

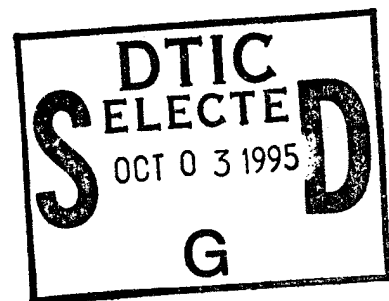
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Normal Mammary Epithelial and Breast Cancer Cells

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28 July 1995

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Role of Epidermal Growth Factor Receptors and Their Ligands
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Kathleen M. Darcy 28 July 1995
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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- α) 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- α 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- α , 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- α 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- α can also act as a dominant transforming gene product in MEC expressing normal levels of EGFR (6). Moreover, TGF- α 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- α 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 the role that mammary gland stromal cells play in regulating the progression and metastatic spread of the breast cancer cells and in 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 maximally 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- α , acting through their common cellular receptor, the EGFR, regulate normal MEC growth, differentiation, morphogenesis, and invasion, as well as the progression of MEC transformed in culture by exposure to a carcinogen (which may function, in part, by mutating the protooncogene ras) and/or by exposure to a tumor promoter known to synergize with certain activities of EGF and TGF- α in MEC. Our model system has been recently upgraded such that MEC can be cultured alone or in combination with different populations of mammary stromal cells (MSC). This upgrade allows for the examination of direct epithelial-specific effects when the MEC are cultured without MSC, and of direct and indirect effects when the MEC are co-cultured with different types of MSC. 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.

One of 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) and transforming growth factor- α (TGF- α), and an intracellular domain with tyrosine kinase activity (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- γ -1, p21^{ras} GTPase-activating protein (GAP), phosphatidylinositol 3'-kinase, src and src-like tyrosine kinases, raf and MAP kinase, lipocortin I, and c-erbB-2 (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).

The signal transduction pathways induced by TGF- α -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 (K_d of 0.1 nM) and low affinity (K_d of 3.6 nM) EGFR are present within cultured mouse MEC (54), and mammary glands from young and mature virgin, pregnant and lactating mice (55,56). 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 (57). Immunohistochemical analysis of normal human breast and benign mammary tumors indicated that EGFR were usually expressed at a low level (58).

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 (59,60), 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 (61). The increased levels of EGFR are generally due an increased level of EGFR mRNA expression (62,63). Gene amplification has only been observed in ~3% of the primary carcinomas studied (63,64). *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 detectable in the absence of EGFR protein (65). 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 (66). 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 (67-69).

2. EGF AND TGF- α

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- α , 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- α 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- α in Elvax pellets *in vivo* can stimulate end bud and ductal growth (end bud branching morphogenesis) in the mammary glands of virgin ovariectomized mice (70). EGF and TGF- α also enhanced lobuloalveolar development in hormonally primed virgin mice (71). TGF- α 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 where it can act as an autocrine and/or paracrine growth factor (72).

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- α transcripts, whereas only EGF transcripts are detected in the mammary glands of mid-lactating mice (70). Using conventional Northern blot analysis, TGF- α mRNA was detected within mammary glands of pregnant rats, but not from mammary glands of virgin or lactating female rats (72). Interestingly the concentration of TGF- α 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 (72). 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 (70). These cells tend to be rather dominant, and cytologically differentiated. In contrast, TGF- α 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- α 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 (73). TGF- α 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 (73). Immunohistochemical studies in the mammary glands of lactating rats localized EGF to the luminal surface of the secretory cells (74). Taken together, endogenous TGF- α 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- α is derived from both mammary gland parenchymal and mesenchymal cell types.

It seems interesting to speculate that TGF- α 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- α is required to determine whether each polypeptide plays a distinct role in normal mammary gland morphogenesis. If TGF- α 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 α 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- α within mammary gland ducts, end buds, and alveoli, as well as precancerous lesions, carcinoma *in situ*, and invasive carcinoma.

EGF and TGF- α have both been implicated as factors which can stimulate the proliferation of various types of tumors. Both EGF and TGF- α have been shown to be potent mitogens for breast cancer cells (1,75-81). Since the salivary gland is the dominant source of biologically active EGF in the body, Oka and co-workers surgically 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- α mRNA can be detected in both benign and malignant breast tumors at a similar frequency and level of expression (82). Immunohistochemical studies revealed minimal levels of TGF- α in normal human breast tissue, and increased levels in ductal hyperplasia, atypical ductal hyperplasia and ductal carcinoma *in situ* (83). TGF- α mRNA expression and immunoreactive TGF- α 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-Ha-ras protooncogene increased the level of TGF- α mRNA and protein expression by 5-10 fold (7). Addition of an anti-TGF- α neutralizing monoclonal antibody to the ras-transformed MEC inhibited anchorage-independent growth of these cells by 50-80% which indicated that TGF- α mediates at least part of the mitogenic effects of an activated ras gene in MEC. Overexpression of TGF- α can act as a dominant transforming gene in NOG-8 mouse cells, and human MCF-10A cells (6,7,84). These cells have previously been shown to express EGFR. Introduction of rat or human TGF- α into the germ line of transgenic mice induces abnormal morphogenesis and proliferation of the mammary glands (85).

3. METALLOPROTEINASES AND INVASION

Normal mammary epithelial development during puberty involves the controlled proliferation, differentiation, and invasion of the mammary gland fatty stroma, whereas breast cancer progression is characterized by a deregulation of cell proliferation, differentiation, apoptosis, and invasion of the mammary gland stroma and the vasculature. The degradative and invasive processes of branching morphogenesis and metastases are thought to initially depend on the activity of metalloproteinase enzymes which target the degradation of type IV collagen. The metalloproteinases, secreted as inactive enzymes, are activated by a mechanism involving either autocatalytic cleavage or by proteases within the microenvironment. Both 72 and 92 kDa inactive metalloproteinase enzymes have been described in breast cancer (86). The invasion of the mammary gland stroma by normal MEC involves the coordinate regulation of matrix degrading metalloproteinases, and the tissue inhibitors of metalloproteinases (TIMPs). Polypeptides and steroids appear to play distinct roles in the regulation of both of these processes (87,88). The details of their expression, mechanism(s) of action, and interactions during normal mammary and breast cancer development are incompletely understood.

4. MODEL SYSTEM UTILIZED FOR OUR STUDIES

The unique and powerful model system utilized for our studies was developed in our laboratory (89-96). 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 has recently been upgraded such that MEC can now be cultured alone or co-cultured with different types of mammary stromal cells (MSC, fibroblasts, pre-adipocytes, and/or adipocytes). This exciting new upgrade of our model system allows for the examination of epithelial cell-specific effects when the MEC are cultured without MSC, and of the actual effects that arise when the MEC are co-cultured with their natural counterparts, the MSC. The co-culture system attempts 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 myoepithelial cells, 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, and composed of cells arranged in a terminal end bud or a blunt end bud type of cellular organizational pattern (91). Electron microscopic examination revealed that most of the isolated mammary colonies were composed primarily of cytologically differentiated or immature epithelial cells. These colonies were classified as mammary epithelial organoids (MEO). The isolated 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 apo-transferrin [5 µg/ml], ascorbic acid [880 ng/ml], fatty acid-free bovine serum albumin [1 mg/ml], and gentamycin [50 µg/ml]) 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. In the presence of ALV Media with *mouse EGF* [10 ng/ml], most of the isolated end bud organoids developed into elaborate lobular, multilobular, and lobuloductal alveolar organoids. The alveolar organoids produce and accumulated abundant quantities of milk protein and lipid (91,93).

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- α are among those factors that stimulate breast cancer cell proliferation and invasion. Studies to directly compare the biological activities of EGF and TGF- α 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- α 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- α 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- α 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- α . The promotional activities of EGF and TGF- α 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- α 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 normal MEC as well as breast cancer cells have been shown to express EGFR as well as TGF- α . The roles that EGFR positive and/or TGF- α 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- α , 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- α . It is important to determine whether the response to EGF or TGF- α 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 (97). Such tight regulation seems to suggest that EGFR expression, the number and affinity of EGF and TGF- α 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- α 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.

1. 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. ³H-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 (MSC) for the Co-Culture Studies
- j. Co-Culture System for the Isolated MEO and MSC
- k. Immunocytochemistry for the Characterization of the Different MSC

2. Special Reagents, Supplies and Equipment utilized thus far:

- a. mouse EGF (mEGF)
- b. human recombinant EGF (hrEGF)
- c. human recombinant TGF- α (hrTGF- α)
- d. ³H-thymidine
- e. rabbit anti-rat polyclonal casein antibody
- f. Bio-tek automatic plate washer
- g. Bio-tek automatic plate reader
- h. Olympus CK2 microscope mounted with a Nikon FX-35A camera
- i. Falcon 24 well and 6 well tissue culture plates
- j. Costar transwell inserts with a transparent polyethylene tetracarboxylate membrane with a 0.45 μ m pore size
- k. BioRad mini-protein II gel system
- l. BioRad Silver Stain Plus Kit
- m. BioRad Ready Gel 4-20% Polyacrylamide Gradient Gels
- n. mouse monoclonal anti-pan cytokeratin antibody
- o. mouse monoclonal anti-vimentin antibody
- p. mouse Vectastain ABC Peroxidase Kit
- q. rabbit Vectastain ABC Peroxidase Kit
- r. hematoxylin, eosin Y, and oil red O stains
- s. Polaroid positive/negative film
- t. densitometer
- u. Beckman LS 1801 Scintillation Counter

Aim 2. To compare the biological activity of exogenous EGF and TGF- α 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.

Aim 3. To examine the temporal changes in the expression of EGFR mRNA, the number and affinity of EGF and TGF- α 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- α 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.

1. 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, MSC, and MDA-MB-468 human breast cancer cells
- e. Immunocytochemistry

2. Special Reagents, Supplies and Equipment utilized thus far:

- a. mouse EGF (mEGF)
- b. human recombinant EGF (hrEGF)
- c. human recombinant TGF- α (hrTGF- α)
- d. histology staining dishes and reagents
- e. rabbit anti-rat polyclonal casein antibody
- f. sheep anti-human polyclonal EGF receptor antibody
- g. Rabbit Vectastain ABC Peroxidase Kit
- h. Sheep Vectastain ABC Peroxidase Kit
- i. Mouse Vectastain ABC Peroxidase Kit
- j. Diaminobenzadine (DAB) tetrachloride
- k. Olympus BH-2 Microscope mounted with a Nikon FX-35A camera

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- α 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.

Aim 5. To compare the functional consequence of EGF- and TGF- α 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.

1. 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 Stromal Cells (MSC) for the Co-Culture Studies
- e. Co-Culture System for the Isolated MEO and MSC
- f. Preparation of Whole Cell Lysates
- g. SDS-Polyacrylamide Gel Electrophoresis
- h. Electrophoretic Transfer of Proteins from an SDS-Polyacrylamide Gel to a Nitrocellulose or an Immobilon Membrane
- i. Western Blot Analysis

2. Special Reagents, Supplies and Equipment:

- a. natural mouse EGF (mEGF)
- b. human recombinant EGF (hrEGF)
- c. human recombinant TGF- α (hrTGF- α)
- d. Falcon 24 well and 6 well tissue culture plates
- e. Costar transwell inserts with a transparent polytetrafluorocarbonate membrane with a 0.45 μ m pore size
- f. sheep anti-human polyclonal EGF receptor antibody
- g. recombinant anti-phosphotyrosine RC20 antibody conjugated to horseradish peroxidase
- h. mouse monoclonal anti-phosphotyrosine antibody, clone 4G10
- i. BioRad mini-protein II gel system
- j. BioRad Silver Stain Plus Kit
- k. BioRad Ready Gel 4-20% Polyacrylamide Gradient Gels
- l. peroxidase-conjugated donkey anti-sheep IgG
- m. peroxidase-conjugated donkey anti-mouse IgG
- n. nitrocellulose
- o. Immobilon P membrane
- p. enhanced chemiluminescence (ECL) Reagent
- q. Kodak X-OMAT AR Film

BODY

A. EXPERIMENTAL METHODS**1. BASIC EXPERIMENTAL DESIGN**

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 Media) 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 Media) which is ALV Media without hydrocortisone.

A series of primary culture studies was undertaken to examine the MEC-specific mitogenic, morphogenic, and lactogenic effects of EGF, EGF-responsiveness in primary rat MEC, and the duration of the EGF-mediated effects following the withdrawal of this growth factor. To more completely define the direct MEC-specific effects of EGF, newly isolated MEC were in the presence of 0, 0.1, 1, 10 or 100 ng/ml mouse EGF (mEGF) from day 0 through day 21 of the experiment. To evaluate the persistence or reversibility of the EGF-mediated effects, and then the ability of the cultured MEC to respond to EGF, newly isolated MEC were cultured without mEGF and/or in the presence of 10 ng/ml mEGF for specific time periods during a 21 day experiment. To determine whether EGF and TGF- α play similar or different roles in regulating the *in vitro* development of the MEC, the newly isolated MEC were cultured in the absence of either growth factor (No GF), or in the presence of 10 ng/ml mouse EGF (mEGF), 0.1, 1, or 10 ng/ml human recombinant EGF (hrEGF), or 0.1, 1, or 10 ng/ml human recombinant TGF- α (hrTGF- α) from day 0 through day 21 of the experiment. A majority of the studies carried out thus far examined the effects of EGF and TGF- α on branching alveolar morphogenesis. A preliminary experiment was undertaken to examine the secretion of matrix-degrading metalloproteinases by MEC cultured in the presence of ALV Media or EB Media without either growth factor or with 10 ng/ml hrEGF or 10 ng/ml hrTGF- α .

For the preliminary mammary stromal-epithelial experiments, a 6 well transwell tissue culture system was used (Falcon and Costar tissue culture products). The transwell inserts contained a transparent polyethylene tetracarboxylate membrane with a pore size of 0.45 µm and fit into a standard 6 well tissue culture plate. The lower culture wells were plated without or with a mixed population of adherent MSC 24 hr prior to the initiation of the co-culture in F12/DMEM with 10% (v/v) fetal bovine serum and were then switched to ALV Media with 10 ng/ml mEGF for the duration of the co-culture experiment. Like the MEC, the MSC were isolated from the mammary glands of 7 week old pubescent female rats. These MSC appeared to exhibit the cytological and biochemical characteristics of mammary fibroblasts and pre-adipocytes, respectively. The newly isolated MEC were cultured within the EHS-derived RBM in the presence of ALV Media with 10 ng/ml mEGF in the upper insert.

2. MTT ASSAY TO QUANTIFY VIABLE CELL NUMBER

Specific changes in the number of viable cells was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (89,92). 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 formazan 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.

3. ³H-THYMIDINE INCORPORATION ASSAY TO MONITOR DNA SYNTHESIS

Specific changes in the rate of DNA synthesis were evaluated using a ³H-thymidine incorporation assay (89). Briefly, individual cultures were incubated with 5 microcuries of ³H-thymidine for 24 hr at 37°C in 1 ml of ALV Media without either growth factor, with 0.1, 1, or 10 ng/ml hrEGF, or with 0.1, 1, or 10 ng/ml hrTGF- α . The RBM surrounding the MEC was digested away with 5 units/ml grade II dispase in F12/DMEM medium. The organoids were recovered, washed and incubated with 5% (v/v) trichloroacetic acid for 16-24 hr at 4°C. The precipitate was washed and solubilized with 0.1 N NaOH with 0.1% (v/v) Triton X-100. Sample pH was adjusted with 1 N HCl and radioactive counts per minute (cpm) were quantified using a Beckman LS 1801 Scintillation Counter.

4. ENZYME LINKED IMMUNOSORBANT ASSAY TO MONITOR CASEIN ACCUMULATION, A MARKER OF MEC FUNCTIONAL DIFFERENTIATION

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 (90,92). 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 harvested independently of the organoid plus RBM sample. PMSF at a final concentration of 0.1 mM was added to the conditioned media and the samples were stored at -20°C. At the end of the experiment, all media samples harvested from an individual culture well were pooled together at equal ratios and were stored at -20°C. The organoid plus RBM samples were harvested with 1 ml of borate-buffered saline (BBS [5 mM boric acid, 1.25 mM sodium tetraborate and 150 mM NaCl at pH 8.45]) containing PMSF, soybean trypsin inhibitor and leupeptin at final concentrations of 0.1 mM, 100 ng/ml and 20 ng/ml, respectively. Alternatively, the organoid plus RBM samples were harvested with 400 μ l of 1% (v/v) NP-40 lysis buffer [150 mM sodium chloride, 50 mM Tris, 2 mM ethylenedinitrilotetraacetic acid (EDTA), 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 phenylmethyl-sulfonyl fluoride (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). Occasionally, some organoids remained intact after the sonication step, and samples were passed through a 25 gauge syringe 10 times. The samples were then centrifuged at 12,000 x g for 15 min at 4°C. The resulting supernatants were stored at -20°C. For the purpose of the data presented below, each individual culture

sample was generated by combining the pooled conditioned medium sample and organoid plus RBM sample at a ratio proportionate to their respective volumes. 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.

5. MORPHOLOGIC CLASSIFICATION

Three culture wells of living colonies for each treatment group were examined using an Olympus CK2 microscope to determine visible changes in colony number, size, coloring, and/or shape at the end of a 21-day culture period. The cultured mammary colonies were classified as end bud, alveolar or end bud/alveolar MEO, adipocyte colonies or squamous colonies. Briefly, end bud MEO had a pale appearance, alveolar MEO had a dark and highly translucent appearance, and end bud/alveolar MEO were a hybrid organoid with some pale lobules and some dark and translucent lobules [Figure 1]. Homogenous end bud and alveolar MEO could be lobular, multilobular or lobuloductal in shape, whereas end bud/alveolar MEO were only multilobular or lobuloductal. The end bud MEO were either non functional or produced modest quantities of milk protein and lipid. In contrast, the alveolar MEO expressed, synthesized, and secreted abundant milk protein and accumulated extensive intracellular lipid. The adipocyte colonies were composed of loosely associated adipocytes-like with a highly refractile appearance and spherical shape, whereas the squamous colonies had a dramatic keratotic whorl imprint on their surface.

6. GELATIN ZYMOGRAM ANALYSIS

Secreted matrix degrading metalloproteinase enzymes were examined by gelatin zymogram analysis of conditioned media samples using a slight modification of the method described by Heussen and Dowdle (98). Recently, the gelatin zymogram method was used by Dr. Ping-Ping Lee in our laboratory to examine metalloproteinase secretion in cultured MEC. Briefly, conditioned medium was collected from individual culture wells, cleared by a 15 min centrifugation at 4°C, and separated by electrophoresis through a 10% SDS-polyacrylamide gel containing 1% gelatin. Gels were washed, incubated in substrate reaction buffer (50 mM Tris, pH8, 5 mM calcium chloride, 0.02% sodium azide) at 37°C for 16-24 h under gentle shaking, and then stained in 0.5% (w/v) Coomassie blue in acetic acid:isopropanol:H₂O (1:3:6) for 1h. Proteins with gelatinase activity were identified by negative staining. Photographs were taken and the negatives scanned using a densitometer.

B. RESULTS

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

1. OVERVIEW

A variety of experiments have been undertaken to examine the biological effects of EGF and TGF- α on the proliferation, differentiation, morphogenesis, and invasion of normal rat MEC. This information provides a more clear understanding of the roles that each of these growth factors play in regulating the normal developmental processes of branching end bud and alveolar morphogenesis. In addition, it also provides the baseline for comparative studies between normal MEC and breast cancer cells (that is carcinogen-transformed and/or tumor-promoted MEC). Thus far, a majority of these studies have focused on establishing the epithelial cell-specific effects of TGF- α in normal MEC, comparing the biological activities of mouse as well as human recombinant forms of EGF with those of human recombinant TGF- α , and determining whether the effects of EGF and TGF- α are additive, synergistic or antagonistic. This whole group of studies was carried out in MEC cultured under conditions that promote branching alveolar morphogenesis. A preliminary study was undertaken to examine matrix-degrading metalloproteinase secretion in MEC cultured under conditions that promote branching alveolar compared to end bud morphogenesis, and to determine whether EGF or TGF- α regulate the secretion of these enzymes. In addition, two preliminary experiments were undertaken to ascertain the feasibility of using a primary mammary epithelial-stromal cell co-culture system to more accurately examine the physiological effects of these two growth factors. The detail of these experiments is described below.

2. EFFECT ON MEC PROLIFERATION

a. COMPARISON BETWEEN AND COMBINATORIAL EFFECT OF MOUSE EGF (mEGF) AND HUMAN RECOMBINANT TGF- α (hrTGF- α) ON CELL NUMBER

To test our hypothesis that TGF- α is a more potent mitogen than EGF and to determine whether the mitogenic activities of these two ligands of the EGFR are additive or synergistic, mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 10 ng/ml mEGF, with 1 or 10 ng/ml hrTGF- α , with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α , or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . When compared to the modest mitogenic response observed when the MEC were cultured in ALV Media without either growth factor, there was a significant increase in cell number at day 7, 14 and 21 of the experiment when the MEC were cultured in ALV Media with 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or either combination of mEGF and hrTGF- α [Figure 2]. Although the mitogenic activity of ALV Media with 1 ng/ml TGF- α was approximately 50% as effective as that of ALV Media with 10 ng/ml mEGF, ALV Media with 10 ng/ml hrTGF- α was as mitogenic as ALV Media with mEGF [Figure 2]. Both combinations of mEGF and TGF- α were also as effective as ALV Media with 10 ng/ml mEGF [Figure 2]. Importantly, the effects of EGF and TGF- α were not additive, providing additional confirmation that both act through the same receptor.

b. COMPARISON BETWEEN THE EFFECT OF hrEGF AND hrTGF- α ON CELL NUMBER

To directly compare the mitogenic activity of the human recombinant forms of these two EGFR ligands, mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . Mammary organoids cultured in ALV Media without either growth factor,

with 0.1 ng/ml hrEGF or with 0.1 ng/ml hrTGF- α underwent very little net cell growth during the 21 day experiment [Figure 3]. Cell number significantly increased when the organoids were cultured in ALV Media with 1 or 10 ng/ml hrEGF, or with 1 or 10 ng/ml hrTGF- α [Figure 3]. ALV Media with 1 ng/ml hrEGF was more mitogenic to the cultured MEO than ALV Media with 1 ng/ml hrTGF- α . Surprisingly, ALV Media with either 1 ng/ml hrEGF, 10 ng/ml hrEGF or 10 ng/ml hrTGF- α stimulated a similar increase in the number of MEC during the 3 week culture experiment [Figure 3].

c. COMPARISON BETWEEN THE EFFECT OF hrEGF AND hrTGF- α ON DNA SYNTHESIS

The MTT assay was used to monitor the effect of these two EGFR ligands on viable cell number. Cell number is not only influenced by the rate of cell growth, but also the rate of cell death. EGF has been shown to enhance cell growth as well as cell survival (99). To begin to test the hypothesis that TGF- α has mitogenic, but not survival activity in cultured MEC, ^3H -thymidine incorporation was used to selectively monitor DNA synthesis and thereby allow the direct comparison of the mitogenic activities of the human recombinant forms of these two EGFR ligands. The effect of TGF- α on MEC apoptosis will have to be evaluated in another type of experiment using methodologies that are able to directly monitor programmed cell death. Mammary organoids were cultured for either 8 or 15 days within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α , and DNA synthesis within individual culture wells was examined using a 24 hr incorporation of ^3H -thymidine starting on either day 7 or day 14 of the experiment. Although similar levels of DNA synthesis were observed when MEO were cultured in ALV Media without either growth factor, with 0.1 ng/ml hrEGF or with 0.1 ng/ml hrTGF- α , significantly higher levels were observed when organoids were cultured in ALV Media with 1 or 10 ng/ml hrEGF or with 1 or 10 ng/ml hrTGF- α [Figure 4]. The mitogenic activities of ALV Media with either 1 ng/ml hrEGF, 10 ng/ml hrEGF, 1 ng/ml hrTGF- α , or 10 ng/ml hrTGF- α were all similar [Figure 4].

3. EFFECT ON MEC FUNCTIONAL DIFFERENTIATION

a. COMPARISON BETWEEN AND COMBINATORIAL EFFECT OF mEGF AND hrTGF- α ON CASEIN ACCUMULATION

Mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 10 ng/ml mEGF, with 1 or 10 ng/ml hrTGF- α , with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α , or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . This experiment was designed to test the hypothesis that TGF- α preferentially supports the outgrowth of non functional MEC and may even directly inhibit the functional differentiation of MEC, and that EGF may have some mitogenic activity, but that this growth factor is a preferential differentiation factor (a lactogen, morphogen and possibly even a survival factor in our system). If this hypothesis is true then TGF- α should either have no effect or an inhibitory effect on casein accumulation. In addition, we were interested in determining whether TGF- α would be able to block the lactogenic activity of EGF. If the latter were so, we wondered whether this would be a concentration dependent effect. When cultured in ALV Media without either growth factor, the MEO were able to

accumulate only a modest amount of casein [Figure 5]. Surprisingly, ALV Media with 1 ng/ml hrTGF- α caused a modest increase in casein accumulation. Moreover, ALV Media with 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or either combination of mEGF and hrTGF- α caused a dramatic and sustained increase in casein accumulation [Figure 5]. The lactogenic activities of ALV Media with 10 ng/ml hrTGF- α or with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α were equivalent to that of ALV Media with 10 ng/ml mEGF, while that observed in the the combination of 10ng/ml mEGF and 10 ng/ml hrTGF- α was slightly less lactogenic than that of ALV Media with 10 ng/ml mEGF [Figure 5].

b. COMPARISON BETWEEN THE LACTOGENIC EFFECTS hrEGF AND hrTGF- α

To directly compare the lactogenic activity of the human recombinant forms of these two EGFR ligands, mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . ALV Media with either 0.1 ng/ml hrEGF or 0.1 ng/ml hrTGF- α had little to no effect on casein accumulation compared to MEO cultured in the presence of ALV Media without either growth factor [Figure 6]. ALV Media with either 1 or 10 ng/ml or 1 or 10 ng/ml hrTGF- α were all found to induce casein accumulation to a similar extent. The day 14 value obtained in ALV Media with 1 ng/ml hrEGF was, however, significantly lower than the values obtained for ALV Media with either 10 ng/ml hrEGF, or 1 or 10 ng/ml hrTGF- α [Figure 6]. The significance of this reduction is called into question since neither the day 7 or the day 21 values were reduced. Moreover, this experiment still needs to be repeated.

4. EFFECT ON IN VITRO MORPHOGENESIS

a. COMPARISON BETWEEN AND COMBINATORIAL MORPHOGENIC EFFECTS OF mEGF AND hrTGF- α

To determine whether TGF- α exerted morphogenic activity in the cultured MEO, to compare the morphogenic activities of mEGF with that of hrTGF- α , and then to ascertain whether the morphogenic effects of these two growth factors were additive, synergistic, or antagonistic, primary mammary organoids were cultured for up to 21 days within the RBM in ALV Media without EGF or TGF- α , or with 10 ng/ml mEGF, 1 hrTGF- α , 10 ng/ml hrTGF- α , the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF α , or the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . After 21 days of culture development, the individual colonies were examined by light microscopic examination and the colonies classified as end bud, alveolar or hybrid end bud/alveolar MEO. When compared to cells cultured in ALV Media without either growth factor for the entire 3 week experiment, there was a lower frequency of end bud organoids and a corresponding higher frequency of alveolar as well as hybrid end bud/alveolar MEO when the cells were cultured with 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or either combination of mEGF and hrTGF- α [Figure 7]. In addition, there was a lower frequency of end bud MEO and a higher frequency of hybrid end bud/alveolar organoids with 1 ng/ml hrTGF- α [Figure 7]. The morphogenic activities of ALV Media with 10 ng/ml mEGF were compared to that of different TGF- α supplemented ALV Media. There was a lower proportion of alveolar MEO when the cells were cultured with 1 ng/ml hrTGF- α [Figure 7]. Furthermore, there was a lower proportion of alveolar MEO and a corresponding higher

frequency of hybrid end bud/alveolar organoids when the cells were cultured with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α when compared to that observed with ALV Media with 10 ng/ml mEGF [Figure 7].

The end bud, alveolar and hybrid end bud/alveolar MEO were also sub-classified based on their shape into lobular, multilobular or lobuloductal organoids. When compared to cells cultured in ALV Media without either growth factor, ALV Media with 10 ng/ml hrTGF- α increased the frequency of lobular and multilobular alveolar as well as end bud/alveolar organoids, whereas ALV Media with 10 ng/ml mEGF only increased the frequency of lobular and multilobular alveolar MEO [Figure 8]. When compared to cells cultured in ALV Media with 10 ng/ml mEGF, ALV Media with 1 ng/ml hrTGF- α reduced the proportion of lobular and multilobular alveolar organoids that developed during the 3 week culture period [Figure 8], while ALV Media with 10 ng/ml hrTGF- α increased the proportion of hybrid multilobular end bud/alveolar organoids [Figure 8]. Moreover, ALV Media with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α increased the proportion of hybrid multilobular as well as lobuloductal end bud/alveolar organoids, whereas ALV Media with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α reduced the proportion of multilobular alveolar organoids and increased the proportion of hybrid multilobular as well as lobuloductal end bud/alveolar organoids when compared to cells cultured in ALV Media with 10 ng/ml mEGF [Figure 8].

b. COMPARISON BETWEEN THE MORPHOGENIC EFFECTS OF hrEGF AND hrTGF- α

To directly compare the morphogenic effects of the human recombinant forms of these two EGFR ligands, mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . After 21 days of culture development, the individual colonies were examined by light microscopic examination and the colonies classified as end bud, alveolar or hybrid end bud/alveolar MEO. ALV Media with 1 ng/ml hrTGF- α supported the outgrowth of a lower frequency of end bud MEO than ALV Media with either 0.1 ng/ml hrEGF or hrTGF- α [Figure 18]. ALV Media with either 0.1 ng/ml hrEGF, 1 ng/ml hrEGF or 0.1 ng/ml hrTGF- α supported the outgrowth of a lower or higher frequency of alveolar organoids than ALV Media with 1 hrTGF- α or with 10 ng/ml hrTGF- α , respectively [Figure 9]. ALV Media with 10 ng/ml hrEGF supported outgrowth of a lower or higher frequency of alveolar organoids than ALV Media with 1 hrTGF- α or with 10 ng/ml hrTGF- α , respectively [Figure 18]. The end bud, alveolar and hybrid end bud/alveolar MEO were also sub-classified based on their shape into lobular, multilobular or lobuloductal organoids. ALV Media with 0.1 ng/ml hrEGF supported the outgrowth of a higher proportion of multilobular end bud organoids than ALV Media with 10 ng/ml hrTGF- α , whereas the distribution of the other end bud, alveolar and hybrid end bud/alveolar MEO based on shape were similar when comparing between the different hrEGF and hrTGF- α supplemented ALV Media conditions [Figure 10].

5. EFFECT ON MATRIX-DEGRADING METALLOPROTEINASE SECRETION

Primary mammary organoids were cultured for 3.5, 7 or 11 days within the RBM either in the presence of ALV Media without either growth factor, with 10 ng/ml hrEGF or with 10 ng/ml hrTGF- α , or in the presence of EB Media without either growth factor, with 10 ng/ml hrEGF or with 10 ng/ml hrTGF- α . Secreted matrix-degrading metalloproteinases were

analyzed by gelatin zymography. The results from the day 3.5 samples are in the Table, with values expressed as the relative band intensity quantified from the densitometric scan of the negative. Similar zymogram profiles were observed for the other time points tested. The most dramatic difference in metalloproteinase secretion was observed when the MEO were cultured in ALV Media compared to EB Media. The only difference in the composition of these two medium is the inclusion of 1 µg/ml hydrocortisone in ALV Media, but not in EB Media. EB Media caused the outgrowth of a higher proportion of extensive and highly branched lobuloductal end bud organoids compared to that observed with ALV Media. It seems reasonable to expect that more matrix-degrading metalloproteinases were secreted under conditions which supported the aggressive invasion of the organoids into the surrounding RBM. There was a general increase in gelatinase activity in the conditioned media from MEO cultured in EB compared to ALV Media. There was a specific 2-3 fold increase in the gelatinase activity of the ~92 kDa metalloproteinase and a 2 fold increase in the activity of the ~150 kDa protease when the MEO were cultured in EB compared to ALV Media. This preliminary study needs to be followed up to determine whether MEC cultured in EB Media with 10 ng/ml hrEGF or with 10 ng/ml hrTGF- α secrete a more significant amount of the ~92kDa metalloproteinase compared to that observed when the MEC are cultured in ALV Media without either growth factor. In addition, the activity of the ~150 kDa needs to be characterized to determine whether it is a true metalloproteinase.

Relative Absorbance (Arbitrary Units)

MMP	ALV Media + No GF	ALV Media + hrEGF	ALV Media + hrTGF-α	EB Media + No GF	EB Media + hrEGF	EB Media + hrTGF-α
~72 kDa	7.9	7.3	9.8	10.6	9.2	9.4
~92 kDa	6.7	7.2	6.7	12.2	15.0	18.6
~150 kDa	4.3	4.4	5.5	9.7	9.4	8.2

6. FEASIBILITY OF USING THE PRIMARY CO-CULTURE SYSTEM

Two preliminary experiments were undertaken to determine whether we could culture primary MEO within the RBM in a transwell insert (with a polyethylene terecarbonate membrane with a 0.45 µm pore size) in the presence of ALV Media with 10 ng/ml mEGF. The transwell insert fits into culture wells that were plated without or with primary mammary stromal cells (MSC, a mixed population of fibroblasts and pre-adipocytes). The MSC are also cultured in the presence of serum-free ALV Media with 10 ng/ml mEGF. In both experiments, MEC proliferation was significantly higher when the MEO were co-cultured with MSC compared to those cultured alone. In the second pilot experiment, colony morphology was also examined between the two culture groups. After 21 days of culture development, 72% of the colonies co-cultured with MSC were classified as alveolar MEO whereas only 37% of the colonies cultured without MSC were alveolar organoids. In the absence of MSC, 33% of the colonies were hybrid end bud/alveolar organoids while only 2% of the colonies were classified as hybrid end bud colonies in the presence of MSC. In addition, MEO co-cultured with MSC preferentially developed into multilobular alveolar organoids (37% of total colonies). In the absence of MSC, the MEO preferentially develop into hybrid multilobular end bud/alveolar organoids (33% of total colonies). Mammary organoids co-cultured with MSC also produced more casein milk protein as revealed by casein Western blot analysis than MEO cultured alone. Finally, colony survival was substantially higher when MEO were

co-cultured with MSC compared to that observed in the absence of MSC (where $n=3$, colony numbers per culture well were 1757.7 ± 32.6 and 983.3 ± 19.8 , respectively).

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

These studies are designed to compare the biological activity of exogenous EGF and TGF- α 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. The studies for this aim will not be initiated until the objectives of aim 1 are satisfied.

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- α .

These studies are designed to examine the temporal changes in the expression of EGFR mRNA, the number and affinity of EGF and TGF- α 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- α . The goal of this aim is to determine the relationship between the expression, number, affinity and/or regional localization of EGFR, and MEC proliferation, differentiation, morphogenesis, and/or invasion. Thus far, the studies have focused on adapting established methodologies to our primary culture samples, and in optimizing protocols for the use of individual antibodies. Specifically, I have focused my attention on modifying the procedure for paraffin-embedding MEO samples for the analysis of hundreds of organoids per treatment per time point, and on adopting immunocytochemistry protocols. The methodology was first worked out using our polyclonal casein antibody and more recently we have been attempting to optimize the immunocytochemistry protocol for the EGFR antibody. In preliminary studies using the human breast cancer cells MDA-MB-468 (known to overexpress EGFR on their surface), strong membrane reactivity was detected using the sheep anti-human EGFR antibody. We therefore know that our technique is working. However, using this same antibody, only weak membrane reactivity was detected in our cultured MEO. In future studies we plan to try a sandwich technique to amplify the weak signal observed in the cultured MEO using the sheep anti-human EGFR antibody. Recently, a rat-reactive mouse anti-human EGFR antibody has become available. The reactivity of this antibody will be tested in the cultured MEO.

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 NMU-treated, PDBu-treated, and NMU- plus PDBu-treated MEC, and then compare these profiles with that observed within normal MEC undergoing either end bud or alveolar branching morphogenesis in response to EGF or TGF- α . The goal of this aim is to determine 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.

AIM 5. TEMPORAL ANALYSIS OF TYROSINE PHOSPHORYLATED SIGNAL TRANSDUCTION PROTEINS IN NORMAL MEC AND VARIOUS TYPES OF TRANSFORMED MEC CULTURED IN THE PRESENCE OF EITHER EGF OR TGF- α .

These studies are designed to compare the functional consequence of EGF and TGF- α 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. Thus far, our studies have focused on adapting established methodologies to analyze rapid tyrosine phosphorylation events in cultured MEO. This has proven to be quite a challenge. Control experiments have demonstrated that two sheep anti-human EGFR antibodies detect a 167 or a 150 kDa protein in control lysates from human epidermoid A431 cells and human breast cancer cells MDA-MB-468, respectively. Both cell lines are known to overexpress EGFR on their surface. The anti-EGFR antibodies were also able to detect a 162 kDa protein in 50 μ g of lysate from purified MEC isolated from mammary glands of lactating female rats. In addition, we have been able to obtain two independent anti-phosphotyrosine antibodies, which we demonstrated could react with a classical variety of tyrosine phosphorylated proteins in A431 cells (150 kDa, 131 kDa, 80 kDa, 66 kDa and 47 kDa proteins). Importantly, we were able to detect phosphotyrosine protein profiles in day 14 cultured MEO lysates (122 kDa, 73 kDa, 50 kDa, 39 kDa, 34 kDa, 32 kDa and 12 kDa proteins). We have tried various methods for ligand-dependent activation of the EGFR within the organoid samples to detect EGFR and tyrosine phosphorylated EGFR in whole cells lysates. Thus far, we appear to have a sensitivity problem using the sheep anti-human EGFR antibody. The RBM within the MEO cultures constitutes a majority of the protein content of the organoid samples. Although EGFR activation in established monolayer cultures is straightforward, it appears to be more complicated using a three-dimensional culture system. The physiological significance of the latter, however, is greater. The general duration of the EGFR activation period ranges from 5 to 15 minutes. We have tried and will continue to try different strategies to get around the technical difficulties of studying EGFR activation in our three-dimensional culture system. For example, dispase digestion of the RBM, prior to ligand-dependent activation of the EGFR in MEO did not work. In addition, increasing the organoid plating density from 3×10^5 cells with a total of 400 μ l of RBM per sample to 5×10^6 cells with 400 μ l of RBM per sample to increase the amount of cellular protein in the sample did not work.

Alternatively, we have attempted to examine EGFR activation by immunocytochemistry. This was done by using immunocytochemical analysis of EGFR

expression and phosphotyrosine activity in serial sections of formalin-fixed and paraffin-embedded MEO. The sheep anti-human EGFR antibody is showing weak membrane reactivity using DAB as the chromogen, whereas two anti-phosphotyrosine antibodies aren't showing any reactivity at this point.

C. DISCUSSION

The main objective of Aim 1 was to define the roles that EGF and TGF- α play in regulating the normal developmental processes of branching end bud and alveolar morphogenesis. In addition, this information would establish the baseline for comparative studies between normal MEC and breast cancer cells (that is carcinogen-transformed and/or tumor-promoted MEC) outlined for Aim 2. The epithelial cell-specific effects of EGF and TGF- α were examined using a three-dimensional primary culture system developed in our laboratory. Non milk producing MEC were isolated as lobular end bud-like mammary epithelial organoids (MEO) from pubescent female rats at a time of development when these rats are maximally sensitive to carcinogen-induced development of mammary tumors. EGF and TGF- α were found to regulate MEC proliferation, functional differentiation, *in vitro* morphogenesis, and possibly matrix-degrading metalloproteinase secretion. My previous data demonstrated that, mouse EGF (mEGF) stimulated MEC proliferation at concentrations of 0.1, 1, 10 or 100 ng/ml. Maximal cell growth was, however, induced when the MEC were cultured in ALV Media with 10 or 100 ng/ml mEGF. ALV Media with 1, 10 or 100 ng/ml mEGF induced MEO to undergo extensive functional differentiation as evidenced by the dramatic increase in casein accumulation in the cultures. Maximal casein accumulation was observed with ALV Media with 10 ng/ml mEGF. EGF at 1, 10 or 100 ng/ml stimulated alveolar morphogenesis and the transition from lobular into multilobular organoids. ALV Media with 10 ng/ml mEGF also induced the development of extensive lobuloductal MEO. Removal of mEGF on day 7 of culture development reduced the mitogenic response of ALV Media with 10 ng/ml mEGF, but did not have any effect on the magnitude of the lactogenic or morphogenic responses. MEC cultured in the absence of EGF remained sensitive to the mitogenic and lactogenic effects of mEGF, but were less responsive to the morphogenic effects of this growth factor when added on day 14 of a 3 week culture experiment.

Preliminary data from other laboratories suggested that TGF- α 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- α should stimulate MEC proliferation, end bud morphogenesis, invasion of the organoids through the ECM (extensive lobuloductal morphogenesis), and the elaboration of matrix-degrading metalloproteinases. In contrast, EGF should induce more differentiated functions in the cultured MEO. In testing this general hypothesis, we demonstrated that ALV Media with 1 ng/ml hrTGF- α was ~ 50% as mitogenic, while ALV Media with 10 ng/ml hrTGF- α was as mitogenic as ALV Media with 10 ng/ml mEGF. The mitogenic activities of both combinations of mEGF and hrTGF- α were neither additive, synergistic nor antagonistic providing additional confirmation that both act through the same receptor. The next study directly compared the abilities of the human recombinant forms of both growth factors to affect viable cell number as well as the rate of DNA synthesis. ALV Media with 1 ng/ml hrEGF increased cell number more than ALV Media with 1 ng/ml hrTGF- α , and was as effective as ALV Media with 10 ng/ml of either hrEGF or hrTGF- α . Interestingly, ALV Media with 1 ng/ml hrEGF, 10 ng/ml hrEGF, 1 ng/ml hrTGF- α , or 10 ng/ml

hrTGF- α stimulated DNA synthesis to the same extent. Additional studies are required to determine whether these two ligands of the EGFR differ in their ability to regulate apoptosis.

We used casein accumulation as a qualitative indicator of functional differentiation, to test the hypothesis that TGF- α inhibits the functional differentiation of MEC, and to determine whether TGF- α was able to block the lactogenic effect of EGF. Unexpectedly, TGF- α was a lactogenic growth factor in our culture system. In fact, ALV Media with 0.1 ng/ml hrTGF- α induced a modest increase in casein accumulation, 1 ng/ml hrTGF- α induced a dramatic, but not sustained lactogenic response, and 10 ng/ml hrTGF- α induced extensive functional differentiation as evidenced by the dramatic and sustained increase in casein accumulation. When compared to the lactogenic activities of ALV Media with 10 ng/ml mEGF or 10 ng/ml hrTGF- α , ALV Media with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α was as lactogenic, while ALV Media with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α was somewhat less lactogenic. The repeat experiment should tell us whether this combinatorial effect is worth following up. Studies were also carried out to directly compare the lactogenic activities of the human recombinant forms of EGF and TGF- α . ALV Media with hrEGF or hrTGF- α at either 1 or 10 ng/ml increased casein accumulation to a similar extent, whereas ALV Media with hrEGF or hrTGF- α at 0.1 ng/ml did not exert any lactogenic activity in the cultured MEO. The day 14 value obtained in ALV Media with 1 ng/ml hrEGF was, however, significantly lower than the values obtained for ALV Media with either 10 ng/ml hrEGF, or 1 or 10 ng/ml hrTGF- α [Figure 6]. The significance of this reduction is called into question since neither the day 7 or the day 21 values were reduced. Moreover, this experiment still needs to be repeated.

Studies were then carried out to determine whether TGF- α exerted an effect on MEO branching morphogenesis, to directly compare the morphogenic effects of EGF with that of TGF- α , and to determine if the morphogenic effects of EGF and TGF- α were additive, synergistic or antagonistic. TGF- α promoted the outgrowth of alveolar as well as hybrid end/bud alveolar MEO, and specifically supported the development of lobular and multilobular alveolar organoids as well as hybrid multilobular end bud/alveolar organoids. When compared to the morphogenic activities of ALV Media with 10 ng/ml mEGF, ALV Media with 1 or 10 ng/ml hrTGF- α or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α supported the outgrowth of a lower proportion of alveolar MEO, whereas ALV Media with 10 ng/ml mEGF or with the combination of mEGF and hrTGF- α at 10 ng/ml also stimulated the outgrowth of a higher frequency of hybrid end bud/alveolar organoids. Furthermore, ALV Media with 1 ng/ml hrTGF- α suppressed the outgrowth of lobular and multilobular alveolar organoids that developed during the 3 week culture period, while ALV Media with 10 ng/ml hrTGF- α stimulated the development of hybrid multilobular end bud/alveolar organoids when compared to cells cultured in ALV Media with 10 ng/ml mEGF. Moreover, ALV Media with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α increased the proportion of hybrid multilobular as well as lobuloductal end bud/alveolar organoids, whereas ALV Media with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α reduced the proportion of multilobular alveolar organoids and increased the proportion of hybrid multilobular as well as lobuloductal end bud/alveolar organoids when compared to cells cultured in ALV Media with 10 ng/ml mEGF when compared to that observed in ALV Media with 10 ng/ml mEGF. When directly comparing the morphogenic activities of the two ligands of the EGFR, ALV Media with 10 ng/ml hrEGF supported the outgrowth of a higher frequency of alveolar organoids than ALV Media with 10 ng/ml hrTGF-

α . There was a similar sub-distribution of lobular, multilobular and lobuloductal end bud, alveolar and hybrid end bud/alveolar MEO when the MEO were cultured in ALV Media supplemented with 1 or 10 ng/ml hrEGF or hrTGF- α .

The most dramatic difference in the secretion of metalloproteinases was observed when the MEO were cultured in ALV Media compared to EB Media. The only difference in the composition of these two medium is the inclusion of 1 μ g/ml hydrocortisone in ALV Media, but not in EB Media. Interestingly, EB Media preferentially supported the outgrowth of extensive and highly branched lobuloductal end bud organoids. It seems reasonable to expect that more matrix-degrading metalloproteinases would be secreted under conditions that also caused the lobuloductal end bud organoids to aggressively invade the surrounding RBM. There was a specific 2-3 fold increase in the gelatinase activity of the ~92 kDa metalloproteinase and a 2 fold increase in the activity of a ~150 kDa protease when the MEO were cultured in EB compared to ALV Media. Our preliminary experiment also suggests that MEO cultured EB Media with 10 ng/ml hrEGF or hrTGF- α secreted more significant amount of the ~92kDa metalloproteinase compared to that observed when the MEC were cultured in ALV Media without either growth factor. However, there weren't significant differences in the secretion of matrix-degrading metalloproteinases when MEO were cultured in hrEGF compared hrTGF- α .

Finally, our preliminary studies suggest that it will be possible to take advantage of the recent upgrade to our primary mammary culture system. MEO co-cultured with mammary stromal cells (MSC) proliferate more extensively, accumulate more casein milk protein, preferentially undergo extensive multilobular alveolar morphogenesis, and secrete a different profile of matrix degrading metalloproteinases than MEO cultured without MSC. Co-culturing MEO with MSC also enhanced colony survival. These preliminary studies demonstrated the importance of stromal-epithelial interactions in regulating various aspects of MEC development. Our upgraded system will, therefore be used to study the epithelial cell-specific effects of EGF and TGF- α on MEC development when the MEO are cultured without mammary stromal cells, and the potentially more physiologically relevant effects of these two ligands of the EGFR on normal MEC development when the MEO are co-cultured with their natural counterparts, mammary stromal cells.

CONCLUSIONS

The studies carried out thus far have demonstrated that both EGF and TGF- α are potent epithelial cell-specific regulators of MEC proliferation, functional differentiation, branching morphogenesis, and possibly secretion of matrix-degrading metalloproteinases. Although the preliminary data from other laboratories suggested that TGF- α was a more dominant stem cell mitogen, and that EGF preferentially supported epithelial cell differentiation, our studies suggest that the biological activities of the two ligands of the EGFR are rather similar. In the studies carried out thus far, EGF and TGF- α are effective mitogens, lactogens and morphogens for MEC cultured within the RBM in the presence of ALV Media. Our future studies will focus on comparing the variety of biological effect of EGF to TGF- α observed in normal MEC cultured alone or with primary mammary stromal cells in the presence of ALV Media and then EB Media. ALV Media promotes extensive branching alveolar morphogenesis and functional differentiation in the presence of either one of the EGFR ligands, whereas EB Media which promotes extensive proliferation, end bud lobuloductal morphogenesis and invasion into the surrounding RBM. By taking advantage of the recent upgrade of our model system we can simultaneously examine the epithelial cell-specific effects when the MEC are cultured without mammary stromal cells (MSC), as well as the actual effects that arise when the MEC are co-cultured with their natural counterparts, the MSC (fibroblasts, pre-adipocytes, and/or adipocytes). The co-culture system attempts to more faithfully reconstitute the complex microenvironment of the mammary gland in a defined culture setting. This type of upgrade to our model system represents a dramatic advancement for those studying the regulation mammary biology and breast cancer.

After completing these baseline studies in normal MEC, we will use our model system to simultaneously monitor effects on the proliferation, cytological and functional differentiation, morphogenesis as well as invasion of normal and carcinogen-transformed and/or tumor-promoted MEC in primary culture under defined, serum-free culture conditions. We hope to determine whether either or both of these polypeptides enhances carcinogen-induced tumor progression and/or invasion in the absence as well as in the presence of the tumor promoter, phorbol 12, 13-dibutyrate (PDBu).

We will continue to adapt, refine and optimize various research methodologies to our mammary culture samples such that we are able to determine the relationship between the expression, number, affinity and/or regional localization of EGFR, and MEC proliferation, differentiation, morphogenesis, and/or invasion in normal as well as carcinogen-transformed and/or tumor-promoted MEC, and whether these two growth factors activate distinct signal transduction pathways during end bud morphogenesis compared to alveolar morphogenesis, and whether EGFR signaling in normal MEC differs from that of carcinogen-transformed and/or tumor-promoted MEC.

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FIGURE LEGENDS

Figure 1. Light Microscopic Appearance of the Cultured End Bud and Alveolar MEO. The intact end bud MEO were pale in color, and spherical (a), multilobular (b,c), or lobuloductal (d) in shape. The alveolar MEO were completely dark (e,g,i) or at least partially dark (f,h,j) in color, and spherical (e), multilobular (f,g) or lobuloductal (h-j) in shape. Alveolar MEO that were only partially dark were often sub-categorized as hybrid end bud/alveolar organoids. The magnification bars = 100 μ m.

Figure 2. Effects of EGF Receptor Ligands on MEO Proliferation - Comparison Between and Combinatorial Effect of mEGF and hrTGF- α . Mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 10 ng/ml mEGF, with 1 or 10 ng/ml hrTGF- α , with the combination or 10 ng/ml mEGF and 1 ng/ml hrTGF- α , or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . 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 $\pm$ the SEM obtained from triplicate culture wells. Compared to cells cultured in ALV Media without either growth factor, there was a statistically significant difference in the day 7, 14 and 21 values obtained when cells were cultured in ALV Media with 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or either combination of mEGF and hrTGF- α . Compared to cells cultured in ALV Media with 10 ng/ml mEGF, there was only a statistically significant difference in the day 21 values obtained when cells were cultured in ALV Media without either growth factor or with 1 ng/ml hrTGF- α . Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

Figure 3. Effects of EGF Receptor Ligands on MEO Proliferation - Comparison Between hrEGF and hrTGF- α . Mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . 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 $\pm$ the SEM obtained from triplicate culture wells. Compared to cells cultured in ALV Media without either growth factor, there was a statistically significant difference in the day 7 and 14 values obtained when cells were cultured in ALV Media with 1 ng/ml hrEGF, 10 ng/ml hrEGF, or 10 ng/ml hrTGF- α , and in the day 21 values obtained when the cells were cultured in ALV Media with 1 ng/ml hrEGF, 10 ng/ml hrEGF, 1 ng/ml hrTGF- α , or 10 ng/ml hrTGF- α . When comparing the concentration-dependent mitogenic activity of hrEGF with that of hrTGF- α , there was a statistically significant difference between the day 21 values obtained when the cells were cultured in ALV Media with 0.1 ng/ml hrEGF and that obtained with 1 ng/ml TGF- α or with 10 ng/ml hrTGF- α , in ALV Media with 1 ng/ml hrEGF and that obtained with 0.1 ng/ml TGF- α or with 1 ng/ml hrTGF- α , and in ALV Media with 10 ng/ml hrEGF and that obtained with 0.1 ng/ml hrTGF- α . Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

Figure 4. Effect of EGF Receptor Ligands on MEO DNA Synthesis - Comparison Between hrEGF and hrTGF- α . Mammary organoids were cultured for either 8 or 15 days

within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . Rate of DNA synthesis within individual culture wells was examined using a 24 hr incorporation of ^3H -thymidine starting on either day 7 or day 14 of the experiment. Each point represents the mean $\pm$ the SEM obtained from triplicate culture wells. Compared to cells cultured in ALV Media without either growth factor, there was a statistically significant difference in the day 7 and 14 values obtained when cells were cultured in ALV Media with 1 ng/ml hrEGF, 10 ng/ml hrEGF, 1 ng/ml hrTGF- α , or 10 ng/ml hrTGF- α . There wasn't any statistically significant differences among the mitogenic activities of ALV Media with 1 or 10 ng/ml hrEGF, and ALV Media with 1 or 10 ng/ml hrTGF- α . Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

Figure 5. Effect of EGF Receptor Ligands on Casein Accumulation - Comparison Between and Combinatorial Effect of mEGF and hrTGF- α . Mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 10 ng/ml mEGF, with 1 or 10 ng/ml hrTGF- α , with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α , or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . Casein accumulation was monitored within individual culture at different times during the 21 day culture period wells using an enzyme linked immunosorbant assay with a polyclonal anti-rat casein antibody. Each point represents the mean $\pm$ the SEM obtained from triplicate culture wells. Compared to cells cultured in ALV Media without either growth factor, there was a statistically significant difference in the day 7, 14 and 21 values obtained when cells were cultured in ALV Media with 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or either combination of mEGF and hrTGF- α , and also in the day 14 values obtained when the cells were cultured in ALV Media with 1 ng/ml hrTGF- α . Compared to cells cultured in ALV Media with 10 ng/ml mEGF, there was only a statistically significant difference in the day 21 values obtained when the cells were cultured in ALV Media with 1 ng/ml hrTGF- α or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

Figure 6. Effect of EGF Receptor Ligands on Casein Accumulation - Comparison Between hrEGF and hrTGF- α . Mammary organoids were cultured for up to 21 days within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . Casein accumulation was monitored within individual culture wells at different times during the 21 day culture period using an enzyme linked immunosorbant assay with a polyclonal anti-rat casein antibody. Each point represents the mean $\pm$ the SEM obtained from triplicate culture wells. Compared to cells cultured in ALV Media without either growth factor, there was a statistically significant difference in the day 7, 14 and 21 values obtained when cells were cultured in ALV Media with 10 ng/ml hrEGF, or 1 or 10 ng/ml hrTGF- α , and also in the day 7 and 21 values obtained when the cells were cultured in ALV Media with 1 ng/ml hrEGF. When comparing the concentration-dependent lactogenic activity of hrEGF with that of hrTGF- α , there was a statistically significant difference between the day 21 values obtained when the cells were cultured in ALV Media with 0.1 ng/ml hrEGF compared to that obtained with 1 ng/ml TGF- α or with 10 ng/ml hrTGF- α ; in ALV Media with 1 ng/ml hrEGF compared to that obtained with 0.1 ng/ml TGF- α or with 1 ng/ml hrTGF- α ; and also in ALV Media with 10 ng/ml hrEGF compared to that obtained with 0.1 ng/ml hrTGF- α . Statistical significance was evaluated using a one way ANOVA with

all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

Figure 7. Effect of EGF Receptor Ligands on Colony Morphogenesis - Comparison Between and Combinatorial Effect of mEGF and hrTGF- α . Mammary organoids were cultured within the RBM in ALV Media without either growth factor, with 10 ng/ml mEGF, with 1 or 10 ng/ml hrTGF- α , with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α , or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . All colonies within individual culture wells were examined by light microscopy and classified on day 21 of the experiment as end bud (EB) MEO, alveolar (ALV) MEO, EB/ALV MEO, adipocyte, and squamous colonies. Values are expressed as a percentage of the total colonies in the well. Each point represents the mean $\pm$ the SEM obtained from triplicate culture wells. When examining the proportion of EB MEO, there was a statistically significant difference observed when the cells were cultured without either growth factor compared to that with any of the other treatments tested. When examining the proportion of ALV MEO, there was a statistically significant difference observed when the cells were cultured without either growth factor compared to that with 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or with either combination of mEGF and hrTGF- α ; with 10 ng/ml mEGF compared to 1 ng/ml hrTGF- α or the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α ; and also with 1 ng/ml hrTGF- α compared to 10 ng/ml hrTGF- α or the combination of 10 ng/ml mEGF with 1 ng/ml hrTGF- α . When examining the proportion of EB/ALV MEO, there was a statistically significant difference observed when the cells were cultured without either growth factor compared to that with any of the other treatments tested; with 10 ng/ml mEGF compared to the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α , and also with 1 ng/ml hrTGF- α compared to that with 10 ng/ml hrTGF- α or either combination of mEGF and hrTGF- α . Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

Figure 8. Effect of EGF Receptor Ligands on MEO Branching Morphogenesis - Comparison Between and Combinatorial Effect of hrEGF and hrTGF- α . Mammary organoids were cultured within the RBM in ALV Media without either growth factor, with 10 ng/ml mEGF, with 1 or 10 ng/ml hrTGF- α , with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α , or with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α . All colonies within individual culture wells were examined by light microscopy and classified on day 21 of the experiment as end bud (EB)-lobular (L), EB-multilobular (ML), EB-lobuloductal (LD), alveolar (ALV)-L, ALV-ML, ALV-LD, EB/ALV-ML, EB/ALV-LD MEO. Values are expressed as a percentage of the total colonies in the well. Each point represents the mean $\pm$ the SEM obtained from triplicate culture wells. When examining the proportion of ALV-L MEO, there was a statistically significant difference observed when the cells were cultured without either growth factor compared to that with 10 ng/ml mEGF or 10 ng/ml hrTGF- α ; with 1 ng/ml hrTGF- α compared to 10 ng/ml mEGF or 10 ng/ml hrTGF- α . When examining the proportion of ALV-ML MEO, there was a statistically significant difference observed when the cells were cultured without either growth factor compared to that with 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α ; with 1 ng/ml hrTGF- α compared to 10 ng/ml mEGF, 10 ng/ml hrTGF- α , or the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α ; and also with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α compared to 10 ng/ml mEGF or the combination of 10 ng/ml mEGF and 1 ng/ml

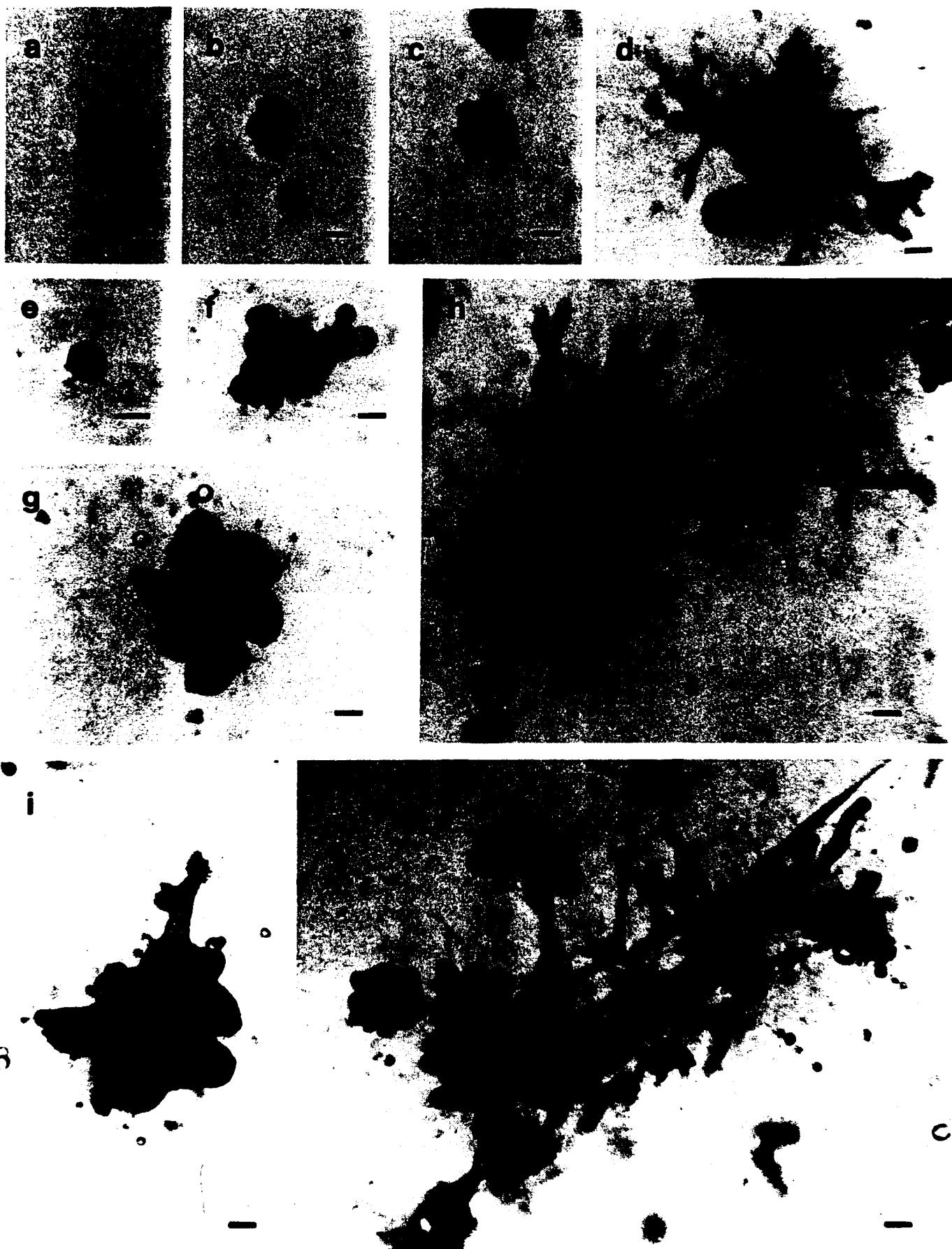
hrTGF- α . When examining the proportion of EB/ALV-ML MEO, there was a statistically significant difference observed when the cells were cultured with 10 ng/ml TGF- α compared to that without either growth factor, with 10 ng/ml mEGF or with 1 ng/ml hrTGF- α ; and also with both combinations of mEGF and hrTGF- α compared to that without either growth factor, with 10 ng/ml mEGF or with 1 ng/ml hrTGF- α . When examining the proportion of EB/ALV-LD MEO, there was a statistically significant difference observed when the cells were cultured with the combination of 10 ng/ml mEGF and 1 ng/ml hrTGF- α compared to that without either growth factor, with 10 ng/ml mEGF, with 1 ng/ml hrTGF- α , or with 10 ng/ml hrTGF- α ; and also with the combination of 10 ng/ml mEGF and 10 ng/ml hrTGF- α compared to 10 ng/ml mEGF, 1 ng/ml hrTGF- α , or 10 ng/ml hrTGF- α . Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

Figure 9. Effect of EGF Receptor Ligands on Colony Morphogenesis - Comparison Between hrEGF and hrTGF- α . Mammary organoids were cultured within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . All colonies within individual culture wells were examined by light microscopy and classified on day 21 of the experiment as end bud (EB) MEO, alveolar (ALV) MEO, EB/ALV MEO, and squamous colonies. Values are expressed as a percentage of the total colonies in the well. Each point represents the mean $\pm$ the SEM obtained from triplicate culture wells. When examining the proportion of EB-MEO, there was a statistically significant difference observed when cells were cultured with 0.1 ng/ml hrEGF compared to 1 ng/ml hrTGF- α ; and also with 0.1 ng/ml hrTGF- α compared to 1 ng/ml hrTGF- α . When examining the proportion of ALV-MEO, there was a statistically significant difference observed when the cells were cultured with either 0.1 ng/ml hrEGF, 1 ng/ml hrEGF or 0.1 ng/ml hrTGF- α compared to that without either growth factor, with 1 hrTGF- α or with 10 ng/ml hrTGF- α ; and also with 10 ng/ml hrEGF compared to that without either growth factor, with 1 ng/ml hrTGF- α , or with 10 ng/ml hrTGF- α . Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

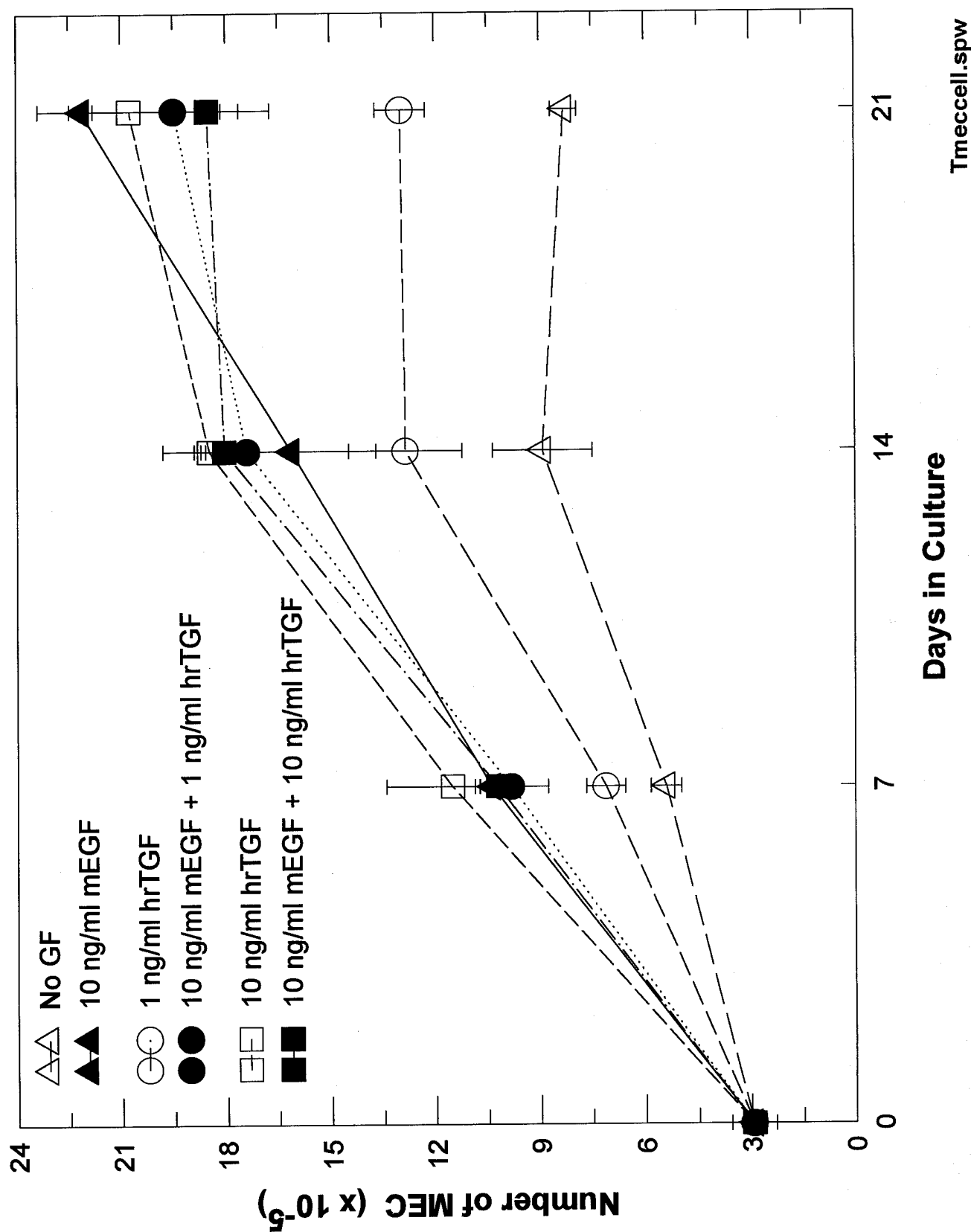
Figure 10. Effect of EGF Receptor Ligands on MEO Branching Morphogenesis - Comparison Between hrEGF and hrTGF- α . Mammary organoids were cultured within the RBM in ALV Media without either growth factor, with 0.1, 1 or 10 ng/ml hrEGF, or with 0.1, 1 or 10 ng/ml hrTGF- α . All colonies within individual culture wells were examined by light microscopy and classified on day 21 of the experiment as end bud (EB)-lobular (L), EB-multilobular (ML), EB-lobuloductal (LD), alveolar (ALV)-L, ALV-ML, ALV-LD, EB/ALV-ML, EB/ALV-LD MEO. Values are expressed as a percentage of the total colonies in the well. Each point represents the mean $\pm$ the SEM obtained from triplicate culture wells. When examining the proportion of EB-ML MEO, there was only a statistically significant difference when cells were cultured with 0.1 ng/ml hrEGF compared to 10 ng/ml hrTGF- α . When examining the proportion of ALV-L MEO, there was a statistically significant difference when cells were cultured without either growth factor compared to that with any concentrations of either growth factor tested. When examining the proportion of EB/ALV-ML MEO, there was a statistically significant difference when cells were cultured without either growth factor compared to that with 1 ng/ml hrEGF or 10 ng/ml hrTGF- α . When examining the proportion of EB/ALV-LD MEO, there was a statistically significant difference when cells were cultured without either growth factor compared to that with 10 ng/ml hrEGF or 10 ng/ml hrTGF- α .

Statistical significance was evaluated using a one way ANOVA with all pairwise multiple comparisons carried out by the Student-Newman-Keuls method ($p < 0.05$).

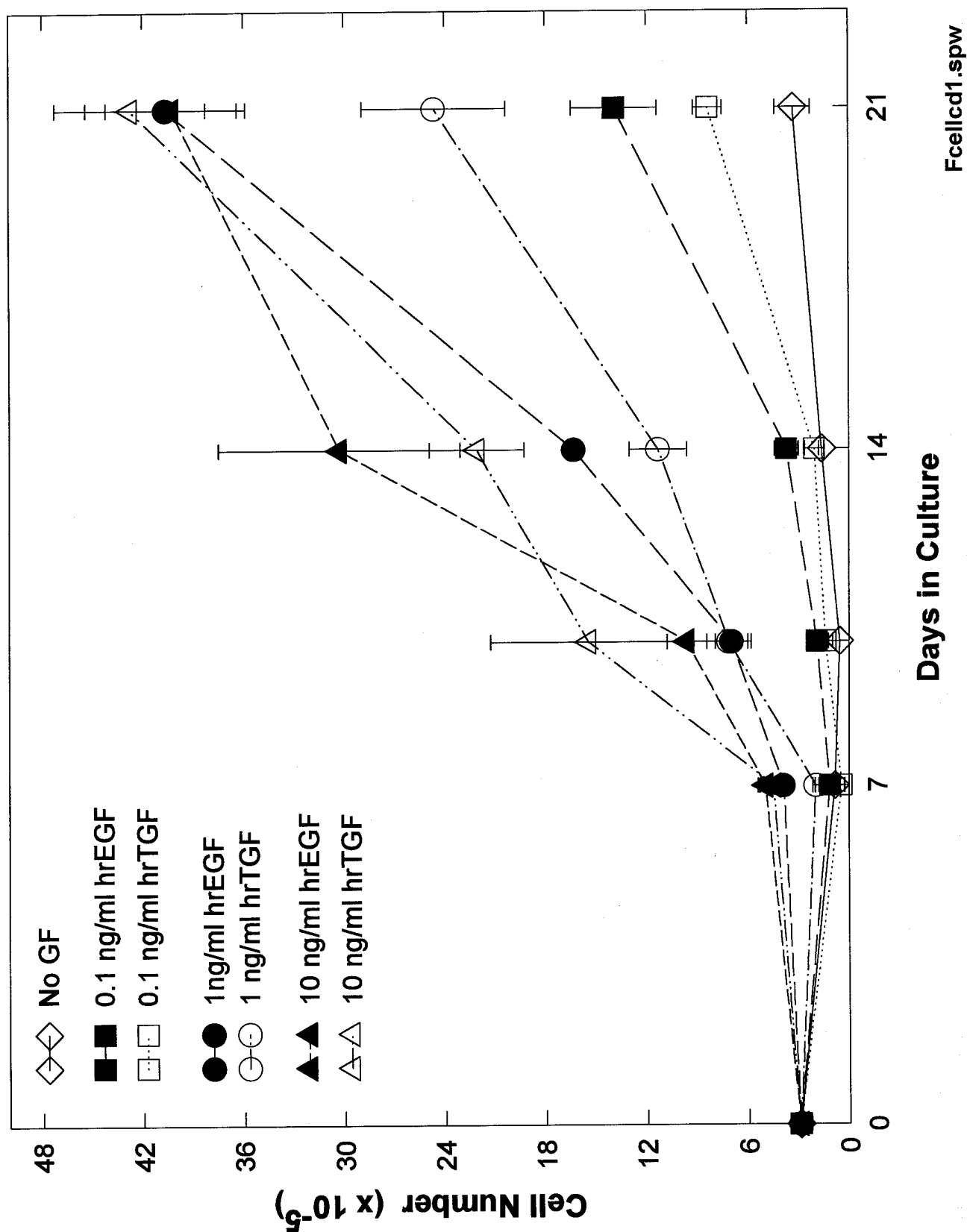
Figure 1.



Effects of EGF Receptor Ligands on MEO Proliferation



Effects of EGF Receptor Ligands on MEO Proliferation



Effect of EGF Receptor Ligands on MEO DNA Synthesis

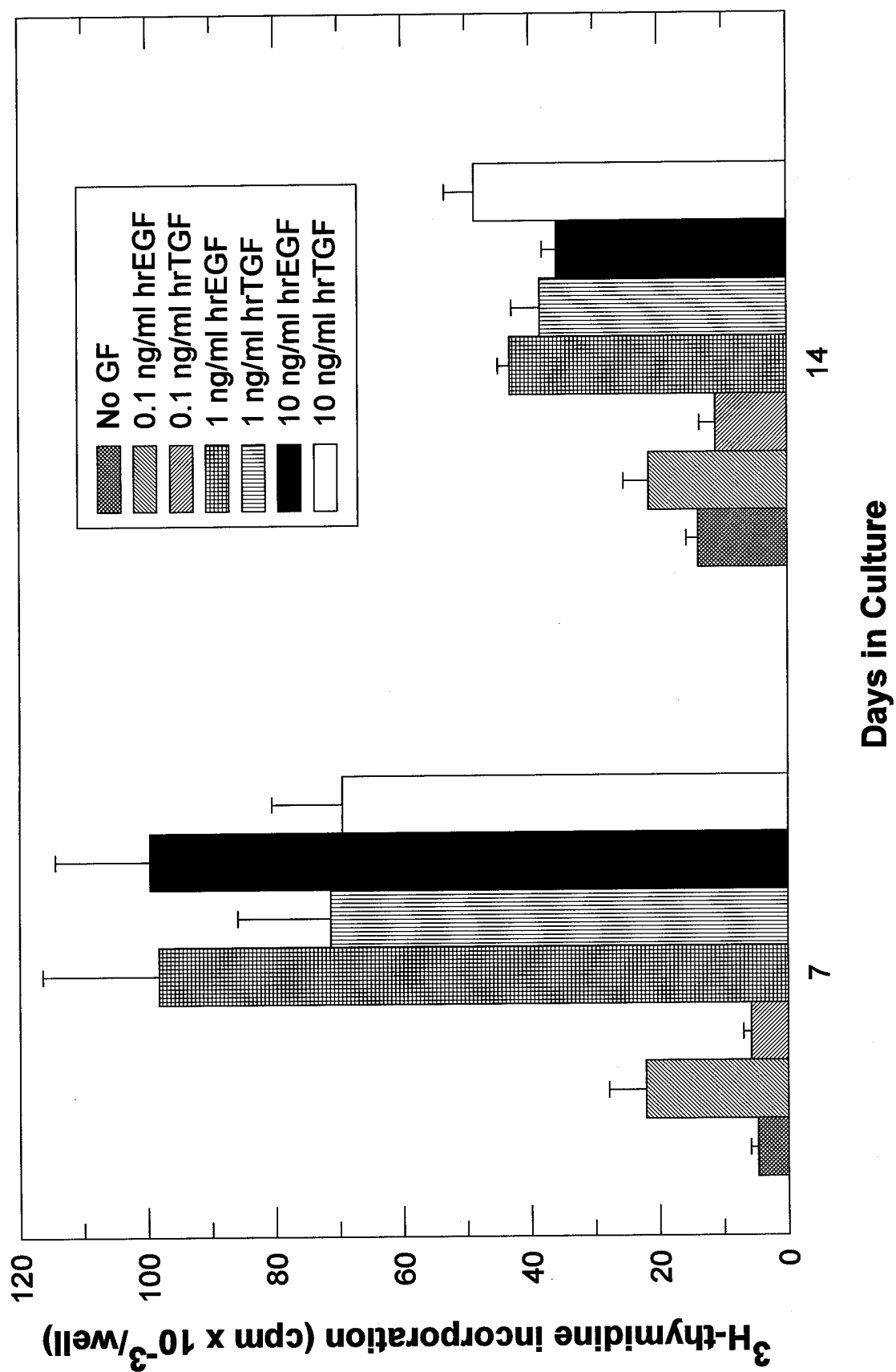


Figure 4.

Effect of EGF Receptor Ligands on Casein Accumulation

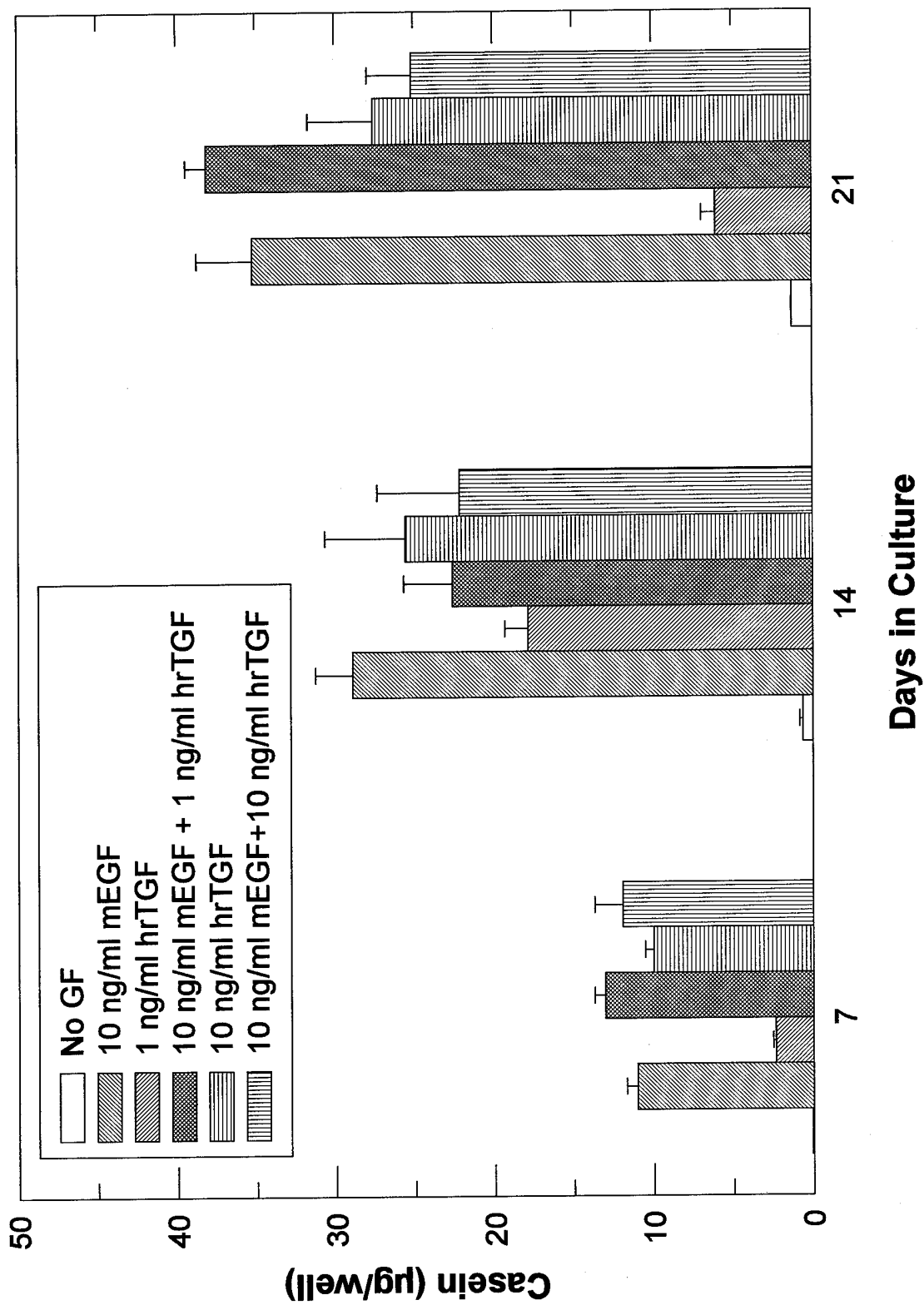
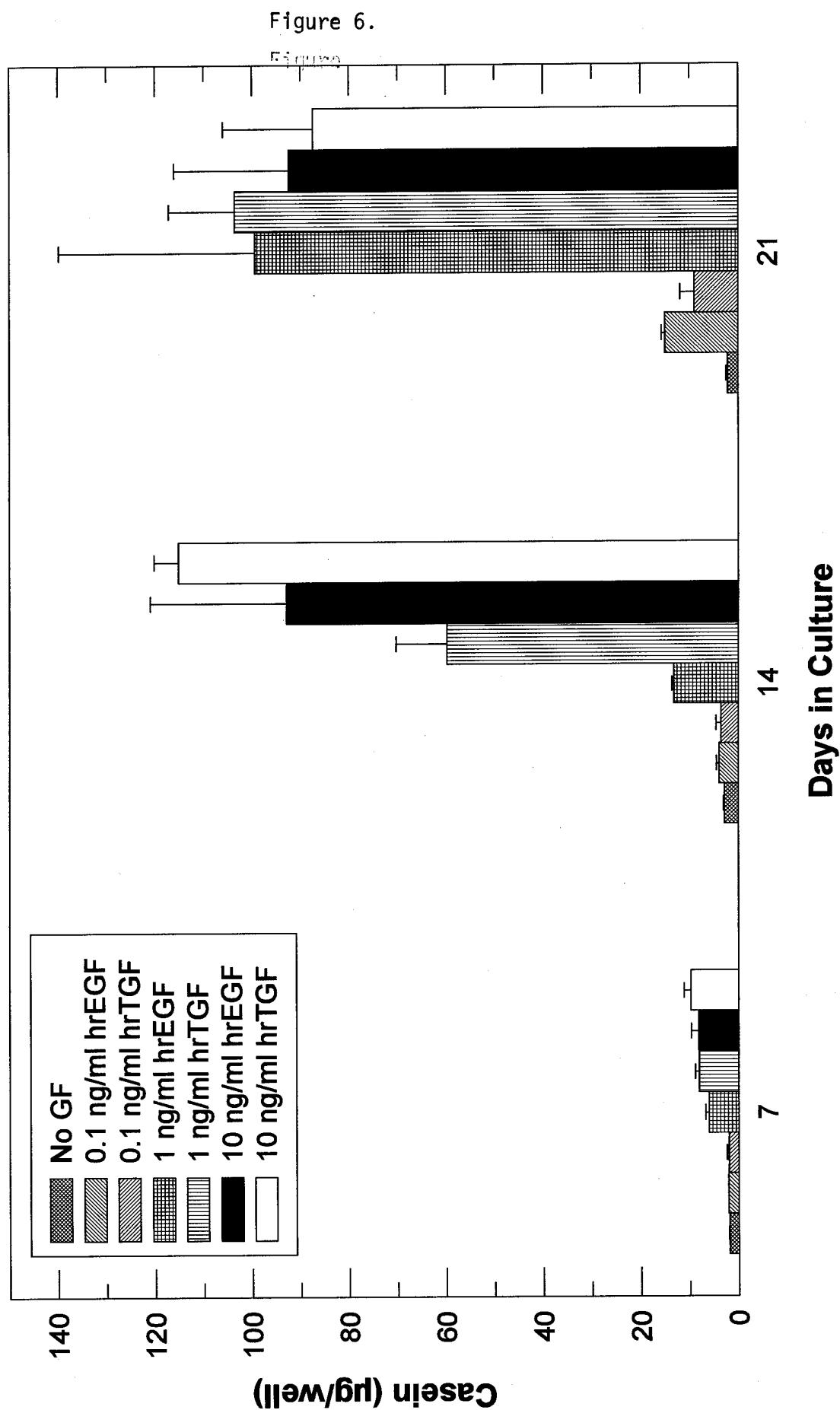


Figure 5.

Effect of EGF Receptor Ligands on Casein Accumulation



Effect of EGF Receptor Ligands on Colony Morphogenesis

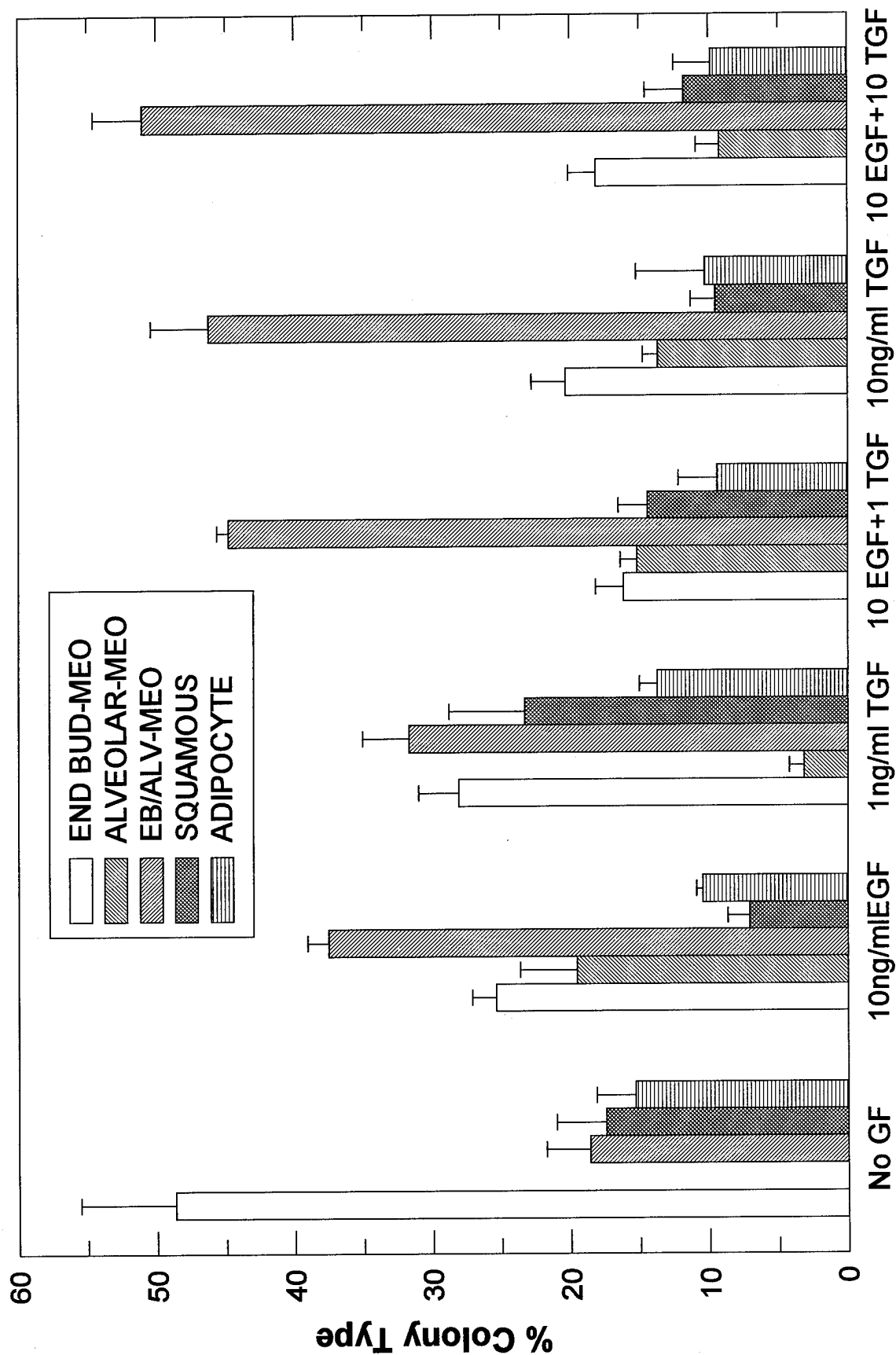
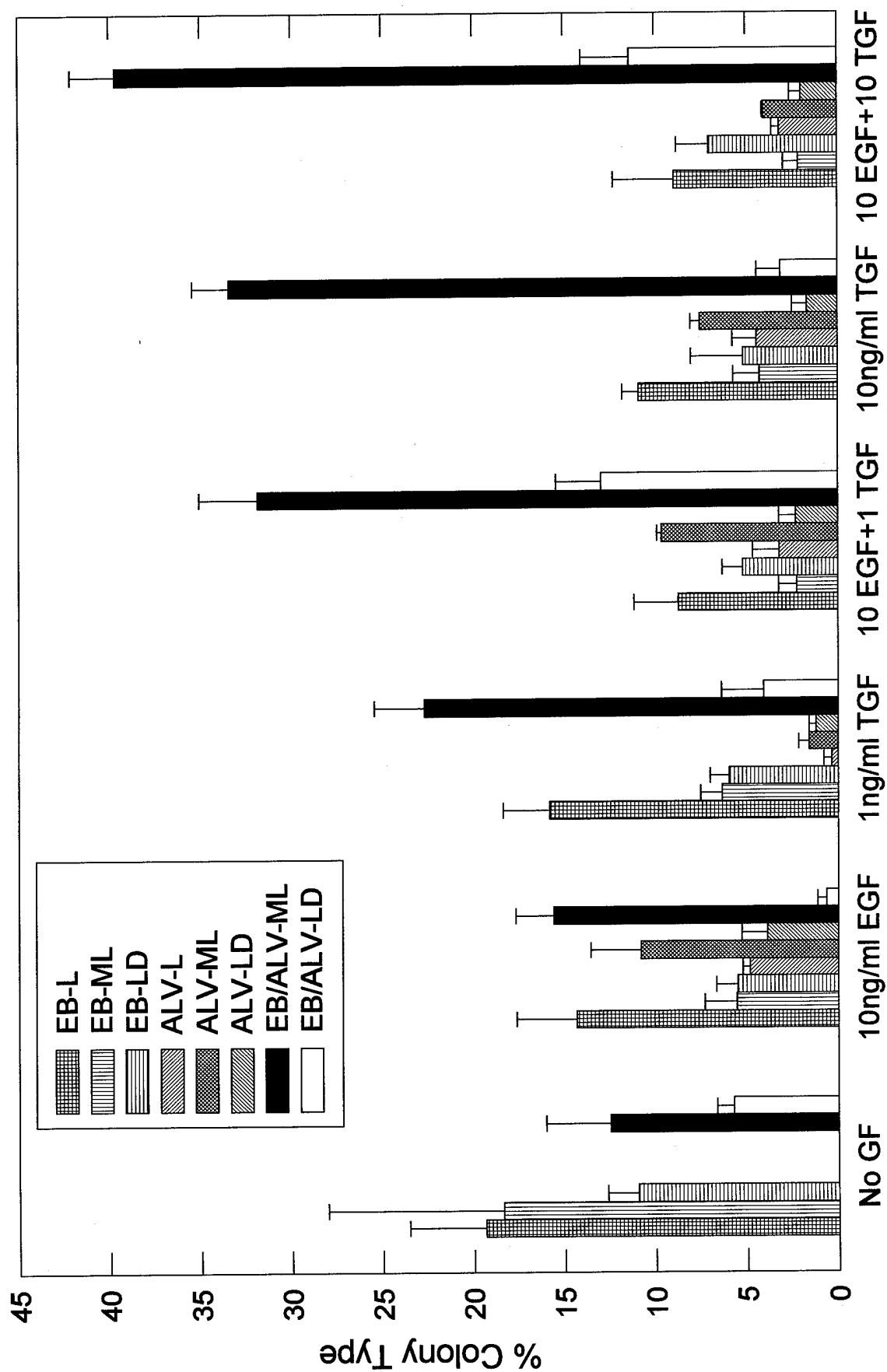


Figure 7.

Effect of EGF Receptor Ligands on MEO Branching Morphogenesis



TMORBM21.spw

Effect of EGF Receptor Ligands on MEO Colony Morphogenesis

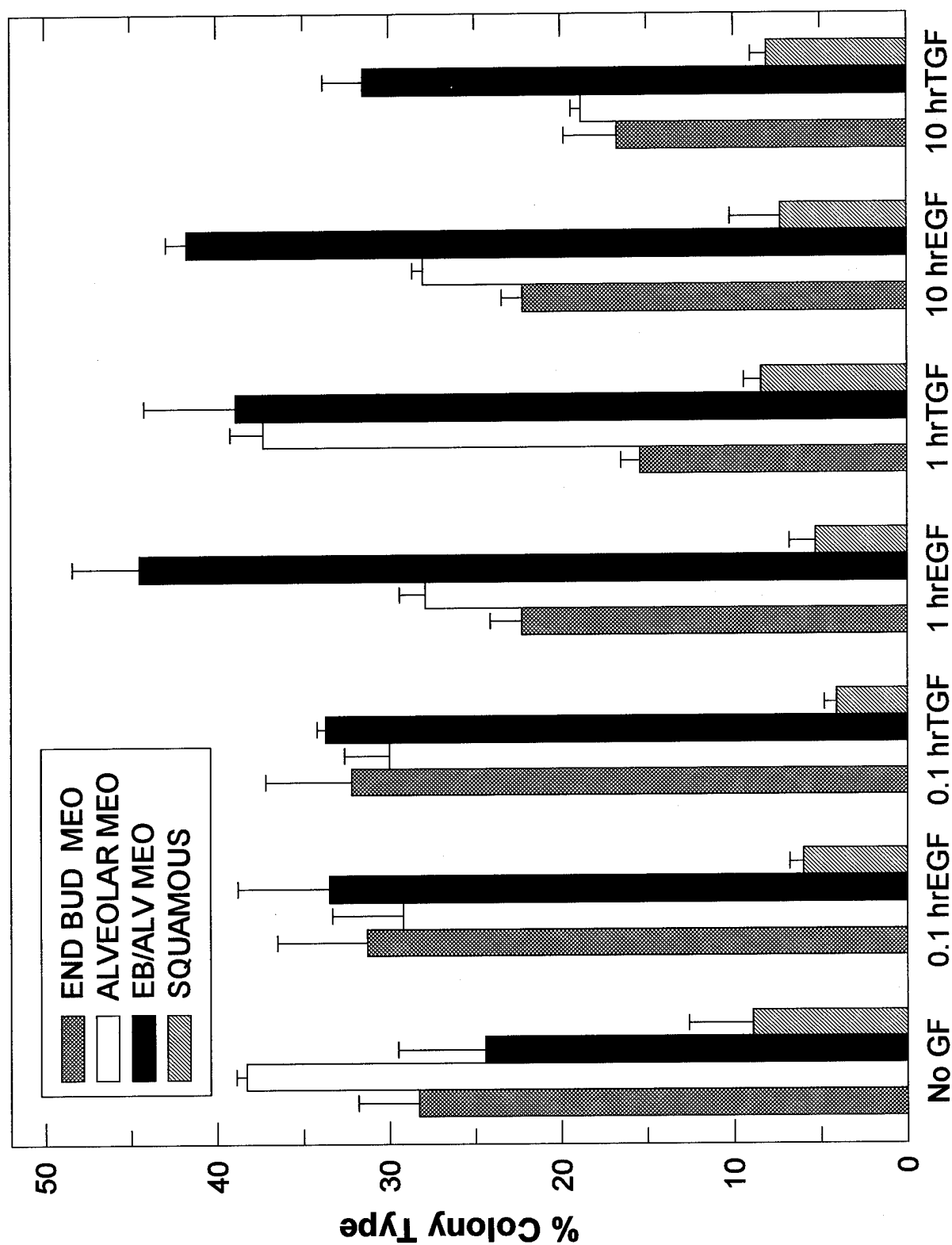


Figure 9.

Effect of EGF Receptor Ligands on MEO Branching Morphogenesis

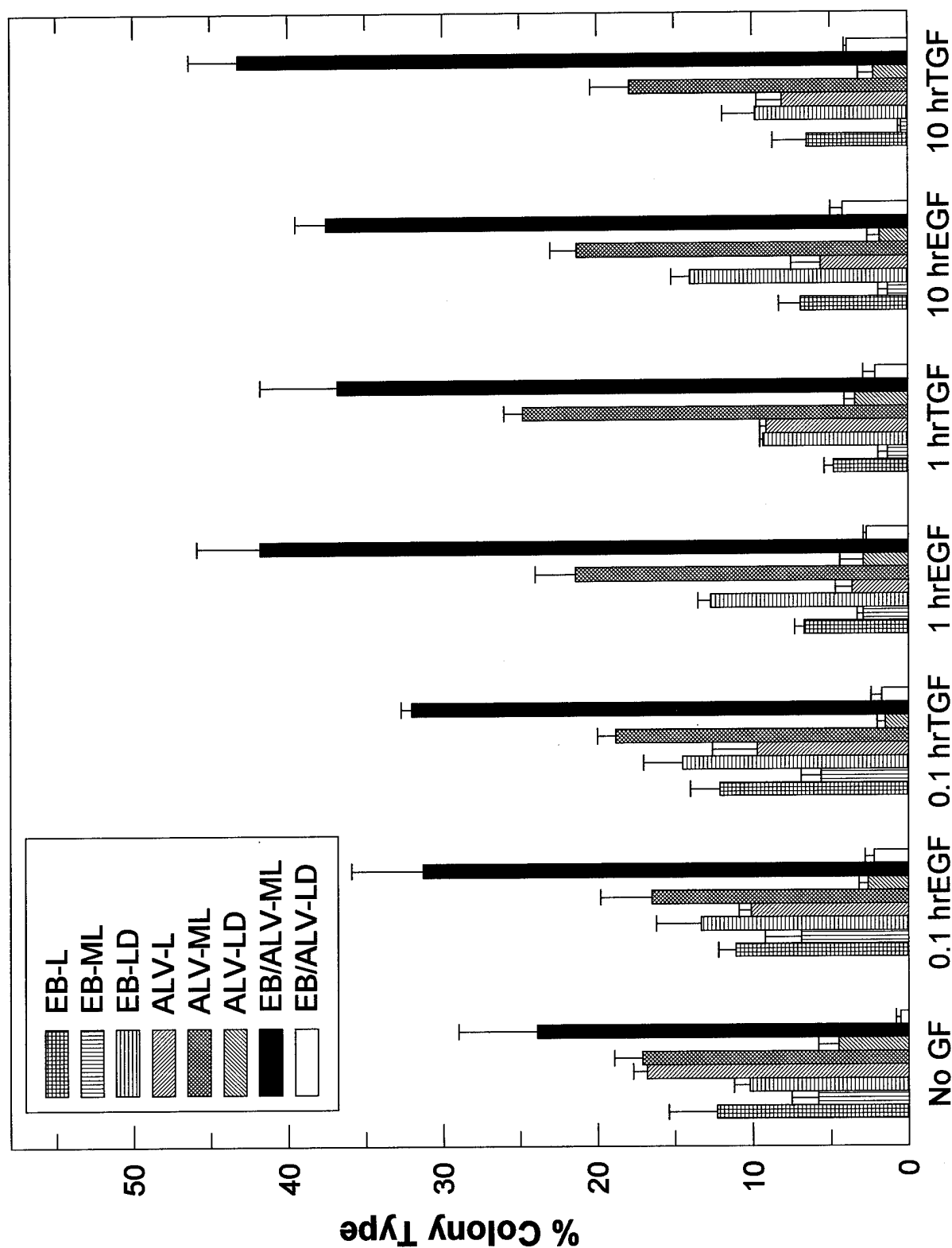


Figure 10.