v) sodium chloride acidified to pH 4.0 with acetic acid. Rats were palpated for mammary tumor at various time points following carcinogen exposure. Rats were sacrificed with carbon dioxide gas at various times post-carcinogen injection with no pain or distress to the animal. Intact cervical-thoracic (mammary glands 1, 2 and 3) and abdominal-inguinal mammary glands (mammary glands 4, 5 and 6) were surgically excised from each side of each rat.

The orientation of the intact mammary glands was maintained by making a sandwich of formalin-soaked Nitex mesh, lens paper, mammary gland, lens paper, and Nitex mesh. The mammary gland sandwich was then inserted into a formalin-soaked lens paper tea bag [MiniMinet Products, Ltd.] along with a embedding label. The individual tea bags were then closed off with a paper clip. Briefly, mammary gland whole mounts were (1) fixed in 10% (v/v) buffered formalin for at least 24 hours, (2) rinsed in *dd*H_2O for 15 minutes, (3) dehydrated for 1 hour each in 70% ethanol, 95% ethanol, 100% ethanol, and then two changes of toluene, (4) rehydrated for 20 minutes each in 100% ethanol, 95% ethanol, 70% ethanol, and then two changes of *dd*H_2O, (5) stained for 1 to 3 days in alum carmine, (6) destained in several changes of *dd*H_2O, (7) photographed using an Olympus SZH microscope and an Olympus OM-4T camera, (8) returned to 10% buffered formalin for 24 hours, (9) dehydrated in a graded alcohol series and then into two changes of toluene, (10) embedded into molten paraffin, (11) 4-5 µm sections cut and placed onto silonated glass

slides, (12) sections stained with hematoxylin and eosin, and (13) histological features of the sections analyzed using an Olympus BH-2 microscope.

3. **RESULTS**

Twenty 23-day-old female Sprague-Dawley rats were given a single intraperitoneal injection of 50 mg/kg 1-methyl-1-nitrosourea. No palpable tumors were detected in 50-60 day old rats (27-37 days post-carcinogen injection). Papable tumors were detected within 43-days post-carcinogen injection (at this time the rats were only 66 days of age). Tumor incidence reached 100% by 50-days post-carcinogen injection (the rats were 73-days old). Most rats developed palpable tumors in at least 4 out of the 12 individual mammary glands within individual rats, and two developed palpable tumors in 6 out of the 12 individual mammary glands within these rats. Both small (< 1 cm) and large (> 1 cm) tumor were identified. The tumor nodules appear as distinct individual masses as well as multiple tumor foci. Mammary glands were excised from the rats 8, 10, 20, and 25 weeks post-carcinogen injection. The sandwich-tea bag processing method was developed to retain tissue orientation throughout the whole mount procedure, to optimize the fixation, dehydration, hydration, and staining steps of Dr. Thompson's procedure, and finally to eliminate the use of the neurotoxic embedded medium (concentrated methyl salicylate) required by Thompson to photograph their alum carmine-stained mammary gland whole mounts. Figures 13-15 illustrate the morphological appearance of different alum carmine-stained mammary gland whole mounts. Note the large mammary tumor indicated by the arrow in Figure 15. Histological examination of the mammary gland whole mounts revealed that intraductal hyperplasias, ductal and lobular carcinoma *in situ*, as well as adenocarcinomas developed in the mammary glands of female rats that we exposed to a single dose of 1-methyl-1-nitrosourea.

4. CONCLUSIONS

It should be emphasized that the work carried out to establish this mammary transformation model system although fundamental in nature was extremely time-consuming and required methodological modifications to adopt Thompson's model to meet our specific needs. This unique *in vivo* rat mammary transformation model system can now be interfaced with our existing primary culture model. Relevant early stage carcinogen-transformed MEC, isolated from 50-60 day old carcinogen-exposed Sprague-Dawley rats, can now be cultured within the RBM in the presence of defined serum-free culture medium. During the next year, carcinogen-transformed MEC will be used in primary culture studies to examine the biological activity of exogenous EGF and TGF, and to determine whether the type and/or the magnitude of the effects induced in transformed cells are different than the effects observed in the normal mammary epithelial cells. Finally we hope to evaluate whether either or both of these polypeptides enhances tumor progression in the absence as well as in the presence of the tumor promoter, phorbol dibutyrate [PDBu].

C. AIM 3. THE TEMPORAL CHANGE IN THE EGFR WITHIN NORMAL MEC INDUCED TO UNDERGO EITHER BRANCHING END BUD OR ALVEOLAR MORPHOGENESIS IN RESPONSE TO EITHER EGF OR TGF.

(Mary Vaughn and Ann Wohlhueter assisted in carrying out these experiments.)

These studies were designed to examine the temporal changes in the expression of EGFR proteins within normal MEC undergoing either end bud or alveolar branching morphogenesis in primary culture in response to EGF or TGF-α. It is hoped that this information will help characterize the relationship between EGFR expression and MEC proliferation, differentiation, morphogenesis, and/or invasion. Thus far, my studies have focused on adapting detection methodologies. I have continued to focus my attention on adopting immunocytochemistry protocols for the detection of EGFR. Very weak membrane reactivity of the sheep anti-human EGFR antibody was detected within MEO cultured in the presence of ALV Medium or EB Medium with 10 ng/ml mEGF using DAB as the chromogen. Strong membrane reactivity was detected in human breast cancer cells MDA-MB-468 using the sheep anti-human EGFR antibody. Various sandwich, microwave archival, and enzyme-pretreatment techniques have been investigated to try to amplify the weak and often non-specific EGFR signal in sections of cultured MEO and in sections of mammary glands from normal female rats at different stages of physiological development (pubescence, sexual maturation, mid-pregnancy, (early-, mid- and late-) lactation as well as post-lactional involution using two of the currently available sheep anti-human EGFR antibodies. These studies have all yielded disappointing results. A new (as of June 1996) "supposed" rat-reactive anti-human EGFR antibody, suitable for immunocytochemistry, just became available from Calbiochem. The reactivity of this antibody will be tested in the various cells and tissues identified above.

Due to the disappointing results obtained from the immunohistochemistry studies, experiments were also carried out to establish a Western blot procedure to detect cellular EGFR using a sheep anti-human EGFR polyclonal antibody. After about 9 months of effort, conditions were finally established to specifically detect a 170 kDa EGFR in as little as 0.5 µg/lane of a A431 cell lysate [Figure 16], 0.5 µg/lane of an MDA-MB-468 cell lysate [data not shown], 2.5 µg/lane of a rat liver lysate [Figure 16], in 10 µg/lane of a primary rat mammary fibroblasts lysate [Figure 16 and Figure 17], and in 110-130 µg/lane of a mammary epithelial cell lysate prepared from a day-10 alveolar as well as a end bud primary culture [Figure 17].

Successful Western blot detection of the 170 kDa EGFR in rat whole cell lysates required the use of a different lysis buffer and the development of a completely new Western blot procedure than recommended by Upstate Biotechnology Inc., the supplier of the rat-reactive sheep anti-human EGFR polyclonal antibody. Whole cell lystates were prepared in a 1% (v/v) triton X-100 rather than a 1% (v/v) nonidate P-40 tyrosine phosphorylation lysis buffer [150 mM sodium chloride, 50 mM Tris, 2 mM ethylenedinitrilotetraacetic acid, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 0.1% (w/v) sodium dodecylsulfate, 0.5% (w/v) sodium deoxycholate, 0.1 mM PMSF, 100 ng/ml soybean trypsin inhibitor and 20 ng/ml leupeptin]. Lysate samples were then sonicated and microfuged at 12,000 x *g*. Lysate supernatants were then mixed with a reducing and denaturing sample buffer rather than simply a denaturing sample buffer. Samples representing an appropriate protein concentration were separated on a 4-20% SDS-polyacrylamide gradient gel, and then electrophoretically transferred for 2 hours to an Immobilon P membrane rather than a nitrocellulose membrane. Membranes were then (1) blocked overnight at 4°C in 5% (w/v) non fat dry milk protein in PBS containing 0.1% (v/v) Tween 20 rather than in 3% (w/v) bovine serum albumin in PBS containing 0.05% (v/v)

Tween 20, (2) washed several times with PBS, pH 7.3, containing 0.1% (v/v) Tween 20 rather than with PBS containing 0.05 % (v/v) Tween 20, (3) incubated for 90 minutes at room temperature with a 1:1000 rather than a 1:2000 dilution of sheep anti-human EGFR polyclonal antibody [Upstate biotechnology Inc.] prepared in PBS with 0.05% (v/v) Tween 20 and 3% (w/v) bovine serum albumin, (4) washed several times with PBS containing 0.1% (v/v) Tween 20 rather than with PBS containing 0.05% (v/v) Tween 20, (5) incubated for 60 minutes at room temperature with a 1:5000 rather than a 1:20,000 dilution of an affinity purified donkey anti-sheep IgG antibody that was preabsorbed against rat, human, sheep and mouse serum proteins and conjugated to horseradish peroxidase [Jackson Immunoresearch Laboratories, Inc.], and (6) washed several times with PBS containing 0.1% (v/v) Tween 20. Immunoreactive proteins were visualized using an enhanced chemoluminescent reagent [Amersham], and then X-ray films exposed in a dark room were developed using an automatic developer.

D. AIM 4. COMPARATIVE ANALYSIS OF THE DISTRIBUTION OF EGFR WITHIN NORMAL AND VARIOUS TYPES OF TRANSFORMED MEC CULTURED WITHIN A COMPLEX RBM IN THE PRESENCE OF DEFINED SERUM-FREE CULTURE MEDIUM.

These studies are designed to examine the temporal changes in the expression, number, affinity, and regional localization of the EGFR within carcinogen-transformed MEC, normal MEC treated *in vitro* with PDBu, and carcinogen-transformed MEC treated *in vitro* with PDBu, and then to compare these profiles with those observed within normal MEC undergoing either end bud or alveolar branching morphogenesis in response to EGF or TGF with the goal of determining the relationship between EGFR and breast cancer development and/or progression, and the difference(s) in the EGFR within normal compared to transformed MEC. Thus far, these studies haven't been initiated due to the technical difficulties associated with aims 2 and 3.

E. AIM 5. TEMPORAL ANALYSIS OF THE SIGNAL TRANSDUCTION PROTEINS THAT ARE TYROSINE PHOSPHORYLATED WHEN NORMAL MEC, VARIOUS TYPES OF MAMMARY STROMAL CELLS, AND CARCINOGEN-TRANSFORMED MEC ARE CULTURED IN THE PRESENCE OF EITHER EGF OR TGF.

1. OVERVIEW

These studies are designed to compare the functional consequence of EGF- and TGF-induced activation of the EGFR within (1) normal MEC undergoing end bud or alveolar branching morphogenesis, (2) various types of primary mammary stromal cells, as well as (3) carcinogen-transformed MEC treated *in vitro* in the absence or in the presence of the tumo-promoter PDBu. The objective of these studies is to determine whether these two growth factors activate distinct signal transduction pathways during end bud morphogenesis and alveolar morphogenesis, and to determine whether EGFR signaling in normal MEC differs from that of the different primary mammary stromal cells and/or carcinogen-transformed MEC. Considerable effort was initially focussed on adapting established methodologies to

detect EGFR and the proteins that are tyrosine phosphorylated in response to EGFR activation.

2. EXPERIMENTAL METHODS

a. EGFR ACTIVATION IN SERUM-STARVED RAT MAMMARY FIBROBLASTS

(Ann Wohlhueter assisted in carrying out this experiment.)

Primary mammary fibroblasts (MFC) were cultured as a monolayer in 100 mm tissue culture plastic dishes in the presence of phenol red-free F12/DMEM medium with 10 % (v/v) fetal bovine serum (FBS) and 50 µg/ml gentamicin for 2 days, and then switched to F12/DMEM medium with 0.5 % (v/v) FBS and 50 µg/ml gentamicin for two consecutive 2 day periods. After the serum-starvation period, the MFC were washed twice with plain F12/DMEM medium and then exposed at 37°C to F12/DMEM medium with 1 mg/ml fatty acid-free bovine serum albumin supplemented with 0, 0.1, 1or 10 ng/ml of either hrEGF or hrTGF for 5, 15, 60 or 120 minutes. Treatment medium was then removed, monolayers rinsed twice with ice-cold PBS, and then lysates prepared at 4°C as described below with a 1 % (v/v) triton X-100 tyrosine phosphorylation lysis buffer which contains a cocktail of phosphatase and protease inhibitors. Lystates were separated on 4-20% gradient SDS-polyacrylamide gels, proteins transferred to Immobilon P membranes and then membranes sequentially immunoblotted with an anti-phosphotyrosine antibody (RC20-HRP), anti-phosphoMAPK, and anti-p44/42 MAPK antibodies as described below.

b. WHOLE CELL LYSATE PREPARATION

(Ann Wohlhueter assisted in preparing these samples.)

Whole cell lysates were prepared in a 1% (v/v) triton X-100 tyrosine phosphorylation lysis buffer [150 mM sodium chloride, 50 mM Tris, 2 mM ethylenedinitrilotetraacetic acid, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 0.1% (w/v) sodium dodecylsulfate, 0.5% (w/v) sodium deoxycholate, 0.1 mM PMSF, 100 ng/ml soybean trypsin inhibitor and 20 ng/ml leupeptin]. Lysate samples were sonicated and microfuged at 12,000 x *g*, and then lysate supernatants were mixed with a reducing and denaturing sample.

c. GEL ELECTROPHORESIS AND WESTERN BLOT PROCEDURES

(Ann Wohlhueter assisted in running these gels.)

Samples representing 20 µg of protein per lane were separated on 4-20% SDS-polyacrylamide gradient gels, and then electrophoretically transferred for 2 hours to Immobilon P membranes. Membranes were then (1) blocked overnight at 4°C in 5% (w/v) non fat dry milk protein in PBS containing 0.1% (v/v) Tween 20, (2) washed several times with PBS, pH 7.3, containing 0.1% (v/v) Tween 20, (3) incubated for 90 minutes at room temperature with a 1:1000 dilution of sheep anti-human EGFR polyclonal antibody [Upstate Biotechnology Inc.] prepared in PBS with 0.05% (v/v) Tween 20 and 3% (w/v) bovine serum albumin, (4) washed several times with PBS containing 0.1% (v/v) Tween 20, (5) incubated for 60 minutes at room temperature with a 1:5000 dilution of an affinity purified donkey anti-sheep IgG antibody that was preabsorbed against rat, human, sheep and mouse serum proteins and conjugated to horseradish peroxidase [Jackson Immunoresearch Laboratories

Inc.], and (6) washed several times with PBS containing 0.1% (v/v) Tween 20. Immunoreactive proteins were then visualized using an enhanced chemoluminescent reagent [Amersham], and X-ray films exposed in a dark room were developed using an automatic developer. Membranes were then stripped with 2% (w/v) SDS and 100 mM 2-mercaptoethanol in 62.5 mM Tris, pH 6.8 at 50°C for 30 minutes, washed several times with PBS containing 0.1% (v/v) Tween 20. Blocked membranes were then incubated with a 1:2,500 dilution of a horseradish peroxidase conjugated recombinant anti-phosphotyrosine antibody, termed HRP-RC20 [Transductions Laboratories Inc.]. Immunoreactive proteins were then visualized using an enhanced chemoluminescent reagent, and X-ray films exposed in a dark room were developed using an automatic developer. Finally, the membranes were stripped again and then a mitogen activated protein kinase (MAPK) Western Blot Detection kit from New England BioLabs was used to detect total (p42/44) as well as phosphorylated MAPK on the Immobilon P membranes. The kit contained two distinct primary rabbit polyclonal antibodies, a secondary anti-rabbit antibody conjugated to alkaline phosphatase and the CPD-Star reagent to detect immunoreactive proteins on X-ray film.

3. **RESULTS**

Even though EGFR detection in cultured rat MEC proved to be quite a challenge, we were able to detect EGFR and tyrosine phosphorylated proteins in various cells and tissues. Specifically, a sheep anti-human EGFR polyclonal antibody was used to detect a 170 kDa protein in whole cell lysates from EGF-induced human epidermoid A431 cells [0.5 µg/lane, Figure 16], human breast cancer cells MDA-MB-468 [0.5 µg/lane, data not shown] and rat livers [2.5-80 µg/lane, Figure 16]. This anti-EGFR antibody was also able to detect a 170 kDa protein within as little as 10 µg of a lysate from primary rat mammary fibroblasts [Figure 16 and Figure 17], but it took as much as 110-130 µg of lystates prepared from purified MEC cultured for 10 days under conditions that supported either end bud or alveolar branching morphogenesis to obtain an immunoreative band at 170 kDa [Figure 17]. Taken together, these studies suggest that the cultured MEC express extremely low levels of EGFR whereas high levels of EGFR are expressed in normal rat livers, rat mammary fibroblasts, as well as in human cell lines known to overexpress EGFR on their cell surface. A recombinant anti-phosphotyrosine antibody was able to detect a number of the classically described tyrosine phosphorylated proteins in A431 cells and in primary rat mammary fibroblasts. For example, a 170 kDa protein that co-migrates with the EGFR and a 125 kDa protein that co-migrates with focal adhesion kinase [FAK] were phosphorylated in response to EGF treatment. In addition, a MAPK detection kit was used to demonstrate that MAPK is tyrosine phosphorylated in primary rat mammary fibroblasts in response to EGF. The apparent low level of EGFR in the cultured MEC helps explain why we have had so much trouble detecting rapid signal transduction responses in the cultured MEC following EGFR activation by EGF or TGF.

In contrast, rat mammary fibroblast cells (MFC) were successfully utilized to examine rapid signal transduction events including tyrosine phosphorylation protein profiles and MAPK phosphorylation in response to EGFR activation by EGF or TGF in normal primary cells at passage 5. Specifically, the signal transduction cascades induced by a 15 minute exposure to different concentrations [0, 0.1, 1 and 10 ng/ml] of human recombinant (hr) EGF and TGF were compared as was the time-dependent (5, 15, 60 and 120 minutes)

transduction in response to a fixed concentration [10 ng/ml] of hrEGF and hrTGF. The selective and potent inhibitor of the tyrosine kinase domain of the EGFR receptor, PD158780, was then evaluated for its ability to block EGF- and TGF-induced signal transduction when exposed simultaneously. Both EGF and TGF were effective at inducing the rapid (observable within 5 minutes, shorter times have yet to be evaluated) tyrosine phosphorylation of a ~170 kDa protein which co-migrates with an EGF receptor immunoreactive protein. The apparent phosphorylation of the EGFR was both concentration- [data not shown] and time-dependent [Figures 18-20]. Maximal EGF receptor phosphorylation was induced with 10 ng/ml of either hrEGF or hrTGF (higher concentrations have yet to be examined). Using either EGF receptor ligand at 10 ng/ml, induction of EGF receptor tyrosine phosphorylation peaked at 5 minutes [Figure 18], and appeared to be downregulated in a time- dependent manner from 15 minutes through the 120 minute treatment [Figures 18-20]. EGF and TGF also induced a rapid (observable within 5 minutes, but shorter time points have yet to be tested) and sustained (through the 120 minute treatment) phosphorylation of mitogen activated protein kinase (MAPK) [Figures 18-20]. PD158780 was able block the apparent phosphorylation of the EGF receptor as well as the phosphorylation of MAPK [Figures 18-20]. Finally, these EGF receptor ligands also induced the phosphorylation of a variety of other proteins. Additional studies are required to identify these tyrosine phosphorylated proteins and study the time-kinetics of their induction and down-regulation.

4. CONCLUSIONS

During the next year, similar EGFR activation studies will be carried out in (1) rat mammary fibroblasts obtained from carcinogen-transformed rat mammary tumors, (2) the other types of normal as well as carcinogen-transformed mammary stromal cells, and (3) a rat mammary tumor cell line (obtained from the American Type Culture Collection) derived from a methyl nitrosurea-induced mammary tumor. This cell line grows as a monolayer on tissue culture plastic. Additional methodological advancements are, however, required to examine rapid signal transduction responses in normal MEC as well as in carcinogen-transformed MEC in primary culture or in co-culture with distinct types of mammary stromal cells. With regard to the immunocytochemical analysis of EGFR expression and phosphotyrosine activity in serial sections of formalin-fixed and paraffin-embedded MEO, the sheep anti-human EGFR antibody and two anti-phosphotyrosine antibodies aren't showing positive reactivity patterns at this point.

FIGURE LEGENDS

Figure 1. Mitogenic effects of EGF and TGF under conditions of End Bud or Alveolar Morphogenesis. Newly isolated MEC were cultured for up to 21 days in the presence of ALV Medium alone [ALV Media + No GF], with 10 ng/ml hrEGF [ALV Media + hrEGF], or with 10 ng/ml hrTGF [ALV Media + hrTGF], or in the presence of EB Medium alone [EB Media + No GF], with 10 ng/ml hrEGF [EB Media + hrEGF], or with 10 ng/ml hrTGF [EB Media + hrTGF]. Viable cell number within individual culture wells was monitored using the MTT assay at different times during the 21 day culture period. Each point represents the mean ± the SEM obtained from triplicate culture wells.

Figure 2. Effect of EGFR Regulation on MEO Proliferation. Newly isolated MEC were cultured for up to 21 days in the continuous presence of ALV Medium either with 0, 0. 05, 0.5, or 5 μM PD158780, with 0 μM PD158780 and 10 ng/ml hrEGF, or with 0 μM PD158780 and 10 ng/ml hrTGF. Viable cell number within individual culture wells was monitored using the MTT assay at different times during the 21 day culture period. Each point represents the mean ± the SEM obtained from triplicate culture wells.

Figure 3. Effect of EGFR Regulation on MEO Proliferation at Day 7 of Culture Development. Newly isolated MEC were cultured for 7 days either in the presence of ALV Medium alone [ALV + No GF], with 10 ng/ml hrEGF [ALV + hrEGF], or with 10 ng/ml hrTGF [ALV + hrTGF], or in the presence of EB Medium alone [EB + No GF], with 10 ng/ml hrEGF [EB + hrEGF], or with 10 ng/ml hrTGF [EB + hrTGF]. The cultured MEC were exposed to 0 [open bars] or 0.5 μM PD158780 [solid bars] from day 3.5 through day 7 of the study. Viable cell number within individual culture wells was monitored using the MTT assay at day. Each point represents the mean ± the SEM obtained from triplicate culture wells.

Figure 4. Effect of EGFR Regulation on MEO Proliferation at Day 14 of Culture Development. Newly isolated MEC were cultured for 14 days either in the presence of ALV Medium alone [ALV + No GF], with 10 ng/ml hrEGF [ALV + hrEGF], or with 10 ng/ml hrTGF [ALV + hrTGF], or in the presence of EB Medium alone [EB + No GF], with 10 ng/ml hrEGF [EB + hrEGF], or with 10 ng/ml hrTGF [EB + hrTGF]. The cultured MEC were exposed to 0 [open bars] or 0.5 μM PD158780 [solid bars] from day 10.5 through day 14 of the study. Viable cell number within individual culture wells was monitored using the MTT assay at day 14. Each bar represents the mean ± the SEM obtained from triplicate culture wells.

Figure 5. Effect of EGFR Regulation on MEO Proliferation at Day 21 of Culture Development. Newly isolated MEC were cultured for 21 days either in the presence of ALV Medium alone [ALV + No GF], with 10 ng/ml hrEGF [ALV + hrEGF], or with 10 ng/ml hrTGF [ALV + hrTGF], or in the presence of EB Medium alone [EB + No GF], with 10 ng/ml hrEGF [EB + hrEGF], or with 10 ng/ml hrTGF [EB + hrTGF]. The cultured MEC were exposed to 0 [open bars] or 0.5 μM PD158780 [solid bars] from day 17.5 through day 21 of the study. Viable cell number within individual culture wells was monitored using the MTT assay at day 21. Each bar represents the mean ± the SEM obtained from triplicate culture wells.

Figure 6. Effects of EGFR Regulation on Casein Accumulation by Day 7 of Culture Development. Newly isolated MEC were cultured for 7 days in the presence of ALV Medium with 0, 0.05, 0.5 or 5 μM PD158780, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF. Total

casein levels within individual culture wells were quantitated by ELISA. Each bar represents the mean ± the SEM obtained from triplicate culture wells.

Figure 7. Effects of EGFR Regulation on Casein Accumulation by Day 14 of Culture Development. Newly isolated MEC were cultured for 14 days in the presence of ALV Medium with 0, 0.05, 0.5 or 5 μM PD158780, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF. Total casein levels within individual culture wells were quantitated by ELISA. Each bar represents the mean ± the SEM obtained from triplicate culture wells.

Figure 8. Effects of EGFR Regulation on Casein Accumulation by Day 21 of Culture Development. Newly isolated MEC were cultured for 21 days in the presence of ALV Medium with 0, 0.05, 0.5 or 5 μM PD158780, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF. Total casein levels within individual culture wells were quantitated by ELISA. Each bar represents the mean ± the SEM obtained from triplicate culture wells.

Figure 9. Effects of EGFR Regulation on Day 7 Casein Isoform Expression. Newly isolated MEC were cultured for 7 days in the presence of ALV Medium alone, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF, and exposed to 0 or 0.5 μM PD158780 from day 3.5 through day 7. Casein isoform expression was analyzed on day 21 using a Western blot procedure.

Figure 10. Effects of EGFR Regulation on Day 14 Casein Isoform Expression. Newly isolated MEC were cultured for 14 days in the presence of ALV Medium alone, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF, and exposed to 0 or 0.5 μM PD158780 from day 10.5 through day 14. Casein isoform expression was analyzed on day 21 using a Western blot procedure.

Figure 11. Effects of EGFR Regulation on Day 21 Casein Isoform Expression. Newly isolated MEC were cultured for 21 days in the presence of ALV Medium alone, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF, and exposed to 0 or 0.5 μM PD158780 from day 17.5 through day 21. Casein isoform expression was analyzed on day 21 using a Western blot procedure.

Figure 12. Effects of EGFR Regulation on Day 14 Casein Isoform. Newly isolated MEC were cultured for 14 days in the presence of EB Medium alone, with 10 ng/ml hrEGF, or with 10 ng/ml hrTGF, and exposed to 0 or 0.5 μM PD158780 from day 10.5 through day 14. Casein isoform expression was analyzed on day 21 using a Western blot procedure.

Figure 13. Alum Carmine-Stained Mammary Gland Whole Mount. Dark field photomicrograph of a portion of an alum carmine-stained abdominal mammary gland excised from a 75 day old Sprague-Dawley female rat injected with 1-methyl-1-nitrosourea when the animal was 23 days of age.

Figure 14. Alum Carmine-Stained Mammary Gland Whole Mount. High power, dark field photomicrograph of a portion of an alum carmine-stained abdominal mammary gland excised from a 75 day old Sprague-Dawley female rat injected with 1-methyl-1-nitrosourea when the animal was 23 days of age.

Figure 15. Alum Carmine-Stained Mammary Gland Whole Mount. Dark field photomicrograph of a portion of an alum carmine-stained cranial mammary gland excised from a 75 day old Sprague-Dawley female rat injected with 1-methyl-1-nitrosourea when the animal was 23 days of age.

Figure 16. Western Blot Detection of EGFR. Whole cell lysates were prepared from rat livers, rat mammary fibroblasts (MFC) and human epidermoid A431 cells. These samples were separated on a 4-20% SDS-polyacrylamide gradient gel and transferred to an Immobilon P membrane. EGFR Westerns were then preformed using a sheep anti-human EGFR polyclonal antibody.

Figure 17. Western Blot Detection of EGFR. Whole cell lysates were prepared from primary rat mammary epithelial cells cultured for 11 days in ALV Medium or EB Medium with 10 ng/ml hrEGF and then dissociated from the RBM, or from rat mammary fibroblasts (MFC). These samples were separated on a 4-20% SDS-polyacrylamide gradient gel and transferred to an Immobilon P membrane. EGFR Westerns were then preformed using a sheep anti-human EGFR polyclonal antibody.

Figure 18. EGFR-Dependent Tyrosine Phosphorylation in Normal Mammary Fibroblasts. Serum-starved rat mammary fibroblast were treated for 5 minutes or 1 hour with basal medium supplemented alone [No GF], with 10 ng/ml hrEGF [hrEGF] or with 10 ng/ml hrTGF [hrTGF] at 37°C. Whole cell lysates, prepared from these different samples, were separated on 4-20% gradient gel, transferred to Immobilon P membrane, and then sequentially immunoblotted using a recombinant anti-phosphotyrosine antibody [anti-*P*-tyrosine], an anti-phospho-MAPK antibody which only recognizes phosphoylated MAPK [anti-*P*-MAPK], and then an anti-p44/42 MAPK antibody which recognizes total MAPK [anti-p44/42-MAPK].

Figure 19. EGFR-Dependent Tyrosine Phosphorylation in Response to a 15 min Treatment. Serum-starved rat mammary fibroblast were treated for 15 minutes with basal medium supplemented alone [No GF], with 0.5 μM PD158780 [No GF + PD158780], with 10 ng/ml hrEGF [hrEGF], with 10 ng/ml hrEGF plus 0.5 μM PD158780 [hrEGF + PD158780], with 10 ng/ml hrTGF [hrTGF], or with 10 ng/ml hrTGF plus 0.5 μM PD158780 [hrTGF + PD158780] at 37°C. Whole cell lysates, prepared from these different samples, were separated on 4-20% gradient gel, transferred to Immobilon P membrane, and then sequentially immuonblotted using a recombinant anti-phosphotyrosine antibody [anti-*P*-tyrosine], an anti-phospho-MAPK antibody which only recognizes phosphoylated MAPK [anti-*P*-MAPK], and then an anti-p44/42 MAPK antibody which recognizes total MAPK [anti-p44/42-MAPK].

Figure 20. EGFR-Dependent Tyrosine in Response to a 2 hour Treatment. Serum-starved rat mammary fibroblast were treated for 2 hours with basal medium supplemented alone [No GF], with 0.5 μM PD158780 [No GF + PD158780], with 10 ng/ml hrEGF [hrEGF], with 10 ng/ml hrEGF plus 0.5 μM PD158780 [hrEGF + PD158780], with 10 ng/ml hrTGF [hrTGF], or with 10 ng/ml hrTGF plus 0.5 μM PD158780 [hrTGF+ PD158780] at 37°C. Whole cell lysates, prepared from these different samples, were separated on 4-20% gradient gel, transferred to Immobilon P membrane, and then sequentially immunoblotted using a

recombinant anti-phosphotyrosine antibody [anti-*P*-tyrosine], an anti-phospho-MAPK antibody which only recognizes phosphoylated MAPK [anti-*P*-MAPK], and then an anti-p44/42 MAPK antibody which recognizes total MAPK [anti-p44/42-MAPK].

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Figure 1.

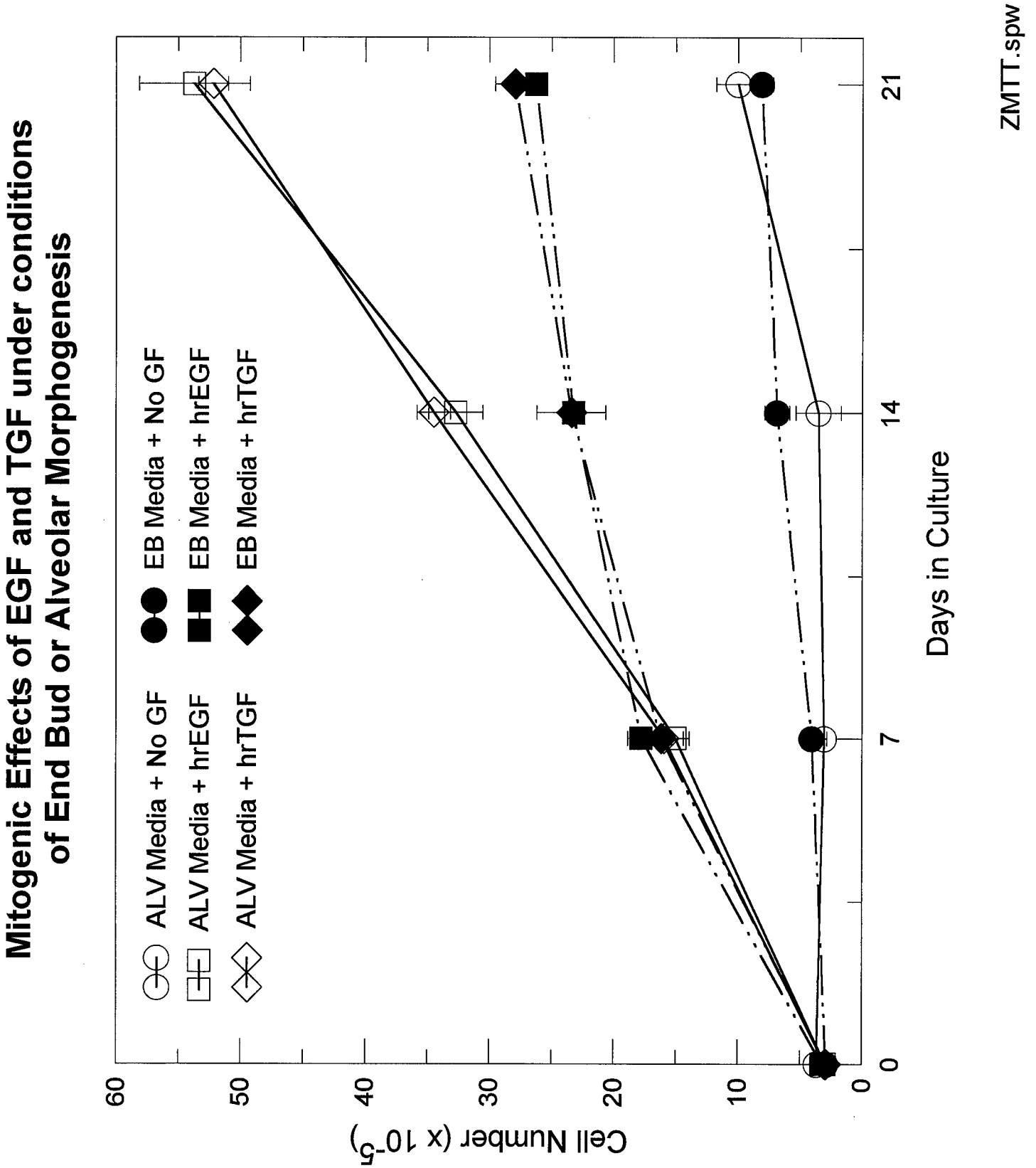

Mitogenic Effects of EGF and TGF under conditions
of End Bud or Alveolar Morphogenesis
ALV Media + No GF
ALV Media + hrEGF
ALV Media + hrTGF
EB Media + No GF
EB Media + hrEGF
EB Media + hrTGF
Cell Number (x 10-5)
Days in Culture
0
10
20
30
40
50
60
0
7
14
21
ZMTT.spw

Figure 2.

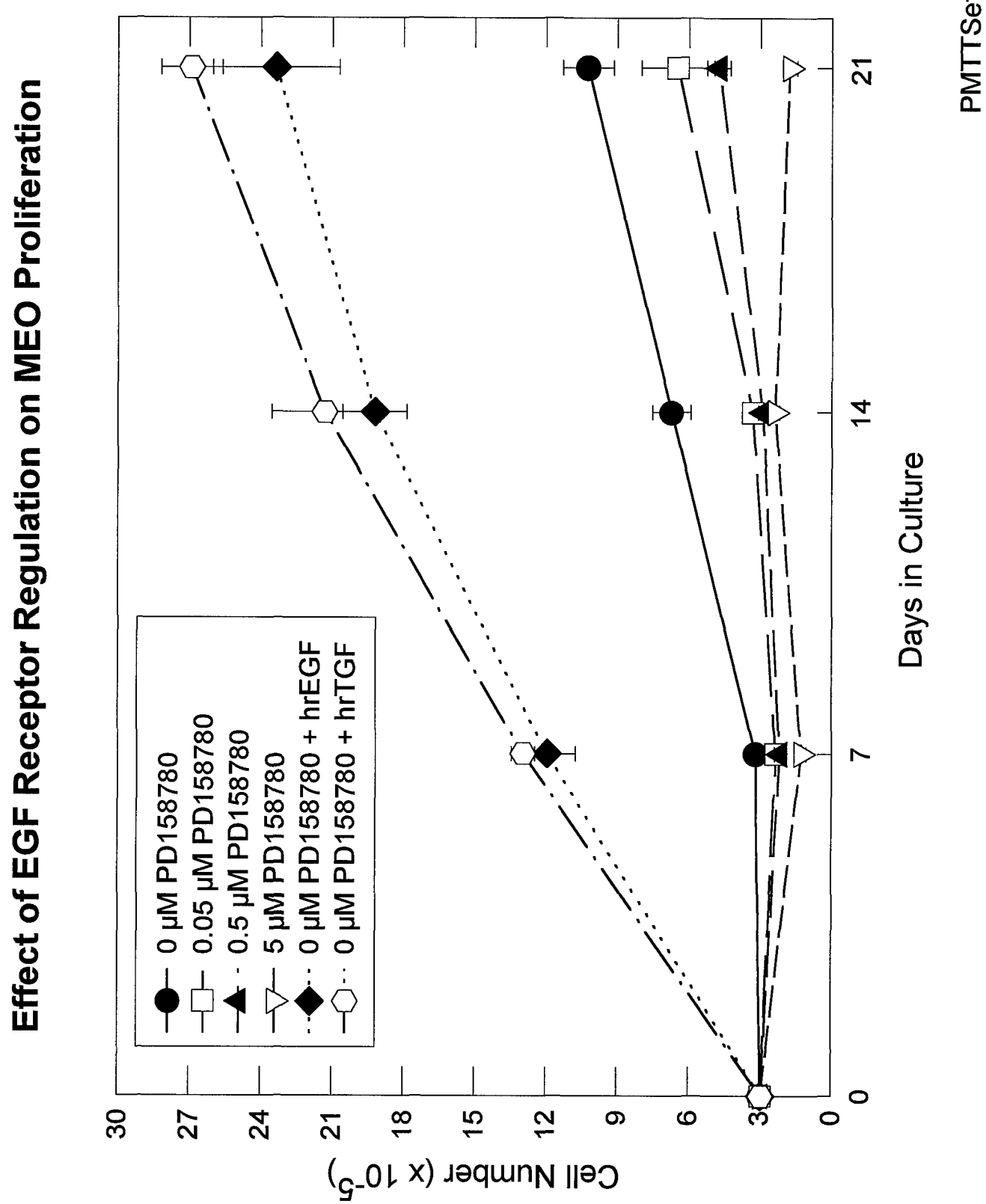

Effect of EGF Receptor Regulation on MEO Proliferation
0 µM PD158780
0.05 µM PD158780
0.5 µM PD158780
5 µM PD158780
0 µM PD158780 + hrEGF
0 µM PD158780 + hrTGF
Cell Number (x 10^-5)
30
27
24
21
18
15
12
9
6
3
0
0
7
14
21
Days in Culture
PMTTSetA.spw

Figure 3.

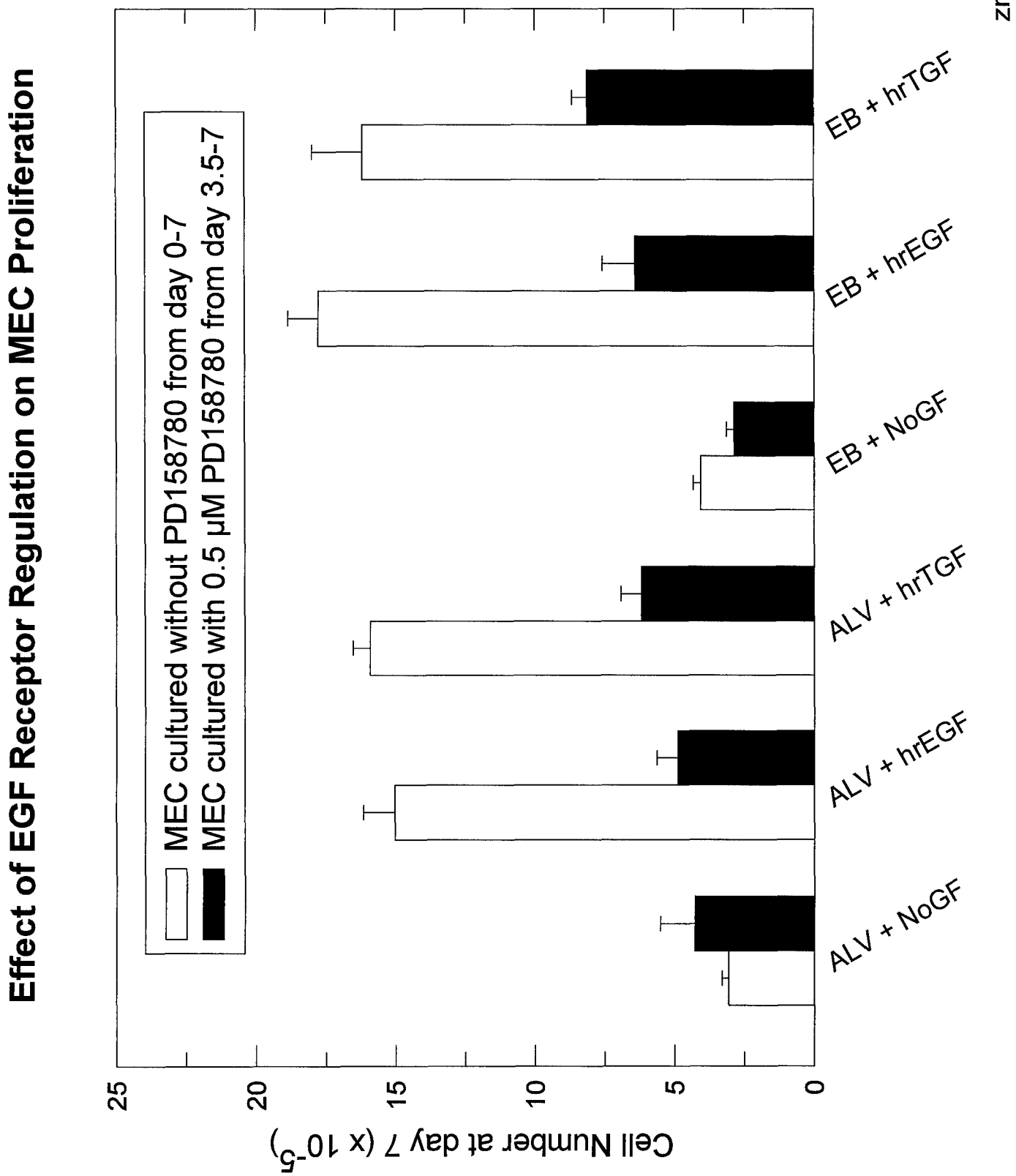

Effect of EGF Receptor Regulation on MEC Proliferation
zmttAB7.spw
MEC cultured without PD158780 from day 0-7
MEC cultured with 0.5 µM PD158780 from day 3.5-7
Cell Number at day 7 (x 10-5)
0
5
10
15
20
25
EB + hrTGF
EB + hrEGF
EB + NoGF
ALV + hrTGF
ALV + hrEGF
ALV + NoGF

Figure 4.

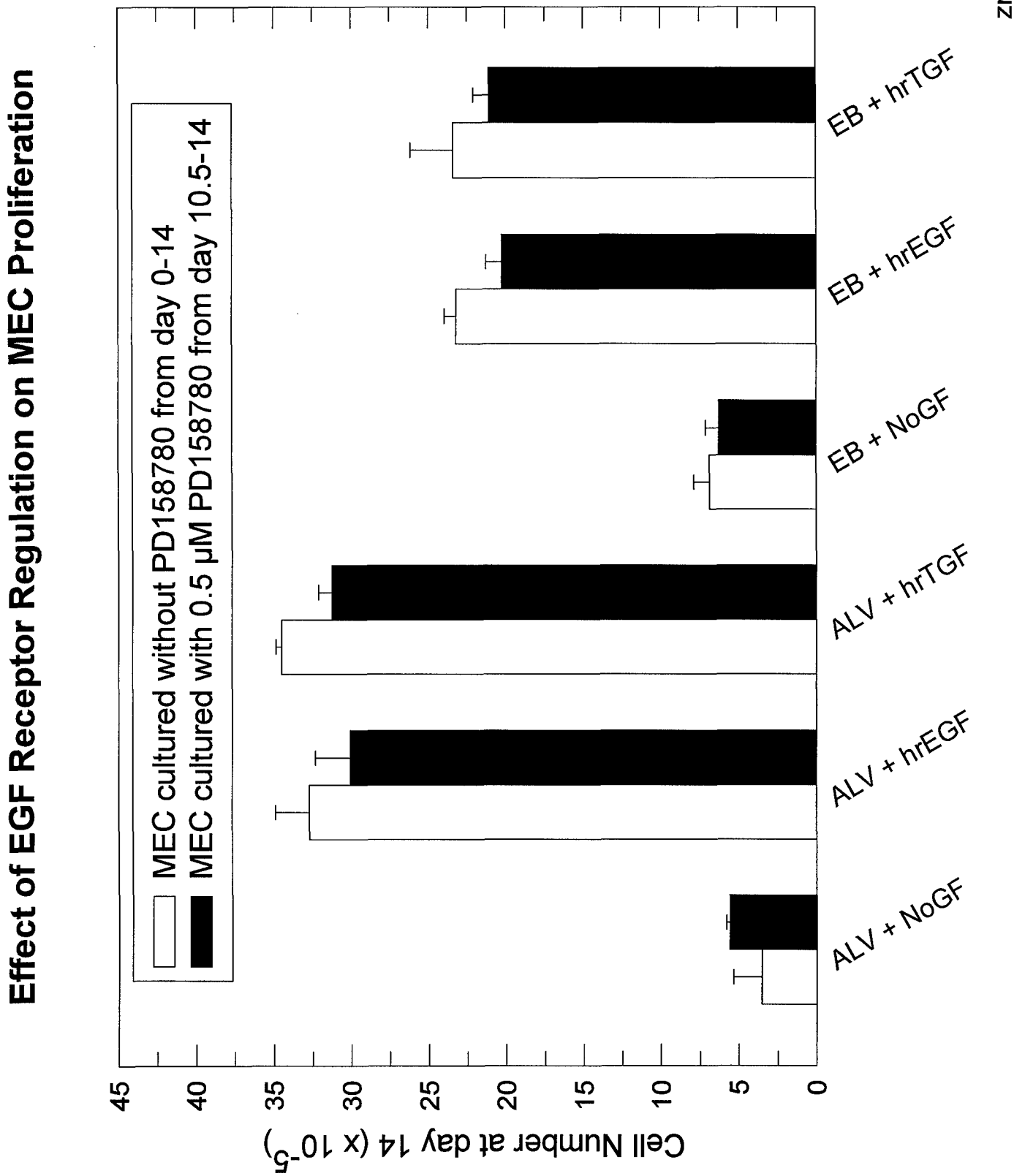

Effect of EGF Receptor Regulation on MEC Proliferation
MEC cultured without PD158780 from day 0-14
MEC cultured with 0.5 µM PD158780 from day 10.5-14
Cell Number at day 14 (x 10-5)
0
5
10
15
20
25
30
35
40
45
EB + hrTGF
EB + hrEGF
EB + NoGF
ALV + hrTGF
ALV + hrEGF
ALV + NoGF
zmttAB14.spw

Figure 5.

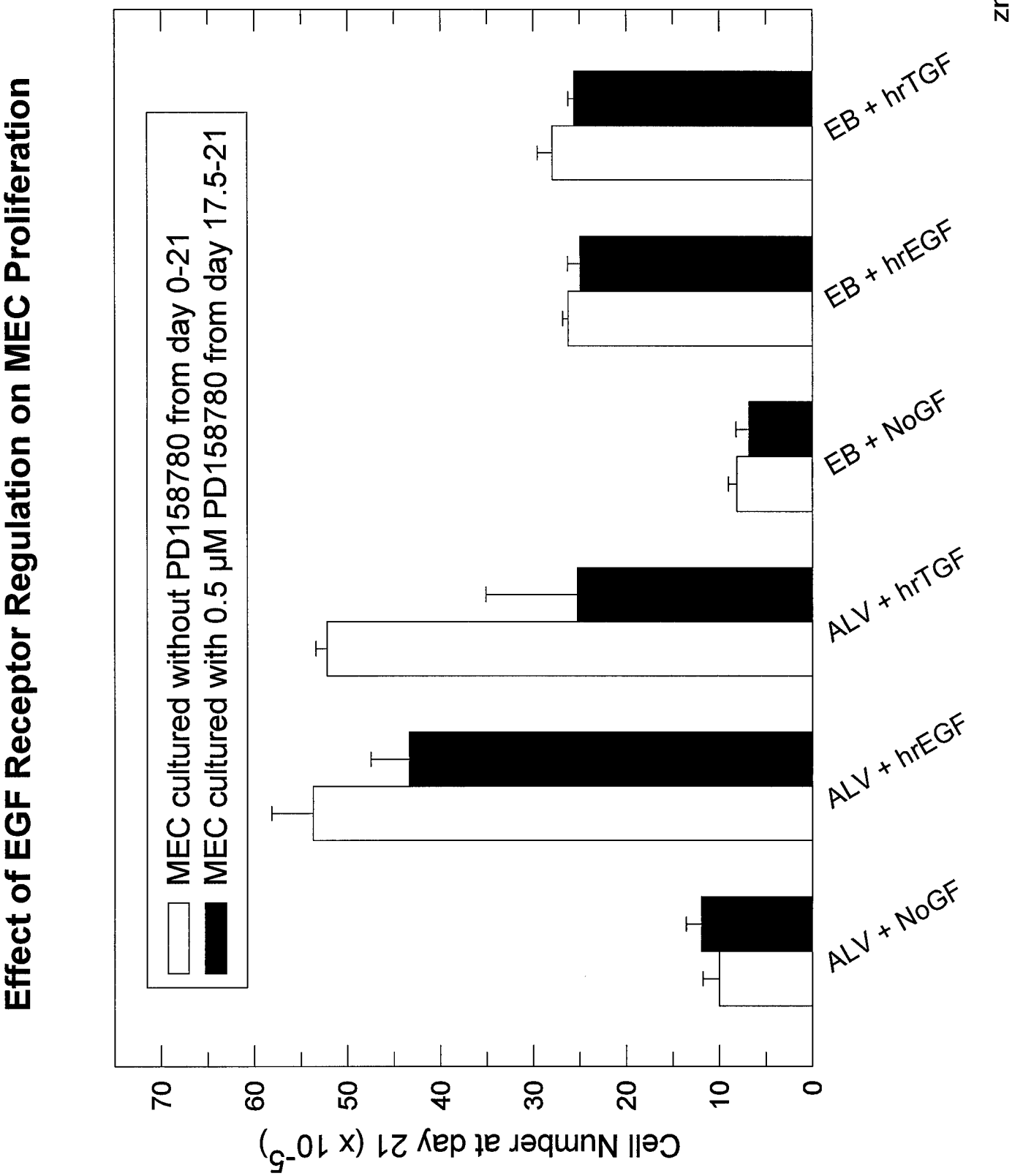

Effect of EGF Receptor Regulation on MEC Proliferation
MEC cultured without PD158780 from day 0-21
MEC cultured with 0.5 µM PD158780 from day 17.5-21
Cell Number at day 21 (x 10-5)
0
10
20
30
40
50
60
70
ALV + NoGF
ALV + hrEGF
ALV + hrTGF
EB + NoGF
EB + hrEGF
EB + hrTGF
zmttAB21.spw

Figure 6.

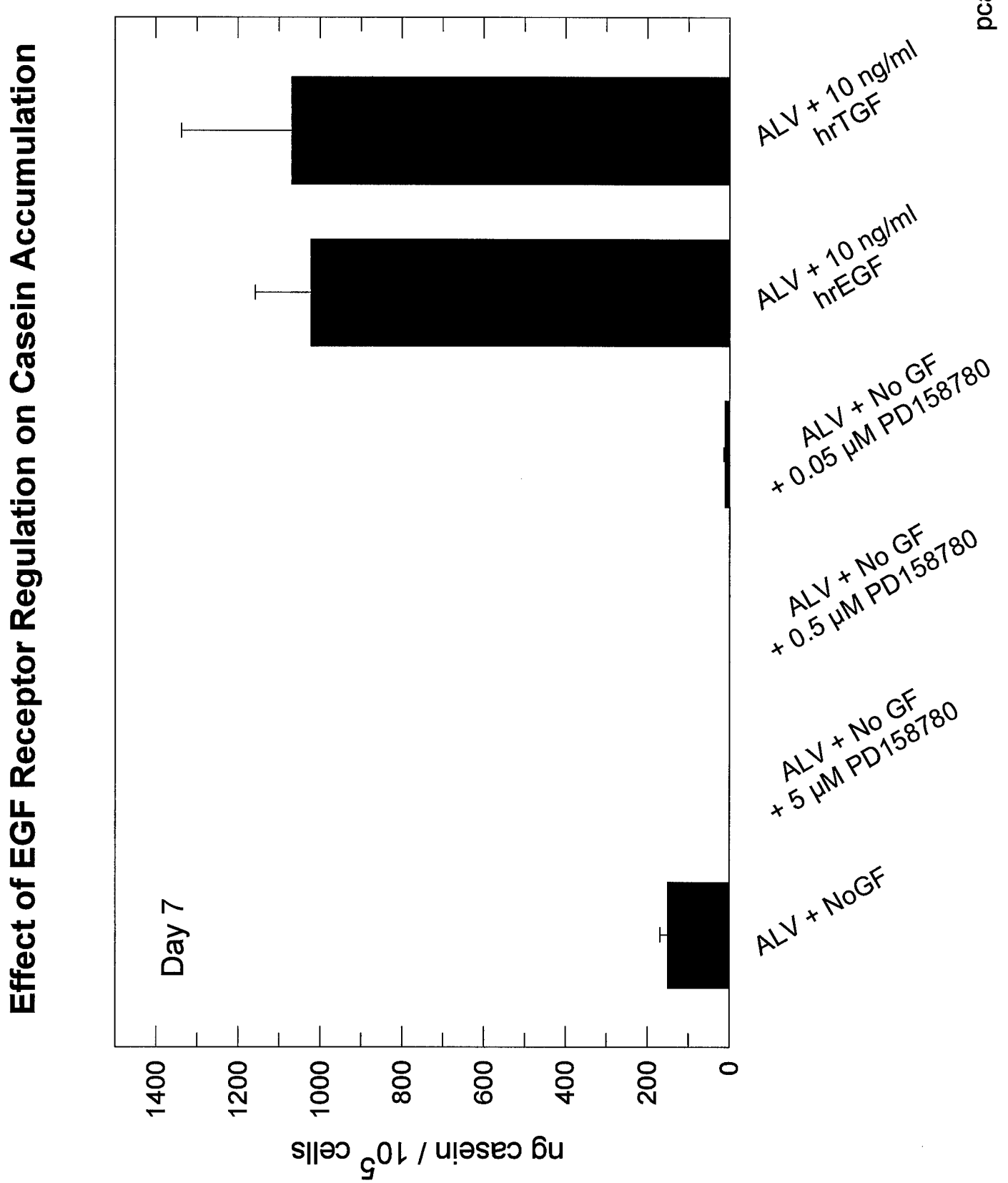

Effect of EGF Receptor Regulation on Casein Accumulation
pcasd7.spw
Day 7
ng casein / 10^5 cells
0
200
400
600
800
1000
1200
1400
ALV + NoGF
ALV + No GF + 5 µM PD158780
ALV + No GF + 0.5 µM PD158780
ALV + No GF + 0.05 µM PD158780
ALV + 10 ng/ml hrEGF
ALV + 10 ng/ml hrTGF

Figure 7.

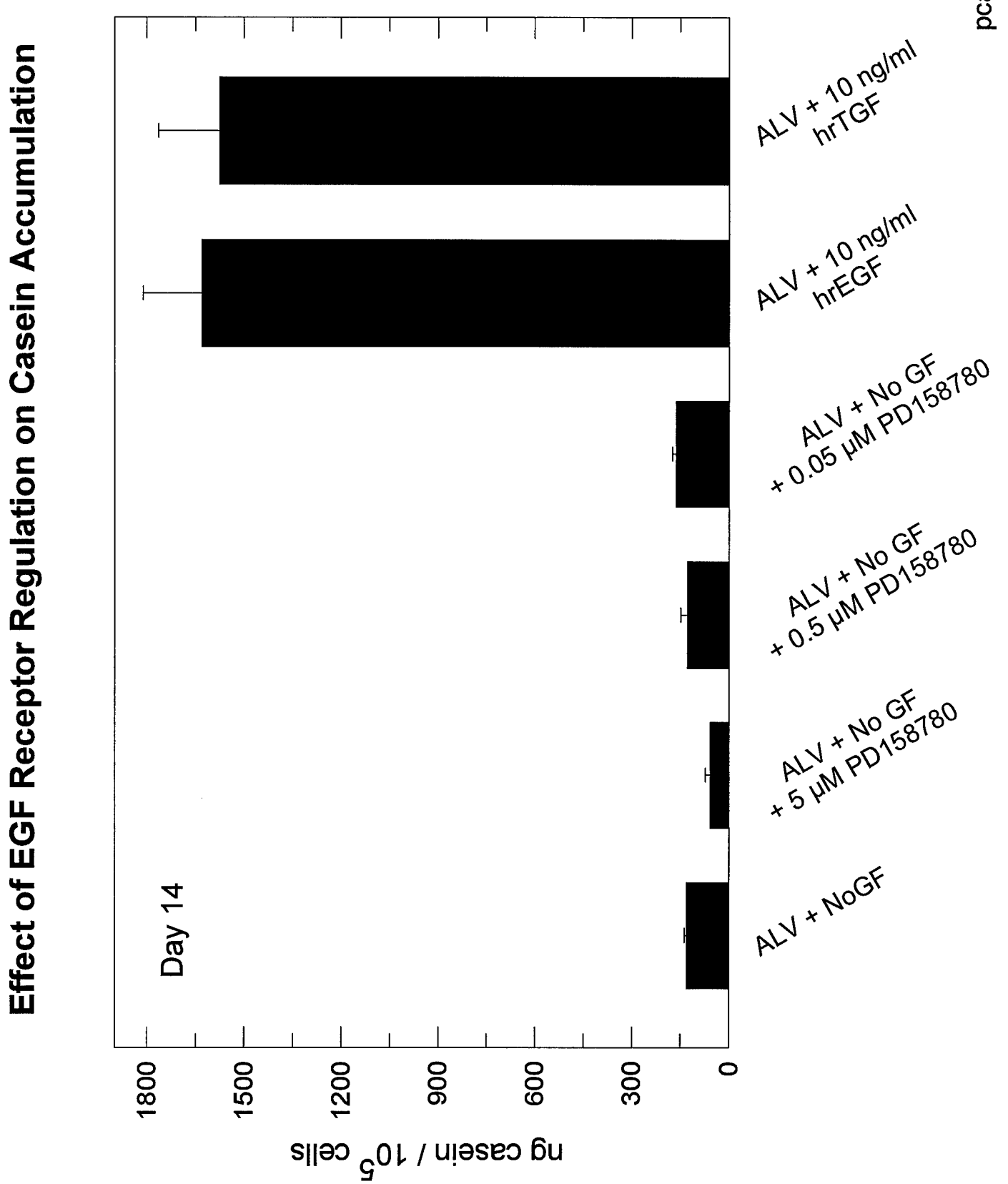

Effect of EGF Receptor Regulation on Casein Accumulation
pcasd14.spw
Day 14
ng casein / 10^5 cells
0
300
600
900
1200
1500
1800
ALV + NoGF
ALV + No GF + 5 µM PD158780
ALV + No GF + 0.5 µM PD158780
ALV + No GF + 0.05 µM PD158780
ALV + 10 ng/ml hrEGF
ALV + 10 ng/ml hrTGF

Figure 8.

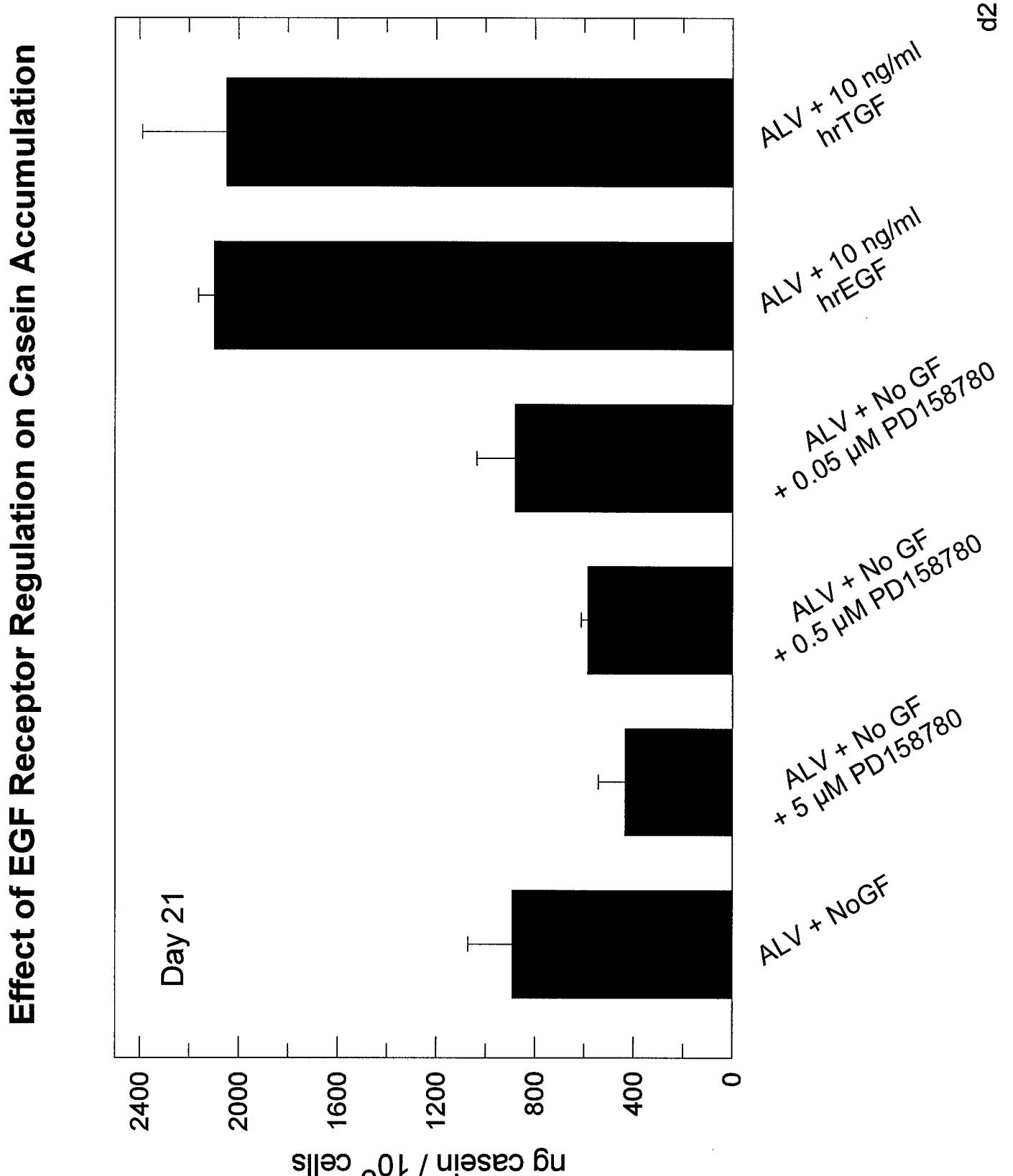
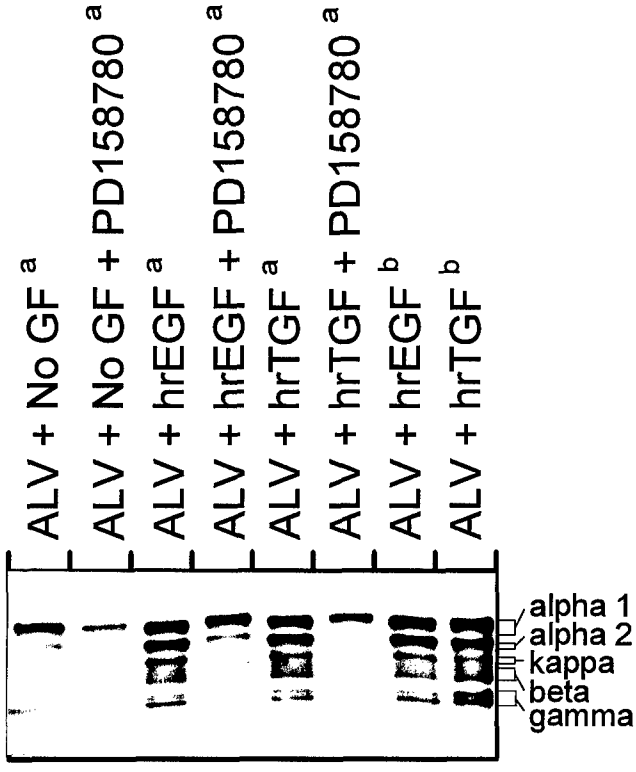

Effect of EGF Receptor Regulation on Casein Accumulation
d21pcas.spw
Day 21
ng casein / 10^5 cells
0
400
800
1200
1600
2000
2400
ALV + NoGF
ALV + No GF + 5 µM PD158780
ALV + No GF + 0.5 µM PD158780
ALV + No GF + 0.05 µM PD158780
ALV + 10 ng/ml hrEGF
ALV + 10 ng/ml hrTGF

Figure 9.

Effect of EGF Receptor Regulation on Day 7 Casein Protein Expression

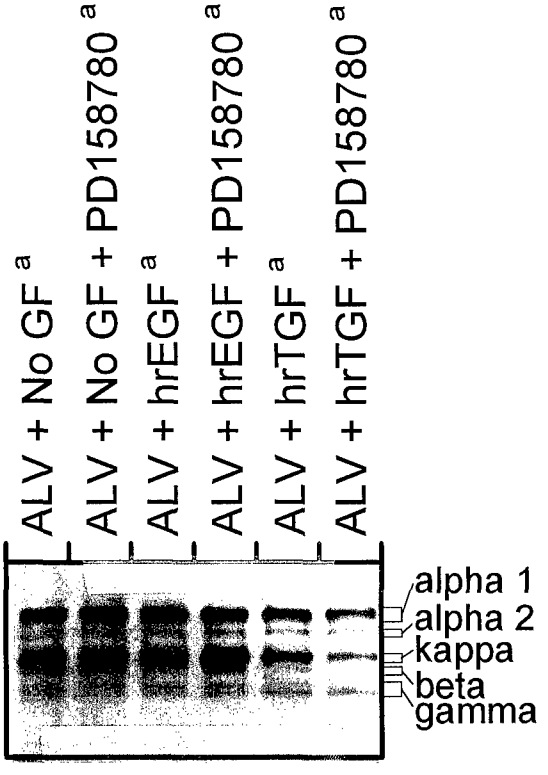


[a] lysate samples represent 7500 cells/lane
[b] lysate samples represent 10,000 cells/lane

71996189.spw

Figure 10.

Effect of EGF Receptor Regulation on Day 14 Casein Protein Expression

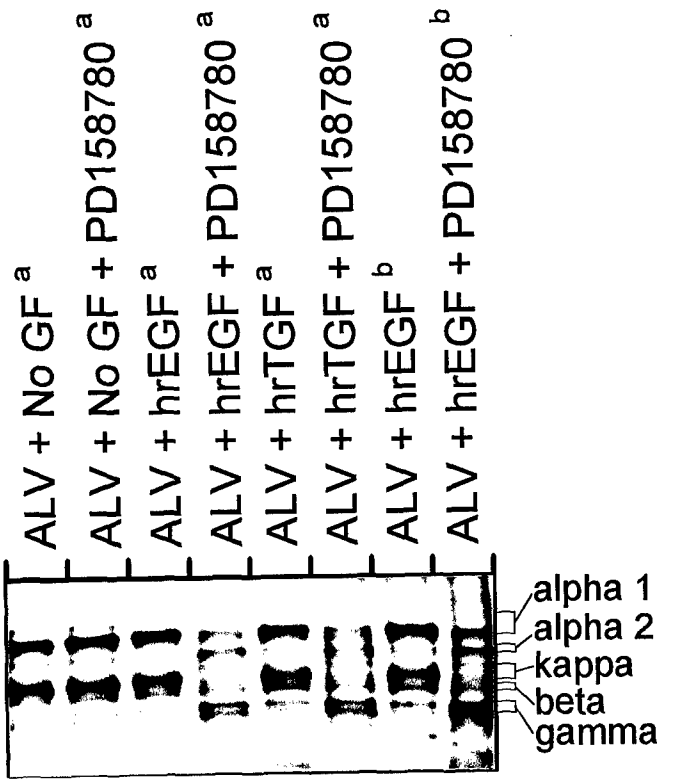


[a] day 14 lysate samples represent 10,000 cells/lane

71996195.spw

Figure 11.

Effect of EGF Receptor Regulation on Day 21 Casein Protein Expression

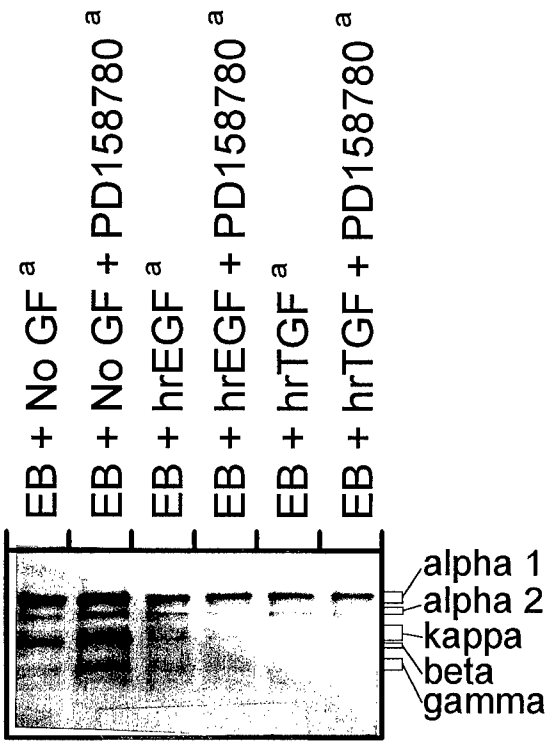


[a] lysate samples represent 10,000 cells/lane
[b] lysate samples represent 20,000 cells/lane

71996192.spw

Figure 12.

Effect of EGF Receptor Regulation on Day 14 Casein Protein Expression



[a] day 14 lysate samples represent 15,000 cells/lane

71996202.spw

Figure 13.

Alum Carmine-Stained Mammary Gland Whole Mount

MGWhMt1.spw

Figure 14.

Alum Carmine-Stained Mammary Gland Whole Mount

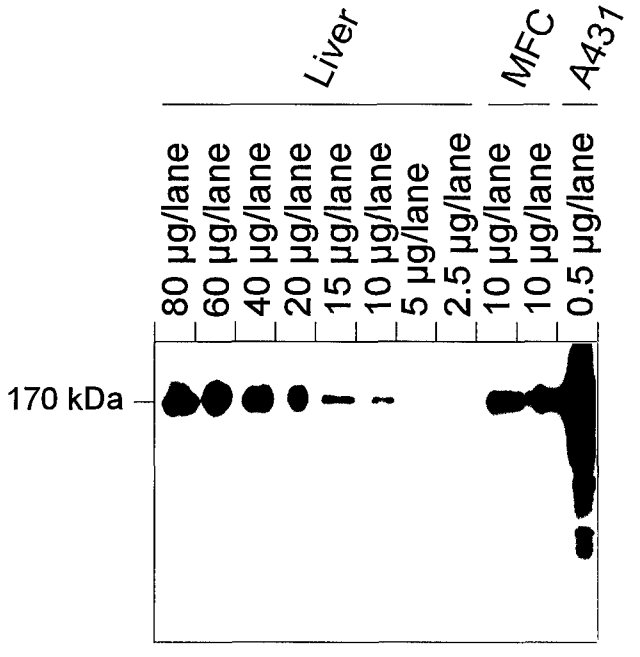
MGWhMt2.spw

Figure 15.

Alum Carmine-Stained Mammary Gland Whole Mount

MGWhMt3.spw

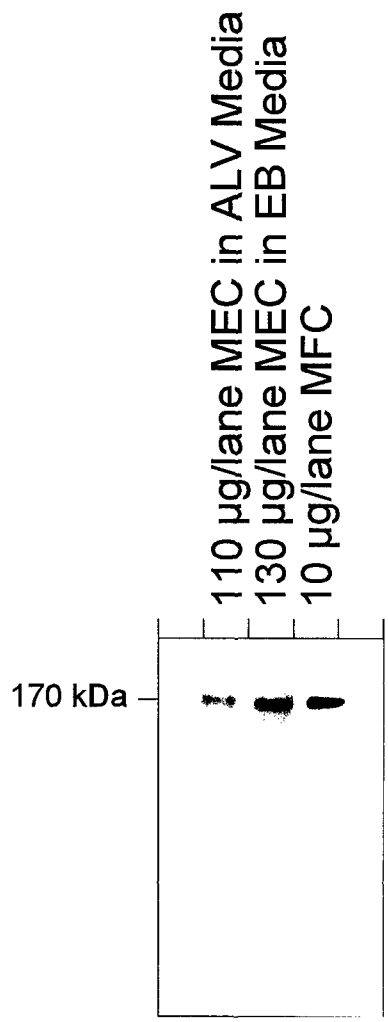
Figure 16.



11039570.spw

Figure 17.

Western Blot Detection of EGFR



02039697.spw

Figure 18.

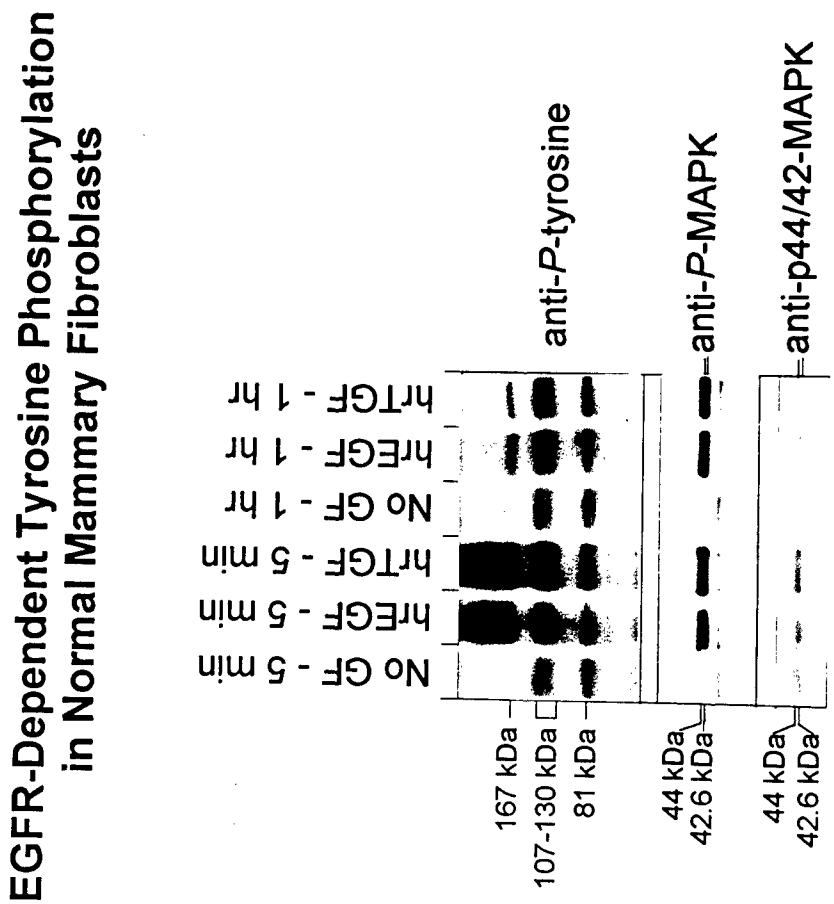

EGFR-Dependent Tyrosine Phosphorylation
in Normal Mammary Fibroblasts
No GF - 5 min
hrEGF - 5 min
hrTGF - 5 min
No GF - 1 hr
hrEGF - 1 hr
hrTGF - 1 hr
anti-P-tyrosine
anti-P-MAPK
anti-p44/42-MAPK
167 kDa
107-130 kDa
81 kDa
44 kDa
42.6 kDa
44 kDa
42.6 kDa

Figure 19.

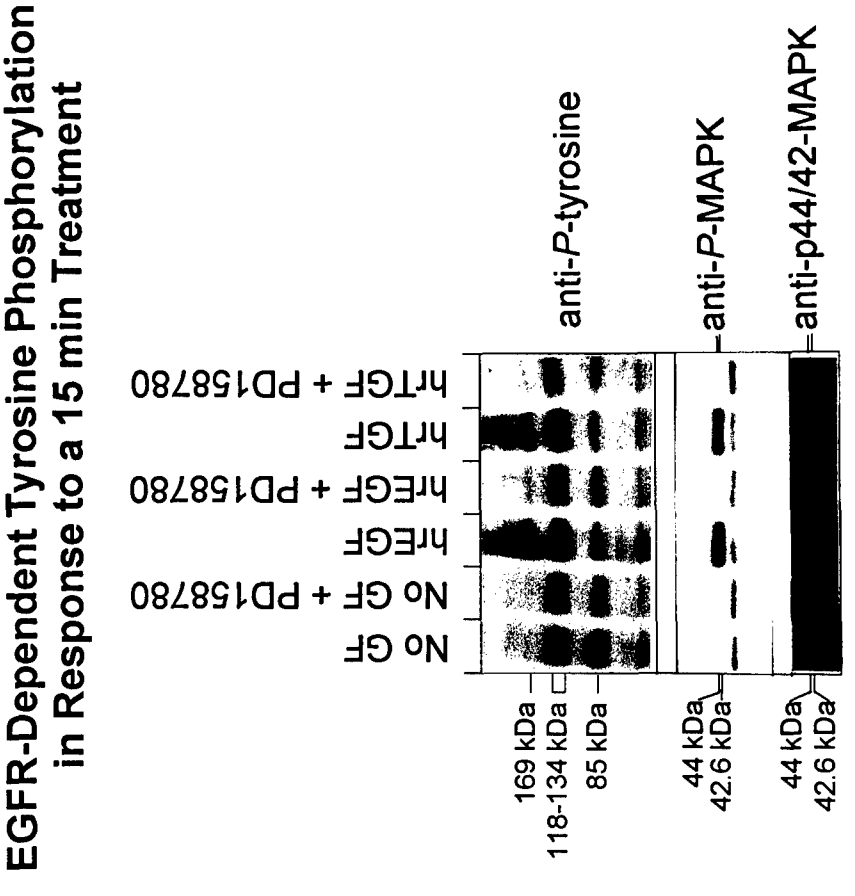
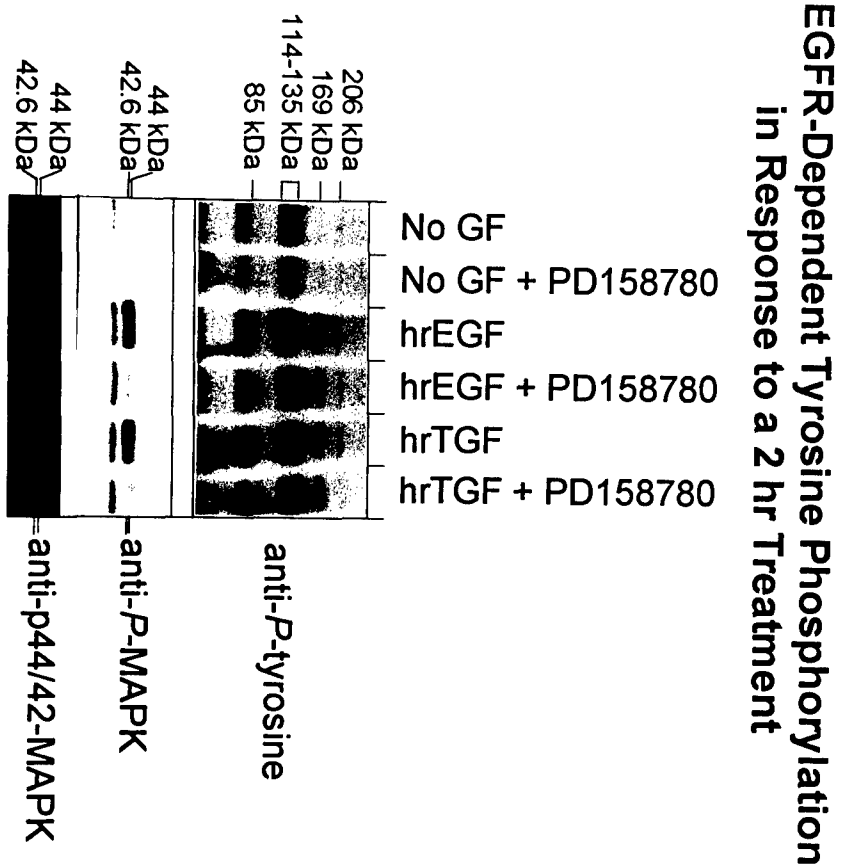

EGFR-Dependent Tyrosine Phosphorylation
in Response to a 15 min Treatment
No GF
No GF + PD158780
hrEGF
hrEGF + PD158780
hrTGF
hrTGF + PD158780
anti-P-tyrosine
anti-P-MAPK
anti-p44/42-MAPK
169 kDa
118-134 kDa
85 kDa
44 kDa
42.6 kDa
44 kDa
42.6 kDa

Figure 20


EGFR-Dependent Tyrosine Phosphorylation
in Response to a 2 hr Treatment
No GF
No GF + PD158780
hrEGF
hrEGF + PD158780
hrTGF
hrTGF + PD158780
206 kDa
169 kDa
114-135 kDa
85 kDa
anti-P-tyrosine
44 kDa
42.6 kDa
anti-P-MAPK
44 kDa
42.6 kDa
anti-p44/42-MAPK

Darcy, Kathleen M.

21 st Meeting of the International Association for Breast Cancer Research

ABSTRACT FORM

TITLE	Role of Epidermal Growth Factor Receptors and their Ligands in the Development of Normal Mammary Epithelial Cells and Mammary Fibroblasts.
AUTHORS ADDRESS	K. Darcy*, A. Wohlhueter, D. Zangani, S. Shoemaker, W. Shea, P.-P. Lee, and M. Ip. Roswell Park Cancer Institute, Buffalo, NY 14263 USA

Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) exert their effects by binding to a membrane-associated EGF receptor (EGFR) and subsequently activating the tyrosine kinase domain of this receptor. Although their mechanisms of action are not completely understood, these ligands and their receptor are physiological regulators of normal mammary gland development, and are often overexpressed in estrogen receptor negative breast cancers. Initially, we examined the biological effects of EGF and TGF-α on normal rat mammary epithelial cell (MEC) development in primary culture, and then used PD158780, a potent and selective inhibitor of the tyrosine kinase domain of the EGFR, to demonstrate that EGFR signaling is required, at least in part, for normal MEC proliferation, functional differentiation (as assessed by casein accumulation), colony survival, branching morphogenesis, as well as secretion of certain matrix-degrading metalloproteinases. In addition, normal rat mammary fibroblast cells (MFC) were used to examine rapid signal transduction events including tyrosine phosphorylation protein profiles and MAP kinase (MAPK) phosphorylation in response to EGFR activation by EGF or TGF-α. PD158780 was then employed to determine which of the tyrosine phosphorylation events induced in the MFC by EGF or TGF-α were EGFR-dependent. We will continue to examine the role that EGFR plays in the development of normal MEC cultured alone as well as MEC co-cultured with the EGFR-responsive MFC, and then examine their role during rat mammary tumor progression and metastasis. Ultimately, we hope that this data will help to identify new therapeutic targets and develop effective therapies to treat patients with estrogen receptor negative breast cancer.

Supported by DAMD17-94-J-4159 and NIH CA 64870.

KEY WORDS: mammary, epithelial, fibroblasts, EGF, TGF-α, EGF receptor, tyrosine phosphorylation

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