REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
Mammary Tumor Development: Stromal-Epithelial Interactions in Oncogenesis

5. FUNDING NUMBERS
DAMD17-94-J-4434

6. AUTHOR(S)
David S. Strayer, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

The purpose of this grant is to define interactions between mammary stroma and epithelium in oncogenesis in transgenic mice expressing Shope growth factor (SGF). Different lines of SGF transgenic mice express this EGF-like cytokine using inducible (metallothionein, MT) or constitutive (RSV-LTR) promoters. We have mostly fulfilled, and in some areas exceeded, proposed goals for the year now ending. We had proposed making sufficient SGF-transgenic mice of RSV-SGF and MT-SGF lines for analysis of mammary differentiation and tumor development. This included assessing expression of SGF and of key cellular genes important in mammary these processes. It also included defining which cells express the transgene and these cellular genes. These studies are completed. We have also interbred RSV- and MT-SGF transgenic mice with p53-/- mice, to assess the effects of a homozygous deletion of this tumor suppressor gene on mammary gland development and tumorigenesis. These mice are just numerous enough for these analyses to be performed. We have been producing reengineered SGF in baculovirus in quantities that can be used for study of SGF activity in cultured cells as proposed. Therefore, our studies of the effects of SGF on mammary gland and tumor development are proceeding as planned, and additional studies are in progress.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Mammary Carcinoma, Stromal-Epithelial Interaction, Growth Factor, Paracrine Stimulation, Transgene, Histopathology/Molecular Biology	25
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

GRANT NUMBER DAMD17-94-J-4434

TITLE: Mammary Tumor Development: Stromal-Epithelial Interactions in Oncogenesis

PRINCIPAL INVESTIGATOR: David S. Strayer, M.D., Ph.D.

CONTRACTING ORGANIZATION: Thomas Jefferson University
Philadelphia, Pennsylvania 19107

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

9/29/97

PI - Signature Date

Table of Contents

Introduction

Nature of the problem/project

In our original application, we proposed to study interactions between stromal and epithelial cells in development of tumors of the mammary gland (MG) in transgenic mice that express Shope growth factor (SGF). SGF is an epidermal growth factor (EGF)-like glycoprotein growth factor that elicits cellular proliferation and in virgin transgenic mice was associated with development of mammary gland tumors. We had found that SGF is produced made mainly in stromal cells of the mammary gland (and other organs), and that proliferative and differentiative responsiveness to SGF were principally seen in the ductal and other epithelial cells of the mammary gland. Thus, we proposed to use this system to study how stromal and epithelial cell interactions produce preneoplasias and tumors in SGF transgenic mice.

Background of Previous Work by ourselves and others

This background section will discuss (i) the roles of EGF-like growth factors (EGFs) in cell activation and growth; (ii) participation of EGFs in oncogenesis, particularly clinical tumor development in the human breast and experimental tumor development in the mammary gland; (iii) what is understood about the interactions between stromal and epithelial cells in oncogenesis; and (iv) tumor development in SGF transgenic mice. The first two subsections of this background reflect work performed by our and other laboratories. The final subsection describes work done on this system in our laboratory.

(i). roles of EGF-like growth factors in cell activation, growth and oncogenesis

There is a family of EGF-like growth factors (EGFs). EGFs stimulate responsive cells to proliferate and, sometimes, to differentiate (1). Therefore, EGFs play roles in cell proliferation and differentiation activities such as oncogenesis, wound healing and organ maturation.

SGF is a glycoprotein related to EGF (2). It is encoded by malignant fibroma virus (MV), which produces malignant tumors of fibroblasts. Epithelial proliferation overlies fibromyxosarcomas in MV-infected animals, and is felt to represent the influence of SGF production by MV-infected cells. (3). When the SGF gene is deleted, MV's virulence is attenuated. Epithelial proliferation and tumor spread are diminished. Instead of dying uniformly, most animals survive (4).

To understand how SGF acts as a growth factor, free of other viral genes we produced transgenic mice that express SGF. In this setting SGF induces mammary differentiation, proliferative preneoplastic lesions, or invasive adenocarcinomas, depending on the promoter construct used and the animal's age when SGF expression begins. In these studies, we proposed to study how stromal and epithelial SGF secretion and responsiveness affects target cells and leads to neoplasia.

The family of EGFs includes EGF, transforming growth factor-α (TGFα), amphiregulin, cripto, three poxviral products (SGF, vaccinia growth factor (VGF) and myxoma growth factor (MGF)) and the potential HER2/neu ligands gp30 and p175. These all differ in primary structure, but generally share a constrained tertiary structure characterized by 3 overlapping disulfide bonds (5). Of these growth factors, EGF and TGFα are the best understood. EGF and TGFα are 53 and 50 amino acids (aa) respectively, and are produced by cleavage of larger pre-

cursors (6). The TGFα precursor may be glycosylated and anchored at the cell membrane, but its post-translational modifications are lost when the secreted form is cleaved from its membrane bound precursor (7). Thus, secreted forms of EGF and TGFα are not glycosylated. The poxviral growth factors and gp30 are larger and are all glycosylated (8). SGF is encoded as an 80 aa polypeptide, then cleaved and glycosylated to 12-16 kDa (1,9,10).

The effects of most of this family on cells depend on interactions with EGF receptor (EGFR). EGF, VGF, SGF and TGFα all bind the extracytoplasmic region of EGFR at different sites (11-14). Human EGFR is a 170 kDa transmembrane glycoprotein that resembles the products of viral oncogene, v-*erb*^{b} and the cellular protooncogene HER2/neu (15). GF-receptor interaction initiates a cascade of events that leads to cell division. When ligand binds EGFR, the receptor oligomerizes and its affinity for ligand increases (16). Cytoplasmic EGFR tyrosine kinase activity is activated, and adjacent receptor molecules trans-phosphorylate each others' cytoplasmic domains (17).

Activated EGFR mobilizes a series of intracellular responses. Many enzymes associate with the cytoplasmic domain of EGFR via SH2 (src-homology-2) domains that bind activated EGFR phosphotyrosine, e.g., PI3 kinase, GTPase activating protein and phospholipase Cγ1 (PLCγ1, 18-20). These enzymes are substrates for EGFR kinase activity. They are activated on phosphorylation by EGFR, and carry the message of growth factor-induced cellular stimulation to the cellular activation apparatus beyond. This leads to activation of PLCγ1, followed by hydrolysis of phosphatidyl inositol phosphates into inositol phosphates, which increase intracellular Ca (21,22). Ras protein is also activated via intermediate proteins, Grb2 and Sos1 (23-25). Ras activation is associated with phosphorylation/activation of raf1 kinase, which leads to activation of MAP kinases (26,27), then of jun-fos AP1 transcription factor. The latter causes increased gene transcription and leads to cell division (28,29).

(ii) participation of EGFs in oncogenesis, particularly mammary and breast oncogenesis

The EGF group of cytokines is important in both organogenesis and oncogenesis. They are needed for normal development and differentiation of many organs, including kidney, GI tract, lung and breast. EGF or TGFα stimulate normal mammary ductal growth, even in the absence of steroids, and are vital to mammary gland differentiation (30-32). We have found that SGF may induce mammary tumors or differentiation in transgenic mice, depending on the timing of its induction. A similar observation has been made for gp30 (33).

Tumor development is also linked to signal transduction via EGFR. EGF, TGFα and EGFR appear to be important for tumorigenesis in several organs via an autocrine loop: tumors make EGF and/or TGFα, plus high levels of EGFR. Tumor cells make EGFR and an EGFR ligand grow more slowly when the ligand is removed with anti-GF antibody (34). Anti-sense RNA that blocks EGFR expression reportedly suppresses transformation (35).

EGF alone does not elicit phenotypic transformation, but it may do so in concert with other agents (36). The v-*erb*^{b} protein may transform cells precisely because it lacks ligand binding sites. This deficiency allows v-*erb*^{b} protein to be constitutively activated in the absence of ligand (37). In addition, cells with high concentrations of EGFR may become phenotypically transformed in response to low concentrations of EGF (38).

The roles of EGFR and HER2/neu in breast cancer have been studied extensively (39). Several investigators have reported that EGFR+ tumors make and/or respond to members of the EGF family (40,41). In addition, EGFR expression is associated with aggressive behavior and poor prognosis. Tumors that produce EGFR (EGFR+) are of higher grade and stage than

EGFR- tumors, and express less estrogen receptor. EGFR+ tumors are also more highly proliferative and aneuploid (40). They tend to recur and kill patients more than EGFR- tumors (42). Detectable EGFR may be the most accurate predictor of survival (43). *C-erb^b* (HER2/neu) protein is an EGFR-like receptor, whose expression has also been associated with poor prognosis in breast and other tumors (44).

A better understanding of the roles of EGFs in oncogenesis has come from the study of transgenic animals expressing EGFs. TGFα induces mammary hyperplasia, and in some cases, differentiation (alveolarization). Aged virgin TGFα-mice mainly showed atypical hyperplasias, while aged multiparous mice may develop secretory tumors (32,45,46). MG tumors are reported in transgenic mice expressing c-*myc*, c-*erbB2/neu*, *int-1*, *int-3* and Ha-*ras*, and in F1 hybrid mice transgenic for both *wnt-1* and *TGFα* (47,48).

The most common mammary preneoplasias are hyperplastic alveolar nodules (HAN) and ductal hyperplasia (DH). These are usually induced by mouse mammary tumor virus (MMTV), chemical carcinogens, hormonal stimulation or radiation (49-52). HAN and DH cells are immortal populations. That is, they can be serially transplanted indefinitely. On transplantation, these preneoplasias show hyperplastic growth patterns and are at high risk for neoplastic transformation (53,54). Studies proposed here will help to define the progression of preneoplasias into tumors and the effects of SGF expressed as a transgene.

We proposed to study transgenic mice expressing SGF, an EGF-like growth factor, as a model of mammary oncogenesis and differentiation. As indicated, we had collaborated to genearate transgenic mice in which SGF expression was controlled by either an inducible promoter or a constitutive promoter. Our choices in this case were the metallothionein (MT) promoter and the constitutive Rous sarcoma virus LTR promoter (RSV-LTR, 55,56). RSV-LTR is a strong, constitutively active regulator. MT is substantially inducible by heavy metal (Zn).

We had reported in our first studies that early generations of virgin RSV-SGF transgenic mice showed marked preneoplastic MG ductal proliferation by 6 mo. By 8 mo., 1/3 of these had developed adenocarcinomas. In contrast, virgin MT-SGF mice that had been induced to express SGF at 2 mo. of age, showed MG differentiation without atypia. By *in situ* hybridization analysis of both sets of transgenic mice, we found that SGF was mainly expressed in mammary stroma, although the observed changes in the mammary gland histologic appearances were all epithelial. These observations suggested that since mouse mammary neoplasias and preneoplasias are typically readily cultured and are transplantable *in vivo*, SGF-mice might represent a model system to examine stromal-epithelial interactions in GF-related oncogenesis.

(iii) what is known about interactions between stromal and epithelial cells in oncogenesis

A peculiar strength of this model is the opportunity it provides to study epithelial responsiveness to growth stimulation by stromal cells. Much evidence implicates interactions between breast stroma and epithelium in the growth of malignant tumors. This interaction involves secretion of and responses to insulin-like growth factors-I and -II (IGF-I, IGF-II). IGF-I and -II have different cell membrane receptors, though responses to IGF-II may be mediated through IGF-I receptor (57-59). Breast cancer cell lines usually respond to both cytokines but do not produce IGF-I. IGF-I is, instead, elaborated by mammary stroma adjacent to the tumor. Thus, IGF-I is a paracrine growth factor for breast tumor cells (60-62). IGF-II is also produced by breast stroma, but is also made by some tumor cell lines. IGF-II, then, acts in both paracrine and autocrine fashions (63).

The interaction between SGF expressed as a transgene and target mammary epithelium resembles these reported data on the IGF's. SGF transgenic mice develop mammary tumors. The growth factor is recognized by EGFR. SGF is expressed in both epithelium and stroma, mainly in the stroma. Therefore, by developing mammary epithelial cell lines from transgenic and normal mice, and then transplanting them into the opposite recipients, we had proposed an experimental model that will allow the study and dissection of mechanisms of stromal-epithelial interactions that promote and sustain tumor and mammary gland growth.

Human breast cancer is a complex disease or group of diseases, involving a variety of independent risk factors such as parity, family history, etc. (64). Understanding human breast tumor development thus requires a number of model systems. The proposed studies of SGF-related mammary carcinogenesis complement other systems of breast carcinogenesis. For example, SGF elicits preneoplasias and invasive tumors in young virgin mice. Multiple parity starting at an early age decreases risk for human breast cancer but increases breast tumors in TGFα transgenic mice (32). SGF transgenic mice should thus provide additional insight into mechanisms of development and progression of breast tumors, and supplement other experimental models of mammary carcinogenesis.

(iv) mammary preneoplasia and neoplasia in SGF transgenic mice

a. Constructs used to produce transgenic mice

SGF constructs were made by cloning the SGF gene into mMT-1, a plasmid construct containing the mouse metallothionein promoter, and pRSVcat as expression vectors (65,66). (Insert orientation was confirmed by DNA sequencing.) These plasmids use the MT promoter and Rous sarcoma virus long terminal repeat (RSV-LTR) respectively as regulators of gene expression. The molecular strategies that were used to make these constructs are described in detail in the appended reprint (67), and are not recapitulated here.

Transgenic mice were made by microinjection of promoter-SGF constructs into (C57Bl/6 x DBA/2)F1 [hereafter, BDF1] embryos using standard techniques. Animals were screened for carriage of SGF transgene by assaying tail DNA. Two founder mice (#8, #9) carried SGF, and both had 3-5 copies of the gene. These mice were backcrossed to normal BDF1's. Offspring were examined for transgene carriage, and lines established from positive animals by sibling mating. We identified 3 founder mice carrying RSV-SGF and established lines from them in the same way.

In our earlier studies, we described generations of mice that were derived from the founders and early backcrosses. Backcrosses to normal parental strain mice and brother-sister matings had produced lines of transgenic mice. Because these were early generations, there were a substantial number of heterozygotes in these crosses. Brother-sister mating at that time produced a substantial percentage of offspring that did not carry the transgene. By this time, however, this does not happen: all matings produce transgenic mice. Although the apparent intensity of dot-blot signals is not always uniform, it is clear at this point that, unlike earlier generations, very few if any heterozygous transgenic mice remain in the lines of pure SGF transgenic animals.

Thus, 5 lines carry the SGF transgene, 2 with SGF as inducible an inducible gene controlled by the MT promoter, and 3 with SGF expressed constitutively under the control of RSV-LTR. The next section describes our clinical and pathologic observations in the early generations of both MT-SGF and RSV-SGF transgenic mice. These findings formed the basis of this application.

b. Histologic, clinical and *in vitro* observations on these transgenic mice.

Histologic findings in mammary glands of virgin transgenic mice are summarized in Table 1 for MT-SGF mice in which SGF expression was induced after sexual maturity, and in Table 2 for the RSV-SGF mice, expressing SGF constitutively. The histology on which these summaries are based is illustrated in ref. #67. RSV-SGF mice are the basis of this application; so this discussion will focus on the preneoplastic and malignant proliferations found in those animals. Other observations are described and illustrated in the accompanying reprint (67). Unless otherwise stated, all observations are made on histologic sections taken near the nipples of the abdominal mammary glands.

Table 1. MAMMARY HISTOLOGY IN VIRGIN MT-SGF TRANSGENIC MICE EXPRESSING SGF FOR 2 MONTHS

Construct	GF Expression	MG Histology		
		Ducts	Lobules	Other
pMTSGF	Uninduced/2 mo.	Normal	None	Normal
pMTSGF	Uninduced/4 mo.	Normal	None	Normal
pMTSGF	Induced at 2 mo. for 2 months Observed at 4 mo. of age	Mild hyperplasia; Abundant protein-rich secretions in ducts, with +++ periductal fibroplasia	Development of lobules with protein-rich secretion	Normal

The findings from MT-SGF transgenic mice can be summarized as follows:

•SGF expression elicits differentiation (alveolarization) and protein secretion in virgin mice when expression is begun at the age of sexual maturity for two months.

By contrast, RSV-SGF transgenic mice develop clear preneoplasia by 6 months of age and invasive secretory carcinomas by 8 months of age in 1/3 of RSV-SGF mice examined.

Table 2. MAMMARY HISTOLOGY IN VIRGIN RSV-SGF TRANSGENIC MICE

Construct	GF Expression	Breast Histology		
		Ducts	Lobules	Other
pRSGF	Constitutively expressed Observed at 2 months of age	Mild hyperplasia & atypia in ducts and ductules	None	Normal
pRSGF	Constitutively expressed Observed at 6 months of age	Highly abnormal. Marked hyperplasia extending through duct walls, into surrounding fat	None	Normal
pRSGF	Constitutively expressed Observed at 8 months of age	Highly abnormal. 1/3 of mice show invasive secretory adenocarcinoma	None	Normal

SGF has effects on other organs as well. These effects, which are not the subject of the current application, are described and illustrated in reference #67, which is appended.

Production of and responsiveness to SGF by stroma and epithelium, and consequent cellular growth *in vitro* and *in vivo*, are important aspects of this application. We studied explanted fibroblasts from SGF transgenic mice *in vitro*. Skin fibroblasts from adult SGF mice transformed spontaneously in culture within 4 weeks: they lost contact inhibition, formed foci in monolayer culture and established colonies in soft agar. Control BDF1 fibroblasts invariably die within 6 weeks. Thus SGF in culture acts as a potent transforming agent.

Thus RSVSGF transgenic mice developed ductal hyperplasias, followed by occasional invasive carcinomas by 8 months of age. The oncogenic potential of SGF as it is produced by fibroblasts, are underscored by the rapid transformation of SGF transgenic fibroblasts *in vitro*.

c. Growth factor transgene expression

SGF transcription in the mammary gland was ascertained by RNA dot and Northern blot analyses, and *in situ* hybridization. SGF expression was studied in MTSGF mice ± Zn for 2 mo., and in RSVSGF mice. MT-SGF and RSV-SGF MG, but not control MG, made mRNA that hybridized with SGF probe (See ref. #67 for *in situ* hybridization data and Northern analysis.)

Cellular patterns of transgene expression were studied by in situ hybridization (ISH) using SGF DNA as a probe. SGF DNA incorporating biotinylated dUTP (Boehringer-Mannheim) was hybridized to MG tissue sections from MTSGF, RSVSGF and normal BDF1 mice. This was followed by avidin, then biotin-alkaline phosphatase, according to established protocols (68). SGF transcript was detected in epithelial and stromal cells of many organs in both transgenic lines, but is expressed most strongly in vascular endothelium and other connective tissue cells, and MG epithelium.

SGF is expressed throughout the body in SGF-transgenic mice, in both stromal and epithelial cells. In the mammary gland SGF is mainly expressed by stromal cells.

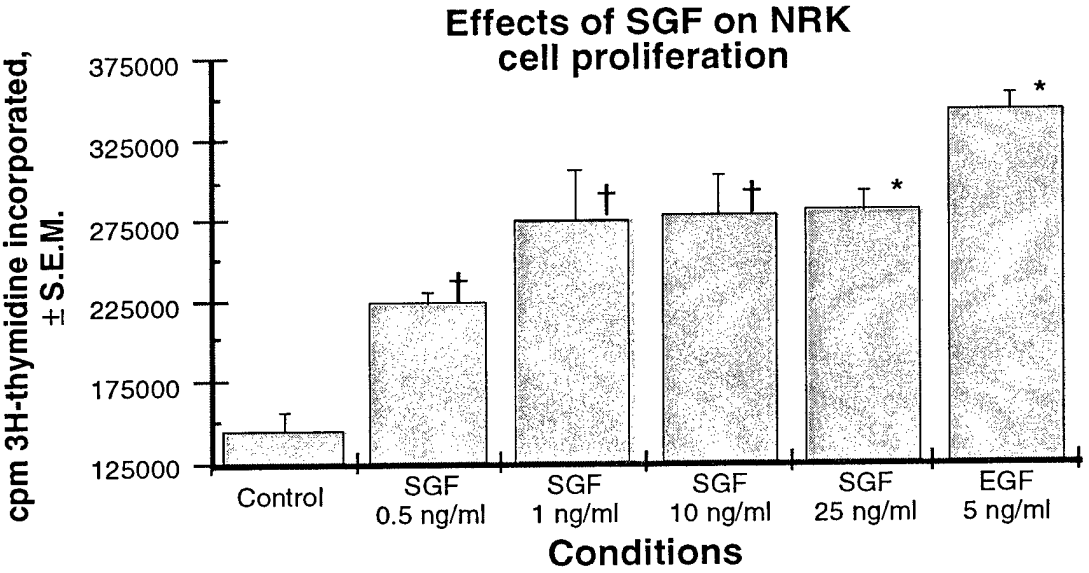
(d) Production and characterization of recombinant SGF

We had proposed to make biologically active recombinant SGF in order to elicit antiserum against the biologically active form of SGF. Other investigators have reported little success in making active SGF, using either prokaryotic expression (2), or chemical synthesis (14). To date, antibodies that can bind to SGF have not been reported. Antibodies vs. SGF peptides (A. Opgenorth, personal communication) or translation products made in E. coli do not recognize native SGF glycoprotein (2).

We have made recombinant glycoprotein SGF (rSGF) using baculovirus (AcMNPV, 69). Sf9 cells infected with SGF-AcMNPV produced a glycoprotein 12-16 kDa not found in Sf9 cells infected with wild type (wt) baculovirus, which matches published descriptions for SGF (2).

We reported that SGF produced in this fashion could be prepared from SDS-PAGE gels and used for functional studies in EGF-responsive cell lines (e.g., NRK cells). Such studies are illustrated in Fig. 1.

Figure 1 (following page). NRK proliferation stimulated by purified SGF or EGF. NRK cells were serum-starved overnight, and cultured for 4 days with SGF or EGF as shown. After 3 days 3H-thymidine was added. Cells were harvested one day later and incorporated radionucleotide counted. *, $P<0.01$; †, $P<0.05$, both compared to Control.



For all experiments described here, we will use recombinant SGF prepared by electroelution from SDS-PAGE of lysates of Sf9 cells infected with SGF-containing baculovirus.

Therefore, SGF produced from baculovirus yielded a glycoprotein similar in size to SGF from SFV-infected mammalian cells. This recombinant SGF has ≈75% of EGF's stimulatory activity. A chemically synthesized SGF peptide is reportedly 10% as active as EGF (14).

SGF is mainly expressed by the glandular stroma in mammary glands of transgenic mice. In this setting, its expression elicits mammary preneoplasia, leading to invasive carcinomas. We proposed in this grant to study tumor development using SGF transgenic mice and biologically active recombinant SGF as tools. This system may help to elucidate cellular, pathologic, and molecular mechanisms involved in mammary oncogenesis.

Purpose of the Present Work

•It is the purpose of the work performed in this grant to study mammary oncogenesis in SGF-transgenic mice as it involves both production of growth factor and responsiveness to it. The studies performed in pursuit of this project are intended to determine the extent to which this requirement may be met by interactions between growth factor-producing stroma and -responsive epithelium.

Methods of Approach

We proposed to use transgenic mice that express SGF as a transgene to study mammary oncogenesis. Cellular, biochemical and molecular parameters of oncogenesis in this system are to be defined, particularly as they relate to epithelial-stromal interactions. Thus, we would:

1. Define the natural history of mammary oncogenesis in SGF transgenic mice

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias

We proposed to use SGF-transgenic mice to study interactions between mammary stromal and epithelial cells and mechanisms of growth factor-related oncogenesis. The experimental approaches proposed, the methods to be applied, and the time frames in which these studies were proposed to be completed are as follows:

1. Define natural history of mammary oncogenesis in SGF transgenic mice (months 1-24)

(a) Transgenic mice will be mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

(b) Expression of recognized genetic markers associated with breast oncogenesis will be quantitated in RNA from mammary tissue.

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines (months 12-30)

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation (months 12-36)

(a) Antibody vs. SGF will be produced

(b) This anti-SGF antibody, in conjunction with cDNA probes, will be used to measure SGF production in the cultured transgenic fibroblasts and epithelial cells

(c) Immunohistochemistry and in situ hybridization will be used to localize SGF production and site of action within the mammary gland

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias (months 24-48)

(a) The ability of cells from transgenic mice to sustain their own growth will be measured by transplanting them into normal mice. The phenotypes of resultant proliferations will be studied by in situ hybridization and immunohistochemistry.

(b) Similarly, the ability of SGF-producing stroma to regulate oncogenesis will be determined by implanting normal cells into transgenic mice and assessing the outcome by in situ hybridization and immunohistochemistry.

Body

The work done in the past year will be described and illustrated with reference to the several tasks proposed in the original application. These will be recapitulated above. Experimental approaches that have to date been described at length in the original application and will not be repeated here, except to note changes that we have made in the original experimental plan. These changes have been made for one of several reasons: (1) to accommodate new data from our own laboratory; (2) to resolve experimental issues raised by new data; or (3) to reflect important contributions to this field described in recently reports from other laboratories.

1. Define natural history of mammary oncogenesis in SGF transgenic mice (months 1-24)

Our studies in the first year concentrated on generating the mice necessary for these studies. This was done by breeding the several lines of SGF-transgenic mice, testing them for

transgene carriage, and grouping them for sacrifice at the time intervals specified in the original proposal.

(a) Transgenic mice were mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

For this purpose, we bred transgenic mice from two different MT-SGF lineages and two different RSV-SGF lineages to provide sufficient numbers to begin our proposed systematic examination of the natural history of SGF-induced mammary gland epithelial proliferation. The proposed studies include the following:

•breeding and testing the SGF transgenic mice for transgene carriage. In the course of these studies, we decided that it was most advantageous to use only mice that were homozygous for SGF transgene carriage. Examination of heterozygotes would potentially complicate the analyses as we found that these mice occasionally express the transgene at lower levels that did homozygotes. Thus, substantial additional breeding was necessary to accommodate this need.

Nonetheless we have succeeded in breeding SGF transgenic mice to produce homozygous animals capable of expressing SGF.

•accumulation of sufficient numbers of transgenic mice to sacrifice the prescribed numbers of animals at the stated intervals (2 mo., 6 mo., etc.) and following the prescribed treatment regimens (Zn^{2+} treatment or control treatment for MT-SGF mice; pregnancy x0, x1, etc.). During the past two years, we had generated and cataloged sufficient numbers of the appropriate lines of mice both to allow breeding to continue, and to begin to sacrifice them in accordance with the proposed protocols.

Thus, we produced control (nontransgenic) mice that were sacrificed following 0 or ≥ 2 pregnancies. Mammary glands and other organs from these animals were saved for histologic examination, RNA extraction, etc., as proposed.

Furthermore we produced sufficient 2 month old virgin homozygous MT-SGF mice not fed Zn^{2+}, which have been sacrificed, again as previously proposed.

Along these lines, we have examined many mammary gland and other organ histologies from RSV-SGF, MT-SGF and normal mice. We have found that a significant percentage of SGF transgenic mice, mostly of the RSV-SGF lineage but occasionally of the MT-SGF lineages, show mammary gland proliferation and differentiation, even when they are virgins. The degree of mammary gland proliferation is highly advanced in some cases. In other cases, mammary differentiation includes milk production and extensive differentiation reminiscent of lactation.

(b) Expression of recognized genetic markers associated with breast oncogenesis will be quantitated in RNA from mammary tissue.

We have made RNA from mammary glands from many of the proposed groups of animals, and have accumulated the appropriate molecular probes with which to analyze these mice. The probes used are cDNA probes for c-myc, β-casein, whey acidic protein (WAP), retinoblastoma protein (Rb), p53, int-3, and Ha-ras, and oligonucleotide probes for α-lactalbumin and gelsolin. All of the RSV-SGF animals proposed in the original application have been studied by these techniques, most have, and the results are summarized below. The analyses shown below are incorporated into a manuscript that is in preparation at this time. This Table is taken from that manuscript.

Previously pregnant mice were sacrificed 2 months following their last pregnancy (for 6 month previously pregnant mice) and 4 months following their last pregnancy (for 12 month

previously pregnant mice). In the table shown below, the numbers of animals tested is indicated in parentheses.

Mice in the MT-SGF lines have all been generated, but a comprehensive analysis of these animals is not yet complete.

Table 1. Gene expression in mammary RNA preparations from SGF-transgenic mice

Genotype(number)	History	Gene tested								
		c-myc	β-cas	WAP	Rb	p53	α-lac	gelsolin	int-3	H-ras
Control										
2 month (2)	virgin	–	–	–	–	–	–	–	±	–
6 month (6)	virgin	±	±	–	–	–	–	–	+	–
6 month (6)	prev. preg.	++	++	–	–	–	–	–	+	–
12 month (6)	virgin	±	–	–	–	–	–	–	+	–
12 month (6)	prev. preg.	+	+/++	–	–	–	–	–	+	–
Experimental										
RSV-SGF										
2 month (2)	virgin	–	–	–	–	–	–	–	–	–
6 month (10)	virgin	+/++	+/++	+	–	–	–	–	+	–
6 month (6)	prev. preg.	+	+++	±/+	–	–	–	–	+	–
12 month (8)	virgin	+/++	±	±	–	–	–	–	+	–
12 month (6)	prev. preg.	++++	+++++	++	–	–	–	–	+	–

The table shown above is remarkable for the following observations:

(1) Expression of c-myc in whole tissue homogenates is highly unusual, yet appears to be a hallmark of SGF transgene expression. Myc is also expressed at detectable levels in control pregnant mouse mammary glands.

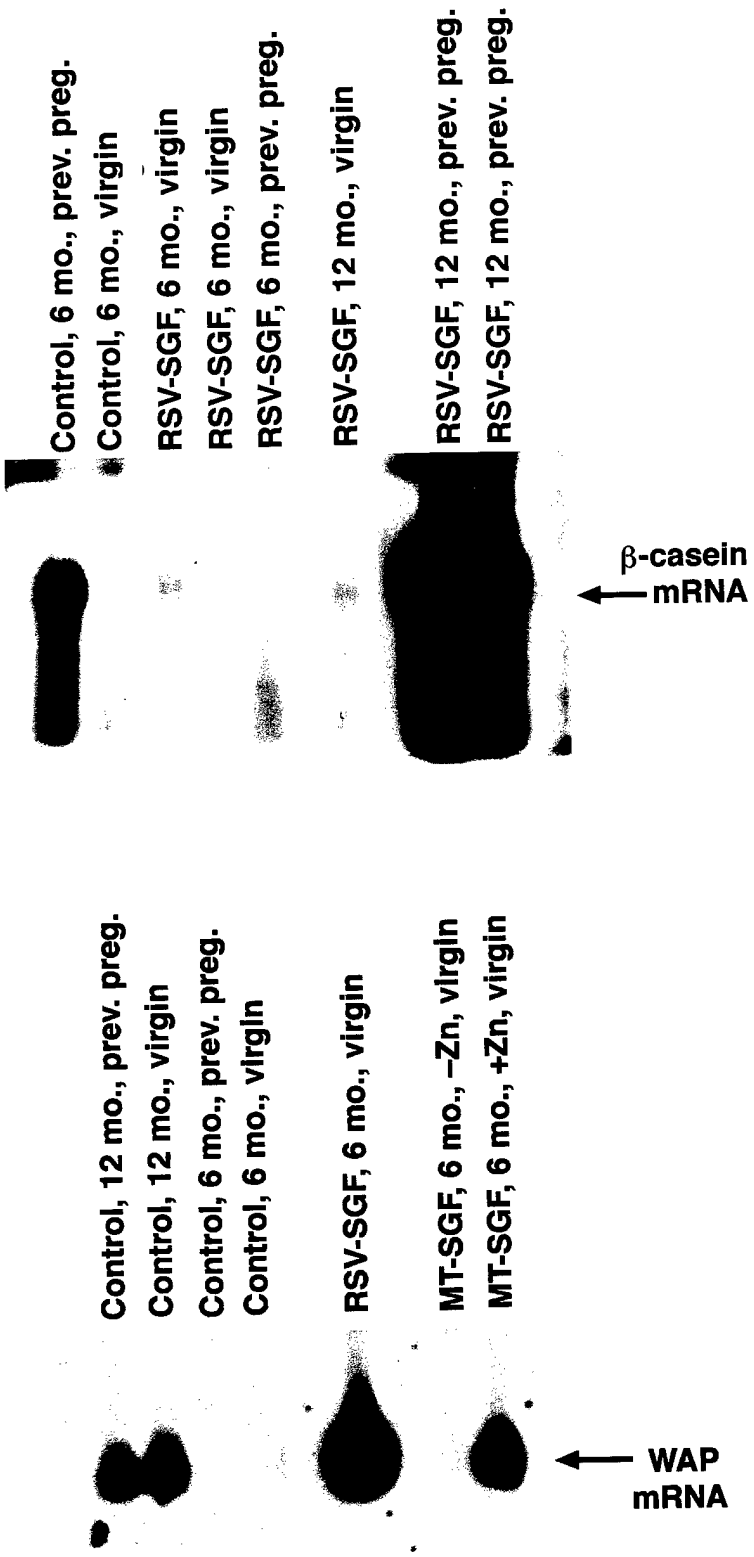
(2) Even more striking is the association of SGF production with expression of differentiation-associated proteins, β-casein and whey acidic protein (WAP).

The lack of detection of α-lactalbumin is of indeterminate significance, since in our hands the sensitivity of oligonucleotide probes is less than that of the cDNA probes used for most of the other cellular genes. (A cDNA probe for α-lac was requested but not provided to us.)

A very extensive histologic analysis has been completed on these animals. The findings of this histologic analysis can be summarized as follows. Compared to control mice, approximately half of virgin RSV-SGF transgenic mice at 6 months of age show mammary glandular histologic changes consistent with early to mid pregnancy: alveolarization of the gland, secretory changes in the alveolar cells, and accumulated or inspissated proteinaceous material in the ducts of the mammary glands. By 12 months of age, the percentage of virgin mice that show these changes is much less.

However, in previously pregnant mice the changes induced by SGF in the mammary glands are striking. Lactational histology in these animals often persists for 4 months following weaning of their last litters. This lactional histology involves very extensive ductal and alveolar proliferation, pronounced secretory cytologic changes in the alveolar cells, engorged alveoli and lobules, and dilated ducts. These glandular and ductal spaces contain

abundant milk-like secretions. By contrast, control animals' mammary gland histologies are normal.



The animals in which histologic analysis showed these described profound differentiative effects of SGF were those in which the most remarkable increases in milk protein gene expression was demonstrated. Thus, it was in these mice that high levels (sometimes extremely high levels) of WAP and β-casein mRNAs were observed, and in which high levels of c-myc expression was also seen.

The most striking feature of these studies is the indication that SGF has a profound differentiative effect on mammary epithelial cells in RSV-SGF mice. That is, in some virgin mice and in many previously (remotely) pregnant mice, continued very high level expression of lactation-associated genes has been observed. This is in strong contrast to our earlier studies in which RSV-SGF mice showed ductal proliferation with substantial atypia, as well as some carcinomas. These findings suggest an effect of the increased inbreeding of the SGF mice on the phenotype displayed: as homozygosity approached 100%, the effect of the transgene tilted towards inducing differentiation rather than inducing proliferation.

Fig. 2. Illustrative Northern analyses of transgenic mice and control mice. As indicated above, 6 mo. old previously pregnant mice are 2 mo. post-pregnancy and 12 mo. old previously pregnant mice are 4 mo. post-pregnancy. The blots illustrated are for total mammary gland RNA preparations hybridized with cDNA probes for β-casein or whey acidic protein, as indicated.

In part because of the surprising directions taken by the analyses generated in the above studies, we began additional breeding to test to the possible effect(s) of combined SGF expression + abnormal p53 on the mammary gland of mice. These additional studies were occasioned in part by the observation that p53 mutation plays such an important role in human cancer, particularly human breast cancer (71-75). Therefore, in collaboration with Larry Donehower, Department of Molecular Virology, Baylor College of Medicine, we began breeding our SGF transgenic mice with p53-knockout mice that he had developed.

We began this breeding program shortly after the onset of the previous grant project. We received a pair of p53 -/+ mice, provided to us by Dr. Donehower. Our first goal with these animals was to produce a stock of p53 -/+ mice with which to breed the SGF mice. This was accomplished. We then identified both p53-/- and p53-/+ mice, and have crossed these animals with SGF mice of both the RSV-SGF and MT-SGF lineages.

The goal of establishing SGF+p53-/- mice, as described in the prior progress report, has proven more difficult than we had expected. We have produced many p53 -/+, SGF+ mice of both sexes, and their phenotypes appear to be indistinguishable from normal mice. Most of these mice are heterozygous for RSV-SGF, perhaps explaining the lack of a more SGF-like mammary gland phenotype. We have made a large number of p53-/- mice, ™ and ¢, which have died at 6-9 months of age. Most of these deaths have been from lymphoma/leukemias. Deaths from osteosarcomas have also occurred. In addition, a number of mice that die spontaneously are consumed by cagemates and do not yield interpretable histologic findings.

We have, as well, noted a very strong preponderance of males among the SGF+p53-/- mice. That is, when pups are analyzed at 2-3 weeks of age, the male:female ratio among these animals is approximately 3:1. The poor fertility of the females of this genotype has further complicated our difficulties in establishing stable p53-/-SGF+ lines for analysis of mammary histologies. (Interestingly, the survival advantage of males during and/or shortly after embryogenesis is reversed in postnatal life. Among the p53-/-SGF+ mice females survive to 6-8 months far more readily than do males. The latter all apper to die within 4-6 months of birth.)

We are currently studying the reasons for the preponderance of males at birth in these animals, and have been in rather close contact with our collaborator in these studies, Dr. Donehower. Currently crosses are in progress to try to define any embryogenetic differences between male and female p53-/-SGF+ mice.

If our proposed lines of these combination transgenic-knockout mice can be developed, we will begin a systematic analysis of their mammary glands and other organs. Initially, the analysis of these animals will parallel analysis of the straight SGF transgenic animals described above. Subsequent analyses will depend upon the nature of the findings in our early studies.

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines (months 12-30)

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

Work on establishment of these cell lines is in progress. Dr. Pilarisetti has worked in the laboratory of Dr. Gilbert Smith (NCI) to learn the necessary procedures for culturing mammary epithelial cells.

At this point, we have successfully established stromal cell lines. These have been well enough established that aliquots have been frozen to preserve them for future analysis. These cells continue to be passaged. Progress has been somewhat slower in producing mammary epithelial cell lines, as several attempts have not yet yielded the expected results. We are currently continuing these studies.

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation (months 12-36)

(a) Antibody vs. SGF will be produced

(b) This anti-SGF antibody, in conjunction with cDNA probes, will be used to measure SGF production in the cultured transgenic fibroblasts and epithelial cells

(c) Immunohistochemistry and *in situ* hybridization will be used to localize SGF production and site of action within the mammary gland

We are working on this specific aim. (Specifically, Aims #3(a) and (c).) The first step in Aim (3(a) was to produce anti-SGF antiserum We have used SGF from our baculovirus system, and have immunized rabbits with 10-25 μg protein intravenously in saline every 3 weeks, bleeding one week after immunization. By Western blot, we have detected antibody activity. Therefore, we have developed antisera with antibody activity against SGF. Continued immunization and testing is being performed to develop sufficiently high anti-SGF activity for the second and third subaims of this specific aim.

With time, however, our stocks of productive recombinant baculovirus have produced increasingly poor yields of SGF. We have found that this is due to the loss of the SGF gene from the virus. Even when the original stock was recloned to produce a stock from the progeny of one SGF+ recombinant virus, we noted difficulties in producing adequate stocks of protein that could be applied effectively to these studies.

Consequently, we have reengineered our SGF-containing baculovirus to contain a his_7 leader sequence. The purpose of this reengineering is to facilitate the identification and purification of the recombinant growth factor. We have succeeded in this endeavor, and are now producing this slightly modified SGF, which can be purified in one step using a Ni affinity column. The levels of SGF production achieved are relatively low, but the relative ease of separation of the recombinant glycoprotein and its preparation compensate in our minds for the low yields.

In situ hybridization studies have been applied to the analysis of SGF production in transgenic mammary glands and other tissues. We have found, as was previously suggested, that the expression of SGF in the mammary gland is principally a function of stromal cells. We are currently applying these approaches to our stromal cell lines.

We had proposed an extensive analysis of SGF production and localization by *in situ* hybridization, and these studies have largely been completed. We have, in addition, done an extensive analysis of expression of several of the key genes studied by Northern analysis, using *in situ* hybridization. Thus, *in situ* hybridization studies of the mammary glands using riboprobes for c-myc, β-casein and WAP have also largely been completed. Expression of these cellular genes in the epithelial cells of the mammary glands is striking, and correlates particularly with the Northern analyses and the histologic analysis (see above).

In addition to the expression of SGF in mammary stroma and epithelium, we have found that epithelia of a number of organs do support SGF expression, however. We have found that epithelium of kidney, liver and gastrointestinal tract express SGF. The relative proportions of stromal: epithelial expression vary from organ to organ, however. The identification of c-myc,

β-casein and WAP transcripts in virgin mammary glands from SGF transgenic mice has provided stimulus for us to expand our *in situ* hybridization studies to these genes as well.

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias (months 24-48)

(a) The ability of cells from transgenic mice to sustain their own growth will be measured by transplanting them into normal mice. The phenotypes of resultant proliferations will be studied by in situ hybridization and immunohistochemistry.

(b) Similarly, the ability of SGF-producing stroma to regulate oncogenesis will be determined by implanting normal cells into transgenic mice and assessing the outcome by in situ hybridization and immunohistochemistry.

We have not yet begun to work on the studies proposed in this aim, but are intending to perform these experiments in the upcoming year.

This grant focuses on the effects of SGF on the mammary gland. In the course of the proposed studies, we expect to define the natural history of murine mammary tumor development as a function of SGF stimulation, and to categorize these effects according to current concepts of mammary development, differentiation and oncogenesis.

Conclusions

The goals of the first years of this project included the generation of a transgenic mouse colony of sufficient numbers to permit the analyses proposed in the later years of this project, and to supply the animals needed to complete the aims of this proposed research. We have largely completed the production of the requisite transgenic mice, the histologic analysis of their mammary glandular tissues, and Northern analyese of these tissues as well. We have further expanded our research to accommodate experimental study of the pathogenetic role that may accompany the clinically established correlation of mutant p53 with aggressive human breast cancer.

The goals of the third year have been to complete the proposed work on the animals of the different transgenic genotypes, to collate our Northern and *in situ* hybridization analyses with the histologic data, and to work on the production and analyses of SGF as we had proposed.

Most of this work has been done. We have performed extensive gene expression studies using MT-SGF and RSV-SGF mice, and have almost entirely completed the analyses of these animals by *in situ* hybridization to localize transgene and other cellular gene expression in the mammary glands of these mice. We have, as well, expanded the scope of our work to include the analysis of mice produced by crossing p53 knockout mice with our SGF transgenic mice.

We have been successful in producing anti-SGF antibody in rabbits, despite difficulties in maintaining the productivity of our recombinant baculovirus expression of SGF. We have addressed this shortcoming by reengineering the SGF gene in these baculovirus stocks. This process, although time consuming, has allowed us to generate an SGF protein that we can purify directly by heavy metal affinity chromatography from Sf9 cell lysates, rather than the less precise size fractionation that we had used before.

Our studies to date have clearly indicated that with the development of homozygous transgene-expressing mice the mammary glandular phenotype has changed, and now strongly favors a differentiative or development-inducing activity on the part of the glycoprotein growth factor, SGF. Although these results are not entirely unanticipated, based on the known

ability of EGFs to elicit both cellular proliferation and glandular differentiation, they underscore the potential applicability of differentiation therapy to the treatment and prevention of tumors. We hope that further studies of these transgenic mice in the context of this grant will define this area more fully and allow us to determine whether this glycoprotein growth factor may eventually find clinical application as an inducer of cellular differentiation and so perhaps as a tumor inhibitor.

References

1. Burgess, AW: Epidermal growth factor and transforming growth factor α. *Br. Med. Bull.*, **45**:401-424, 1989.

2. Chang, W, Macaulay, C, Hu, S-L, Tam, J, McFadden, G: Tumorigenic poxviruses: characterization of the expression of an epidermal growth factor related gene in Shope fibroma virus. *Virol.*, **179**:926-930, 1990.

3. Strayer, DS, Cabirac, GF, Sell, S, Leibowitz, JL: Malignant rabbit fibroma virus: Observations on the cultural and histopathologic characteristics of a new virally-induced rabbit tumor. *JNCI*, **71**:91-104, 1983.

4. Opgenorth, A, Strayer, DS, Upton, C, McFadden, G: Tumorigenic Poxviruses: Deletion of a growth factor gene reduces virulence of malignant rabbit fibroma virus. *Virology*, **186**:175-191, 1992.

5. Prestrelski, SJ, Arakawa, T, Wu, C-SC, O'Neal, KD, Westcott, KR, Narhi, LO: Solution structure and dynamics of epidermal growth factor and transforming growth factor α. *J. Biol. Chem.*, **267**:319-322, 1992.

6. Laurence, DJR, Gusterson, BA: The epidermal growth factor. *Tumor Biol.*, **11**:229-261, 1990.

7. Wong, ST, Winchell, LF, McCune, BK, Earp, HS, Teixidó, J, Massagué, J, Herman, B, Lee, DC: The TGFα precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. *Cell*, **56**:495-506, 1989.

8. Stroobant, P, Rice, AP, Gullick, WJ, Cheng, DJ, Ker, IM, Waterfield, MD: Purification and characterization of vaccinia virus growth factor. *Cell*, **42**:383-393, 1985.

9. Chang, W, Upton, C, Hu, S, Purchio, AF, McFadden, G: The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with a sequence similarity to those encoding epidermal growth factor and transforming growth factor alpha. *Mol. Cell. Biol.*, **7**:535-540, 1987.

10. Upton, C, Macen, JL, McFadden, G: Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. *J. Virol.*, **61**:1271-1275, 1987.

11. Matsunami, RK, Campion, SR, Niyogi, SK, Stevens, A: Analogs of human epidermal growth factor which partially inhibit the growth factor-dependent protein-kinase activity of the epidermal growth factor receptor. *FEBS Lett.*, **264**:105-108, 1990.

12. Defeo-Jones, D, Tai, JYU, Vuocolo, GA, Wegrzyn, RJ, Schofield, TL, Riemen, MW, Oliff, A: Substitution of lysine for arginine at position 42 of human transforming growth factor-alpha eliminates biological activity without changing internal disulfide bonds. *Mol. Cell. Biol.*, **9**:4083-4086, 1989.

13. Eppstein, DA, Marsh, YV, Schreiber, AB, Newman, SR, Todaro, GJ, Nestor, JJ, Jr.: Epidermal growth factor occupancy inhibits vaccinia virus infection. *Nature*, **318**:663-665, 1985.

14. Lin, Y-Z, Caporaso, G, Chang, P-Y, Ke, X-H, Tam, JP: Synthesis of a biological active tumor growth factor from the predicted DNA sequence of Shope fibroma virus. *Biochemistry*, **27**:5640-5645, 1988.

15. Downward, J, Yarden, Y, Mayes, E, Scrace, G, Totty, N, Stockwell, P, Ullrich, A, Schlessinger, J, Waterfield, MD: Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature*, **307**:521-527, 1984.

16. Hurwitz, DR, Emanuel, SL, Nathan, MH, Sarver,N, Ullrich, A, Felder, S, Lax, I, Schlessinger, J: EGF induces ligand binding affinity and dimerization of soluble epidermal growth factor (EGF) receptor extracellular domain. *J. Biol.Chem.*, **266**:22035-22043, 1991.

17. Lammers, R, Van Obberghen, E, Ballotti, R, Schlessinger,J, Ullrich, A: Transphosphorylation as a possible mechanism for insulin and epidermal growth factor receptor activation. *J. Biol. Chem.*, **265**:16886-16890, 1990.

18. Hu, P, Margolis, B, Skolnik, EY, Lammers, R, Ullrich, A, Schlessinger, J: Interaction of phosphotidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell. Biol.*, **12**:981-990, 1992.

19. Vega, QC, Cochet, C, Pilhol, O, Chagn, CP, Rhee, SG, Gill, GN: A site of tyrosine phosphorylation in the C terminus of the epidermal growth factor receptor is required to activate phospholipase C. *Mol. Cell. Biol.*, **12**:128-135, 1992.

20. Liu, XQ, Pawson, L: The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at tyr-460, adjacent to the GAP SH2 domains. *Mol. Cell. Biol.*, **11**:2511-2516, 1991.

21. Meldolesi, J: Multifarious IP3 receptors. *Curr. Biol.*, **2**:393-394, 1992.

22. Lückhoff, A, Clapham, DE: Inositol 1,3,4,5-tetrakisphosphate activates and endothelial Ca2+-permeable channel. *Nature*, **335**:356-358, 1992.

23. Egan, SE, Giddings, BW, Brooks, MW, Buday, L, Sizeland, AM, Weinberg, RA: Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*, **363**:45-51, 1993.

24. Baltensperger, K, Kozma, LM, Cherniack, AD, Klarlund, JK, Chawla, A, Banerjee, U, Czech, MP: Binding of the Ras activator Son of Sevenless to insulin receptor substrate-1 signaling complexes. *Science*, **260**:1950-1952, 1993.

25. Li, Batzer, A, Daly, R, Yajnik, V, Skolnik, E, Chardin, P, Bar-Sagi, D, Margolis, B, Schlessinger, J: Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signaling. *Nature*, **363**:85-88, 1993

26. Bruder, JT, Heidecker, G, Rapp, UR: Serum, TPA and ras induced expression from AP-1/Ets driven promoters requires raf-1 kinase. *Genes Dev.*, **6**:545-556, 1992.

27. Kyriakis, JM, App, H, Zhang, X-F, Banerjee, P, Brautigan, DL, Rapp, UR, Avruch, J: Raf-1 activates MAP kinase-kinase. *Nature*, **358**:417-421, 1992.

28. Binetruy, B, Smeal, T, Karin, M: Ha-ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature*, **351**:635-638, 1991.

29. Cutry, AF, Kinniburgh, AJ, Krabak, MJ, Hui, S-W, Wenner, CE: Induction of c-fos and c-myc proto-oncogene expression by epidermal growth factor and transforming growth factor a is calcium-independent. *J. Biol.Chem.*, **264**:19700-19705, 1989.

30. Snedeker, SM, Brown, CF, DiAugustine, RP: Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc. Natl. Acad. Sci. (USA)* **88**:276-280, 1991.

31. Taverna, D, Groner, B, Hynes, NE: Epidermal growth factor receptor, platelet-derived growth factor receptor, and c-erbB-2 receptor activation all promote growth but have distinctive effects upon mouse mammary epithelial differentiation. *Cell Growth Diff.*, **2**:145-154, 1991.

32. Sandgren, EP, Luetteke, NC, Palmiter, RD, Brinster, RL, Lee, DC: Overexpression of TGFα in transgenic mice: Induction of epithelial hyperplasia, pancreatic metaplasia and carcinoma of the breast. *Cell*, **61**:1121-1135, 1990.

33. Bacus, SS, Huberman, E, Chin, D, Kiguchi, K, Simpson, S, Lippman, M, Lupu, R: A ligand for the erbB-2 ondogene product (gp30) induces differentiation of human breast cancer cells. *Cell Growth Diff.*, **3**:401-411, 1992.

34. Monaghan, P, Ormerod, MG, O'Hare, MJ: Epidermal growth factor receptors and EGF-responsiveness of the human breast carcinoma cell line PMC42. *Int. J. Cancer*, **46**:935-943, 1990.

35. Moroni, MC, Willingham, MC, Beguinot, L: EGF-R antisense RNA blocks expression of the epidermal growth factor receptor and suppresses the transforming phenotype of a human carcinoma cell line. *J. Biol.Chem.*, **267**:2714-2722, 1992.

36. Yasui, W, Takekura, N, Kameda, T, Oda, N, Ito, M, Ito, H, Tahara, E: Effect of epidermal growth factor on rat stomach carcinogenesis induced by N-methyl-N'nitro-N-nitrosoguanidine. *Acta Pathol. Jpn.*, **40**:165-171, 1990.

37. Ullrich, A, Coussens, L, Hayflick, JS, Dull, TJ, Gray, A, Tam, AW, Lee, J, Yarden, Y, Libermann, TA, Schlessinger, J, Downward, JH, Mayes, ELV, Whittle, N, Waterfield, MD, Seeburg, PH: Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*, **309**:418-425, 1984.

38. Riedel, H, Massoglia, S, Schlessinger, J, Ullrich, A: Ligand activation of overexpressed epidermal growth factor receptors transforms NIH 3T3 mouse fibroblasts. *Proc. Natl. Acad. Sci. (USA)*, **85**:1477-1481, 1988.

39. Klijn, JG, Berns, PM, Schmitz, PI, Foekens, JA: The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review of 5232 patients. *Endocr. Rev.*, **13**:3-17, 1992.

40. Omekita, Y, Enokizono, N, Sagara, Y, Kuriwaki, K, Takasaki, I, Yoshida, A, Yoshida, H: Immunohistochemical studies on oncogene products (EGF-R, c-erbB-2) in human breast cancer: their relationship to oestrogen receptor status, histological grade, mitotic index and nodal status. *Virchows Archiv A*, **420**:345-351, 1992.

41. Kraus, MH, Fedi, P, Starks, V, Muraro, R, Aaronson, SA: Demonstration of ligand-dependent signaling by the erbB-3 tyrosine kinase and its constitutive activation in human breast tumor cells. *Proc. Natl. Acad. Sci. (USA)*, **90**:2900-2904, 1993.

42. Nicholson, S, Richard, J, Sainsbury, C, Halcrow, P, Kelly, P, Angus, B, Wright, C, Henry, J, Farndon, JR, Harris, AL: Epidermal growth factor receptor (EGFr): results of a 6 year follow-up study in operable breast cancer with emphasis on the node negative subgroup. *Br. J. Cancer*, **63**:146-150,1991.

43. Hainsworth, PJ, Henderson, MA, Stillwell, RG, Bennett, RC: Comparison of EGFR, c-erbB-2 product and ras p21 immunohistochemistry as prognostic markers in primary breast cancer. *Eur. J. Surg. Oncol.*, **17**:9-15, 1991.

44. Lundy, J, Schuss, A, Stanick, D, McCormack, ES, Kramer, S, Sorvillo, JM: Expression of neu protein, epidermal growth factor receptor, and transforming growth factor alpha in breast cancer. Correlation with clinicopathologic parameters. *Am. J. Pathol.*, **138**:1527-1534, 1991.

45. Jhappan, C, Stahle, C, Harkins, RN, Fausto, N, Smith, GH, Merlino, GT: TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell*, **61**:1137-1146, 1990.

46. Matsui, Y, Halter, SA, Holt, JT, Hogan, BLM, Coffey, RJ: Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. *Cell*, **61**:1147-1155, 1990.

47. Jhappan, C, Gallahan, D, Stahle, C, Chu, E, Smith, GH, Merlino, G, Callahan, R: Expression of an activated Notch-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev.*, **6**:354-355, 1992.

48. Ernberg, IT: Oncogenes and tumor growth factors in breast cancer. *Acta Oncol.*, **29**:331-334, 1990.

49. DeOme, KB, Faulkin, LJ, Jr, Bern, HA, Blair, PB: Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res.*, **19**:515-520, 1969.

50. Medina, D, DeOme, KB: Effects of various oncogenic agents on tumor-producing capabilities of D series BALB/c mammary nodule outgrowth lines. *JNCI*, **45**:353-363, 1970.

51. Huseby, RA, Soares, MJ, Talamantes, F: Ectopic pituitary grafts in mice: Hormone levels, effects on fertility and the development of adenomyosis uteri, prolactinomas and mammary carcinomas. *Endocrinology*, **116**:1440-1448, 1985.

52. Medina, D: Preneoplastic lesions in murine mammary cancer. *Cancer Res.*, **36**:2589-2595, 1976.

53. Medina, D: Mammary Tumors, pp. 373-396 in, *The Mouse in Biomedical Research*, vol. IV, ed. by HJ Foster, JD Small, JG Fox. Academic Press, New York, 1982.

54. Medina, D: Preneoplastic lesions in mouse mammary tumorigenesis, pp. 3-53 in *Methods in Cancer Research*, vol. 7, ed. by H Busch, Academic Press, New York, 1973.

55. Stuart, GW, Searle, PF, Chen, HY, Brinster, RL, Palmiter, RD: A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc. Natl. Acad. Sci. (USA)*, **81**:7318-7322, 1984.

56. Gorman, CM, Merlino, GT, Willingham, MC, Pastan, I, Howard, BH: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. (USA)*, **79**:6777-6781, 1982.

57. Osborne, CK, Coronado, EB, Kitten, LJ, Arteaga, CI, Fuqua, SAW, Ramasharma, K, Marshall, M, Li, CH: Insulin-like growth factor-II (IGF-II): A potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Molec. Endocrinol.*, **3**:1701-1709, 1989.

58. Cullen, KJ, Yee, D, Sly, WS, Perdue, J, Hampton, B, Lipmann, ME, Rosen, N: Insulin-like growth factor receptor expression and functino in human breast cancer. *Cancer Res.*, **50**:48-53, 1990.

59. Mathieu, M, Rochefort, H, Barenton, B, Prebois, C, Vignon, F: Interactions of cathepsin-D and insulin-like growth factor-II (IGF-II) on the IGF-II/Mannose-6-Phosphate receptor in human breast cancer cells and possible consequences on mitogenic activity of IGF-II. *Molec. Endocrinol.*, **4**:1327-1335, 1990.

60. Rosen, N, Yee, D, Lippman, ME, Paik, S, Cullen, KJ: Insulin-like growth factors in human breast cancer. *Breast Cancer Res. Treat.*, **18**(Suppl. 1):S55-S62, 1991.

61. Yee, D, Paik, S, Lebovic, GS, Marcus, RR, Favoni, RE, Cullen, KJ, Lippman, ME, Rosen, N: Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Molec. Endocrinol.*, **3**:509-517, 1989.

62. Yee, D, Rosen, N, Favoni, RE, Cullen, KJ: The insulin-like growth factors, their receptors and their binding proteins in human breast cancer. *Cancer Treat. Res.*, **53**:93-106, 1991.

63. Cullen, KJ, Allison, A, Martire, I, Ellis, M, Singer, C: Insulin-like growth factor expression in breast cancer epithelium and stroma. *Breast Cancer Res. Treat.*, **22**:21-29, 1992.

64. Sellers, TA, Kushi, LH, Potter, JD, Kaye, S, Nelson, CL, McGovern, PG, Folsom, AR: Effect of family history, body-fat distribution, and reproductive factors on the risk of postmenopausal breast cancer. *N. Engl. J. Med.*, **326**:1323-1329, 1992.

65. Stuart, GW, Searle, PF, Chen, HY, Brinster, RL, Palmiter, RD: A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc. Natl. Acad. Sci. (USA)*, **81**:7318-7322, 1984.

66. Gorman, CM, Merlino, GT, Willingham, MC, Pastan, I, Howard, BH: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. (USA)*, **79**:6777-6781, 1982.

67. Strayer, DS, Yang, S-J, Schwartz, MS: Epidermal growth factor-like growth factors. 1. Breast malignancies and other epithelial proliferations in transgenic mice. *Lab. Invest.*, **69**:660-673, 1993.

68. Guitteny, A-F, Bouque, B, Mougin, C, Teoule, R, Bloch, B: Histological detection of messenger RNAs with biotinylated synthetic oligonucleotide probes. *J. Histochem. Cytochem.*, **36**:563-571, 1988.

69. Summers, MD, Smith, GE, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, College Station, Texas A&M University, 1987.

70. Strayer, DS, Leibowitz, JL: Inhibition of epidermal growth factor-induced cellular proliferation. *Am. J. Pathol.* , **128**:203-209,1987.

71. Ozbun, MA, Butel, JS: Tumor suppressor p53 mutations and breast cancer: a critical analysis. *Adv. Cancer Res.*, **66**:71-141, 1995.

72. Rosanelli, GP, Steindorfer, P, Wirnsberger, GH, Klimpfinger, M, Ratschek, M, Puerstner, P, Auner, H, Berhold, A: Mutant p53 expression and DNA analysis in human breast cancer: comparison with conventional clinicopathological parameters. *Anticancer Res.*, **15**:581-586, 1995.

73. Tsuda, H, Hirohashi, S: Association among p53 gene mutation, nuclear accumulation of the p53 protein and aggressive phenotypes in breast cancer. *Int. J. Cancer*, **57**:498-503, 1994.

74. Friedrichs, K, Gluba, S, Eidtmann, H, Jonat, W: Overexpression of p53 and prognosis in breast cancer. *Cancer*, **72**:3641-3647, 1993.

75. Silvestrini, R, Benini, E, Daidone, MG, Veneroni, S, Boracchi, P, Cappelletti, V, DiFronzo, G, Veronesi, U: p53 as an independent prognostic marker in lymph node-negative breast cancer patients. *JNCI*, **85**:965-970, 1993.

ANIMAL USE REPORTING

Activity Name & Address: D. STRAYER, MD
JEFFERSON MEDICAL COLLEGE

Grant number: DAMD17- J-4434

Animal Type Genus/Species	Animals Purchased or Bred	Animals Used	USDA Pain Column C	USDA Pain Column D	USDA Pain Column E	AAALAC Accreditation
Transgenic mice	714	510	0	0	0	9/23/97

USAMRAA FORM 52-R (NOVEMBER 1994)