Mammary Epithelial Cells Treated Concurrently with TGF-α and TGF-β Exhibit Enhanced Proliferation and Death

T. M. Casey,*,†,[1] T. M. Mulvey,† T. A. Patnode,† A. Dean,† E. Zakrzewska,† and K. Plaut*,†

*Department of Animal Science, Michigan State University, East Lansing, Michigan 48824; and †Department of Animal Science, University of Vermont, Burlington, Vermont 05405

Transforming growth factor-α (TGF-α) stimulates while TGF-β inhibits mammary epithelial cell growth, suggesting that when cells are treated concurrently with the growth factors their combined effects would result in no net growth. However, combined treatments stimulate proliferation and cellular transformation in several cell lines. The objective of this paper was to describe the effect of long-term (6 days) concurrent TGF-α and TGF-β treatment on normal mammary epithelial cell growth pattern, morphology, and gene expression. Growth curve analysis showed that TGF-α enhanced while TGF-β suppressed growth rate until Day 4, when cells entered lag phase. However, cells treated concurrently with both growth factors exhibited a dichotomous pattern of growth marked by growth and death phases (with no intermittent lag phase). These changes in growth patterns were due to a marked induction of cell death from Day 2 (16.5%) to Day 4 (89.5%), resulting in the transition from growth to death phases, even though the combined treated cultures had significantly more ($P < 0.05$) cells in S phase on Day 4. TGF-β stimulated epithelial to mesenchyme transdifferentiation (EMT) in the presence of TGF-α, as characterized by increased expression of fibronectin and changes in TGF-β receptor binding. Expression patterns of genes that regulate the cell cycle showed significant interaction between treatment and days, with TGF-β overriding TGF-α–stimulated effects on gene expression. Overall, the combined treatments were marked by enhanced rates of cellular proliferation, death, and transdifferentiation, behaviors reminiscent of breast tumors, and thus this system may serve as a good model to study breast tumorigenesis. Exp Biol Med 232:1027–1040, 2007

Funding for this project was provided by the University of Vermont Agricultural Experiment Station.

[1] To whom correspondence should be addressed at Department of Animal Science, B290 Anthony Hall, Michigan State University, East Lansing, MI 48824. E-mail: ande1218@msu.edu

Received September 3, 2006.
Accepted April 2, 2007.

DOI: 10.3181/0609-RM-218
1535-3702/07/2328-1027$15.00
Copyright © 2007 by the Society for Experimental Biology and Medicine

Key words: transforming growth factor-alpha (TGF-α); transforming growth factor-beta (TGF-β); mammary; epithelial cell

Introduction

Transforming growth factor-alpha (TGF-α) and TGF-β were initially partially purified together and described as sarcoma growth factor (SGF; Ref. 1). SGF was characterized as a TGF that induced both proliferation and phenotypic transformation of rat kidney fibroblast cells (1–3). When the growth factors were isolated and their individual effects defined, it was quickly recognized that, in general, TGF-α stimulates while TGF-β inhibits epithelial cell growth. In the mammary gland, TGF-α and TGF-β influence ductal and alveolar morphogenesis during normal development. However, aberrant expression of TGF-α and TGF-β and/or their respective receptors is associated with mammary neoplasia (4–12). Although the opposing effects of these growth factors on cell proliferation suggests that the net combined effect on cell growth would be zero, the early studies demonstrated that together they produced cellular transformation and proliferation that was greater than the individual effects of the growth factors alone. Therefore, it is critical to understand how the growth factors work in concert to affect cell growth.

TGF-α is a potent mitogen and induces mammary epithelial cell proliferation by binding to the epidermal growth factor receptor (EGFR; Refs. 13, 14). Overexpression of TGF-α is associated with cellular transformation *in vitro* and *in vivo*. A normal mouse mammary epithelial cell line transfected with TGF-α cDNA exhibited increased growth rates and anchorage-independent growth in soft agar (15, 16). Transgenic mice that overexpress TGF-α in mammary epithelium form preneoplastic structures, hyperplastic alveolar nodules (HANs), that can develop into mammary carcinomas (17–19). Expression of TGF-α has been identified in invasive ductal carcinoma (9) and correlates with increased neoangiogenesis in breast tumors

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

(20). Carcinomas of the breast that have higher levels of expression of TGF-α also express higher levels of EGFR (21); thus, blockade of EGFR activation by using anti-EGFR monoclonal antibodies has been shown to be a potent anti–breast cancer therapy (22–24).

TGF-β decreases growth rates of epithelial cells by causing cell cycle arrest and/or inducing apoptosis. These cellular responses are elicited through its binding to the type II receptor (TGF-βR2), which in turn dimerizes with the type I receptor (TGF-βR1) and activates signaling pathways (25). Overexpression of TGF-β leads to mammary ductal (26) and alveolar (27) hypoplasia and is associated with resistance to 7, 12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor formation in mice (28). In contrast, conditional knockout of TGF-βR2 in mammary epithelium results in lobuloalveolar hyperplasia (29). When TGF-βR2 conditional knockout mice were mated with PyVmT transgenic mice, tumor latency was shortened and pulmonary metastases increased. These studies demonstrate that the growth-inhibitory effects of TGF-β play an important role in suppressing mammary tumorigenesis and progression.

TGF-β also induces epithelial to mesenchymal trandifferentiation (EMT) in culture. EMT is described as an entire series of events involved in the transition from an epithelial to a mesenchymal phenotype that include (i) decreased expression of the epithelial markers E-cadherin, ZO-1, and desmoplakins I and II; (ii) increased expression of mesenchymal markers, such as fibronectin; and (iii) a fibroblast-like reorganization of actin fibers (30, 31). During embryonic development EMT allows cells to detach from the epithelial tissue where they originate and to migrate. EMT is also believed to be the mechanism by which epithelium-derived tumor cells invade the stroma and metastasize throughout the body. In fact, EMT has been correlated with a more aggressive tumor phenotype (32, 33). Thus, it is important to define and characterize TGF-β–regulated EMT, as the dissociation of the growth-inhibitory effects of TGF-β from its effects on EMT and the extracellular matrix (ECM) appears to be the turning point in its role as a tumor suppressor to its role as a breast cancer progression and metastatic factor (34).

Although the early history of discovery of the TGFs and their functions focused on their combined effects, little work has continued in this area. More recent investigations have focused on how each of these factors affects epithelial cell growth and cell cycle gene expression alone. Furthermore, the effect of these growth factors on the regulation of the cell cycle has largely been limited to early response genes in the culture system (35, 36). It is clear from previous work that the interplay between these growth factors has an effect on cell growth that is not merely additive, but results in a transformed phenotype. Additionally, the combined effect may vary over time depending on the stage of cell cycle, presence of other factors, and period of exposure. For this study we used a normal mouse mammary epithelial cell line, NOG-8, to investigate the combined effects of TGF-α and TGF-β on cell growth and morphology. Further, we used cDNA miniarray analysis to determine whether treatment and day affected expression of cell cycle–related genes. Real-time quantitative polymerase chain reaction (PCR) was used to validate differences in gene expression among the treatments and between days that fell into one of the three broad categories of cell cycle progression, differentiation, and cell death: Cdc25b, Trp63, and Mre11A, respectively.

Materials and Methods

Cell Culture. The normal mouse mammary epithelial cell line, NOG-8, derived from NMuMG (37), was used to characterize the effect of TGF-α and TGF-β1 on cell growth. NOG-8 cells were maintained at 37°C in a humidified chamber with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Corp., St. Louis, MO), pH 7.2 to 7.3, supplemented with 100,000 U/l penicillin G, 100 mg/l streptomycin (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS; Invitrogen), and 1 μg/ml insulin (maintenance media; Sigma Chemical). The cells were passaged when they reached approximately 90% confluence by treatment with 0.25 m*M* trypsin and 0.03% EDTA (Invitrogen), and they were seeded at 10,000 cells/cm^2 for every experiment. All treatments were done in duplicate, and experiments were repeated at least two times.

Determination of Optimal Serum and Growth Factor Concentrations and Growth Curve Analysis. To determine the optimal concentration of TGF-α (in 4 m*M* HCl with 1 mg/ml bovine serum albumin [BSA]; R&D Systems, Minneapolis, MN), TGF-β1 (in 10 m*M* acetic acid with 0.1% BSA; R&D Systems) and TGF-α plus TGF-β1 (the combined factors) on the growth of NOG-8 cells, cells were incubated in DMEM with 10% FBS supplemented with 0, 10, 25, or 100 ng/ml TGF-α, 0, 0.25, 2.5, or 10 ng/ml TGF-β1. Cells were seeded in six-well dishes in maintenance media, allowed to plate overnight, and synchronized by removing maintenance media and incubating for 24 hrs in serum-free DMEM. After 24 hrs, media were replaced with DMEM supplemented with serum and growth factors. Preliminary data showed that neither 10 m*M* acetic acid nor 4 m*M* HCl with respective amounts of BSA affected NOG-8 cell growth, so vehicle control treatments were not necessary. Media were changed and growth factors were replenished on Days 2 and 4. Cells were harvested 24 hrs after serum starvation (Day 0) or at 2, 4, and 6 days and were resuspended in 0.4% trypan blue to stain dead cells. Live and dead cells were counted on a hemacytometer to obtain an independent measure of numbers of cells and the ratio of live to dead cells. Statistical analysis was performed using SAS General Linear Models (Statistical Analysis System V8; Cary, NC) with growth factor, day, and growth factor by day as main effects. If the overall F was significant, multiple comparisons were analyzed using Bonferroni t test (38).

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

To determine optimal serum concentration, media were changed to DMEM supplemented with 0, 1%, 2%, or 10% FBS after synchronization.

Cell Cycle Analyses. Flow cytometry was used to determine the proportion of cells in each phase of cell cycle during the 6-day growth period. NOG-8 cells were seeded in 75 cm^2 flasks (Corning Inc., Corning, NY) with maintenance media, synchronized, and treated as described above for the 6-day experimental period.

At Days 0, 2, 4, and 6 the cells were harvested, washed in phosphate-buffered saline (PBS) and stain solution (3% polyethylene glycol 6000, 50 μg/ml propidium iodide [PI; Sigma Chemicals, St. Louis, MO], 720 U/ml RNase [Worthington Biochemical Corp., Lakewood, NJ], 0.1% Triton X in 4 m*M* sodium citrate (pH to 7.8), and PBS + 0.1% sodium azide and 0.1% BSA), was added to the cell suspension to a final concentration of 2 million to 4 million cells/ml and was incubated at 37°C for 30 min. The proportion of cells in each phase of the cell cycle was determined with flow cytometry using a BD LSRII flow cytometer equipped with DIVA software v4.1.1 (both from BD Bioscience, San Jose, CA). Cells were analyzed at a flow rate of 800 cells/sec. Data were evaluated using analysis of variance with treatment and day as main effects using SAS. If overall F was significant, multiple comparisons were analyzed using Bonferroni t-test (38). Data were expressed as percentage of the total analyzed population.

Bromodeoxyuridine (BrdU) Pulse-Chase Labeling and TUNEL Assay. To determine whether cells underwent DNA synthesis prior to cell death, a BrdU pulse-chase experiment was used, followed by the TUNEL assay to measure apoptotic cells. Before the pulse-chase experiment was begun, the length of the NOG-8 cell cycle was determined. Cells were pulse labeled with 50 μ*M* BrdU for 2 hrs and then were harvested every hour for 48 hrs (39). BrdU incorporation was detected using the BrdU staining kit from Zymed (Zymed Laboratories Inc., San Francisco, CA). Regression of the percentage of labeled mitoses against time was then used to determine the length of the cell cycle and S phase (40). From these data we determined that an 18-hr chase would follow the 2-hr pulse labeling with BrdU for the pulse-chase experiments.

NOG-8 cells were seeded in DMEM plus 10% FBS, synchronized, and treated as above. At 20 hrs prior to harvesting, cells were pulse labeled with 50 μ*M* BrdU in serum-free DMEM (for Day 0) or DMEM plus 10% FBS and appropriate growth factors (for Days 2, 4, and 6) for 2 hrs. Cells were washed with PBS, media were replaced, and cells were incubated for an additional 18 hrs. After 18 hrs, chase media were collected, and cells were harvested on Days 0, 2, 4, and 6, and BrdU incorporation was measured using flow cytometry (41) and the percentage of dying cells determined with a TUNEL flow cytometry assay (42).

Evaluation of EMT and TGF-β1 Binding Assay. NOG-8 cells were photographed using a light microscope daily to monitor for change in morphology. Day of transdifferentiation was identified by a distinct visible change in morphology, characterized by a change from an epitheliallike cobblestone morphology to a fibroblastlike morphology (EMT) in more than 75% of the cells. To determine whether EMT was influenced by the ECM, NOG-8 cells were plated on plates coated with laminin, fibronectin, or collagen IV (Collaborative Biomedical, Bedford, MA) in the presence of maintenance media plus 0 or 2.5 ng/ml TGF-β1.

Fibronectin expression was evaluated using immunocytochemistry. Cells were plated in eight-well chamber slides (Nunc, Naperville, IL) in the presence of 0 or 2.5 ng/ml TGF-β1, grown to 90% confluence, and then fixed in 3% paraformaldehyde. Cells were incubated for 1 hr at room temperature with a 1:400 dilution of a rabbit anti-human fibronectin antibody (Sigma, St. Louis, MO), followed by immunodetection of the primary body using a Zymed Histostain kit with the chromogen 3-amino-9-ethyl carbazole substrate for the streptavidin–horseradish peroxidase.

To determine whether TGF-β altered receptor binding during EMT, TGF-β1 binding to NOG-8 cells was measured after cells underwent a noticeable morphologic change on each of the matrices. Cells were incubated with ^{125}I-TGF-β1 (Amersham, Arlington Heights, IL) and 0 or 10 ng/ml unlabeled TGF-β1 as described by Plaut and Maple (43). To determine receptor subtypes, samples from the binding assays were suspended in an equal volume of 2× sample buffer (125 m*M* Tris base, 20% glycerol, 138 m*M* sodium dodecyl sulfate, 10% β-mecaptoethanol, and 10% bromophenol blue) boiled for 2 mins, cooled, and then loaded into an 8% polyacrylamide gel and run at 10 mA for stacking and 20 mA for the separating gel. Gels were fixed in 50% methanol–10% acetic acid, amplified with Amplify (Amersham, Piscataway, NJ), dried on a Savant gel drier (Savant Instruments Inc., Farmingdale, NY), and exposed to Kodak XAR film (Rochester, NY) at −70°C for 2 weeks. Film was developed, and the density of each band was evaluated by scanning autoradiographs.

Miniarray. To determine the effect of growth factor treatment on cell cycle–related gene expression, NOG-8 cells were seeded in a 25-cm^2 flask, synchronized by serum starvation, and treated as described above for cell cycle analysis. Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and was used as a template for $^{\alpha 32}$P-dCTP-labeled (PerkinElmer Life Sciences, Boston, MA) cDNA probe synthesis (using the GEArray Q series procedure; SuperArray, Bethesda, MD). Probes were hybridized to mouse cell cycle genes using Mouse Cell-cycle GEArray *Q series* (SuperArray). Image and data analyses were performed using the Storm Phosphoimager system (Molecular Dynamics, Sunnyvale, CA). Analysis of variance was used to evaluate control genes. The control gene Pp1a was spotted in quadruplicate on each assay, and the intraassay coefficient of variance was 14.2%. Since there was a significant increase in expression in Ppa1 in response to growth factor treatment, it was not compared across day

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

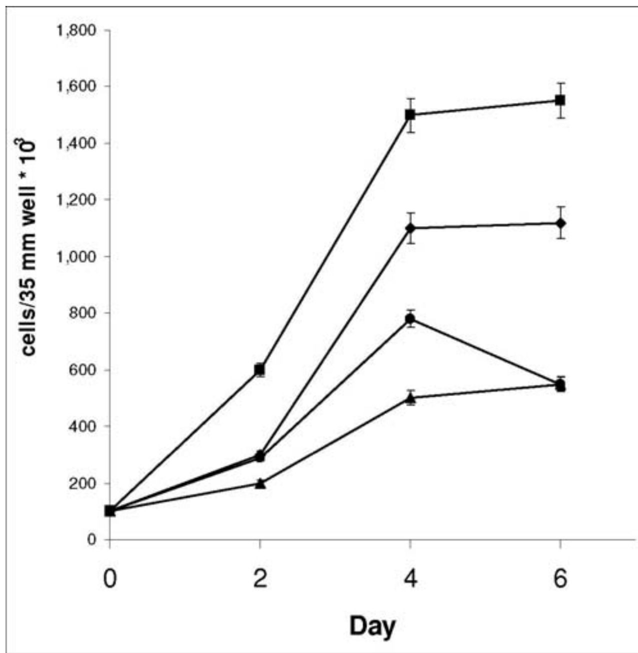


Figure 1. To determine the effects of TGF-α and TGF-β1 on the growth rate of the NOG-8 cell line, cells were seeded in six-well dishes at 10,000 cells per cm^2 in DMEM + 10% FBS (black diamonds) supplemented with 10 ng/ml TGF-α (black squares), 2.5 ng/ml TGF-β1 (black triangles), or 10 ng/ml TGF-α plus 2.5 ng/ml TGF-β1 (black circles). Media were changed and growth factors replenished every 2 days; cells were collected and resuspended in 0.4% trypan blue, and live cells were counted on a hemacytometer on Days 2, 4, and 6. Data points are the mean ± SE of duplicate experiments.

or treatment. GAPDH, which was spotted in duplicate on each array, did not vary by day or by treatment. The interassay coefficient of variance for GAPDH was 12.8%; therefore, GAPDH was used to correct for blot variability. Gene expression was normalized to the control (10% FBS with no growth factors) on each day.

Real-Time Quantitative PCR (RTq-PCR). RTq-PCR was used to validate miniarray analysis of the cell cycle genes Cdc25b, Mre11a, and Trp63. NOG-8 cells were seeded in T-75 flasks, serum starved, and treated with TGF-α, TGF-β1, and TGF-α plus TGF-β1 for 2, 4, or 6 days as described above. Total RNA was prepared from cells using TRIzol (Invitrogen) and treated with DNase (Ambion, Austin, TX), and quantity was estimated using the Nano-drop (NanoDrop Technologies, Wilmington, DE). The quality of the RNA was assessed on a Bioanalyzer 2100 (Agilent Inc., Palo Alto, CA). High-quality RNA was reverse transcribed into cDNA from 1 μg total RNA using the hexamer reaction from the GeneAmp RNA PCR kit (catalog no. N808-0017; Applied Biosystems, Foster City, CA). Assays-On-Demand, the TaqMan Gene Expression Assays (Applied Biosystems) were used for the RTq-PCR reactions for the following genes: mre11a (Mm00450600_m1), cdc25b (Mm00499136_m1), and trp63 (Mmoo495788_m1), following manufacturer specifications. The 18S (hs99999901_s1) rRNA was used to normalize the sample (44).

Results

Optimal Experimental Serum and Growth Factor Concentrations. To determine the concentration of serum needed to measure the experimental effects of the growth factors alone or in combination, growth curves were performed with varying concentrations of FBS. Cells grown in media supplemented with 0, 1%, or 2% FBS were growth inhibited by approximately 70% or more on Day 6 compared with cells grown in media supplemented with 10% FBS (data not shown). Thus, to prevent masking of the growth inhibitory effects of TGF-β1 by serum deprivation, all subsequent experiments were performed with a control medium of DMEM supplemented with 10% FBS.

To determine the optimal growth factor concentrations to use for the studies, growth curve analyses were run with varying concentrations of growth factors alone. All three TGF-α concentrations (10, 25, and 100 ng/ml) significantly increased NOG-8 cell growth rate compared with controls (data not shown). Growth rates of cultures treated with 0.25 ng/ml TGF-β1 were not different from controls. However, cultures treated with 2.5 ng/ml and 10 ng/ml TGF-β1 significantly inhibited NOG-8 cell growth through Day 6 relative to controls ($P < 0.0007$; data not shown). Thus, 10 ng/ml TGF-α and 2.5 ng/ml TGF-β1 were used for all subsequent experiments, as these were the minimal concentrations that significantly affected epithelial growth rate and fell within reported physiologic concentrations (45, 46).

Cell Cycle Analysis. To determine the effect of the combined growth factor treatments on long-term cell culture growth patterns, 6-day growth curves were performed by counting the total number of live and dead cells on a hemacytometer (Fig. 1). As expected, when TGF-α was added in the presence of serum it enhanced the growth rate of NOG-8 cells, whereas the addition of TGF-β suppressed growth relative to serum-treated controls. When the growth factors were added together the pattern of growth was marked by two phases: growth and death. During the first 2 days of culture there was an apparent equilibrium between the two transforming growth factors, as the rate of growth was equal to that of the control cells. Between Days 2 and 4 the rate of cell growth increased in the combined treatment; however, it was intermediate between control and TGF-β. Growth of cultures treated with TGF-α plus TGF-β apparently "peaked" at Day 4 and then crashed into death phase between Days 4 and 6 (Fig. 1). This was in contrast to all other treatments, which appeared to enter a stationary or lag phase, as indicated by the lack of change in slope of the growth curve between Days 4 and 6 (Fig. 1).

Flow cytometry was used to determine how the proportions of cells changed in each phase of the cell cycle during this 6-day period. Figure 2 shows that following 24

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

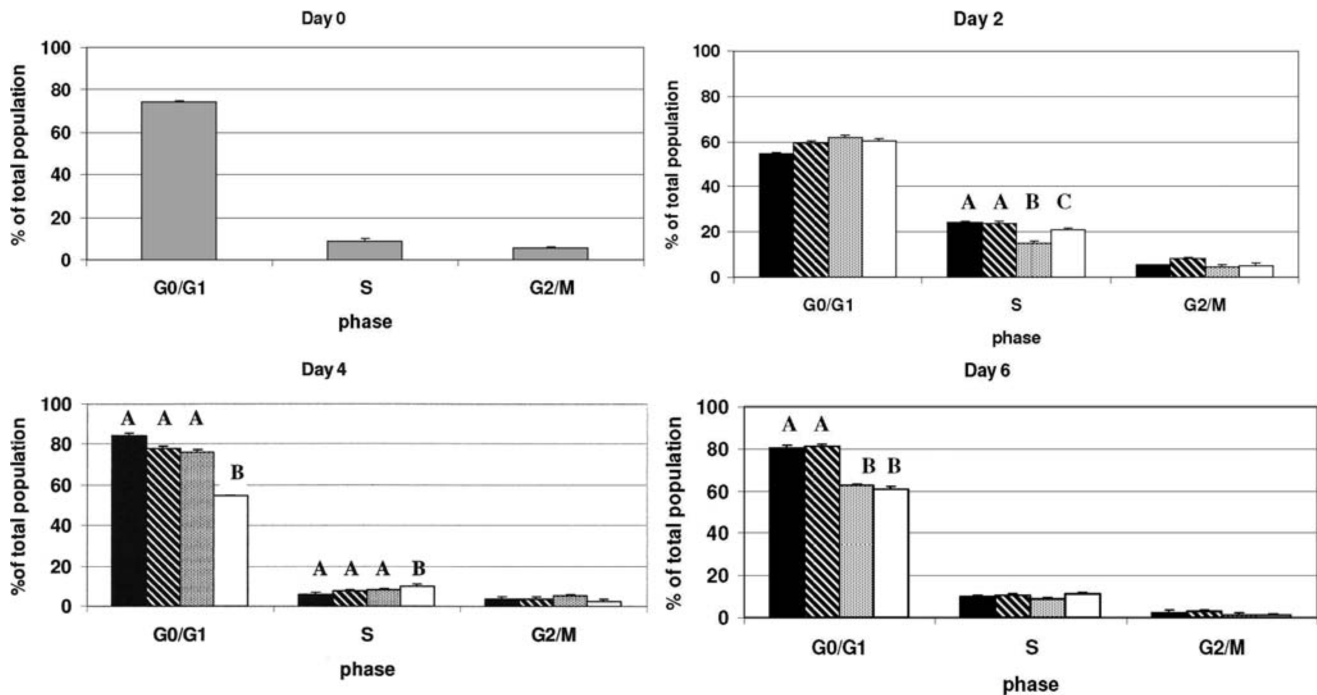


Figure 2. Flow cytometry was used to determine the proportion of cells in each phase of cell cycle during the 6-day experimental period. NOG-8 cells were seeded at 10,000 cells per cm^2 with DMEM supplemented with 10% FBS and were synchronized in the cell cycle by serum starvation for 24 hrs (gray bars; Day 0). After synchronization, maintenance media (DMEM and 10% FBS) was added to cells alone (control; black bars) or supplemented with 10 ng/ml TGF-α (striped bars), 2.5 ng/ml TGF-β (dotted bars), or 10 ng/ml TGF-α and 2.5 ng/ml TGF-β (white bars). The proportion of cells in each phase of the cell cycle was determined with flow cytometry, and data were evaluated using analysis of variance, with treatment and day as main effects using SAS. Experiments were done in duplicate; the data are expressed as mean percentage of the total analyzed population ± SE. Means with a different letter indicate treatment difference at $P < 0.05$ within each phase of the cell cycle.

hrs of serum starvation, cells were synchronized, with 73% of cells arrested in G_0/G_1 phase of the cell cycle. After 2 days of culture, control cells were cycling, with approximately 20% fewer cells in G_0/G_1 and 15% more cells in S phase relative to Day 0 (Fig. 2). Cells treated with TGF-α were cycling at a rate similar to that of control cultures (Fig. 2). When cells were cultured with TGF-β1, they also began cycling, but at a slower rate: 68% of TGF-β1–treated cells were in G_0/G_1 versus 52% of control cells ($P < 0.05$), and 12% of TGF-β1–treated cells were in S phase versus 23% of control cells ($P < 0.05$), indicating that TGF-β1 does inhibit DNA synthesis. Cells in the combined treatment also entered the cell cycle at a slower rate relative to controls, but the effect was less pronounced, with 21% of these cells versus the 23% of the control cells ($P < 0.05$; Fig. 2) in the S phase of the cell cycle. On Day 4 the combined treated cultures had a significantly lower proportion of cells in G_1/G_0 phase and a significantly greater proportion of cells in S phase relative to all other treatments ($P < 0.05$), and on Day 6 both the TGF-β–treated and the combined treated cultures had a significantly lower proportion of cells in G_1/G_0 relative to control and TGF-α–treated cultures.

A significant change in the percentage of PI-stained cells with less than 2 N nuclear content was evident during flow cytometry analysis, and thus led to the examination of the dying population. Centrifugation of media and cells and subsequent use of trypan blue exclusion verified that the whole population of dead cells was being recovered. To determine the rate of cell death in cultures, a TUNEL flow cytometry assay was combined with a BrdU pulse-chase experiment. The BrdU pulse-chase experiment was used to determine whether cells underwent apoptosis prior to or after S phase. Following serum starvation, approximately 33% of the population died, indicating that serum starvation had a negative effect on the cell population. By Day 2, only 6%, 4%, and 17% of control, TGF-α–, and combined growth factor–treated cells were dying. However, 40% of TGF-β–treated cells were dying at Day 2. For all treatments, the dying cell population went through the cell cycle prior to death (Fig. 3).

On Day 4, TGF-α–treated cultures were protected against death compared with the other treatments. However, the population of dying cells in the combined growth factor treatment was increased from approximately 16% to 89% from Day 2 to Day 4. The increase in the dying population for other treatments was much smaller; 15%, 10%, and 22% more cells were dying by Day 4 in the control, TGF-α, and TGF-β treatments, respectively (Fig. 3). For the control and TGF-α treatments, two thirds of the dying cell population was not cycling prior to death, and one third was cycling before cell death. In contrast, the dying cell population was split equally between the cycling and noncycling populations in both the TGF-β and the combined treatments.

By Day 6, 50% of the TGF-β–treated cells were dying

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

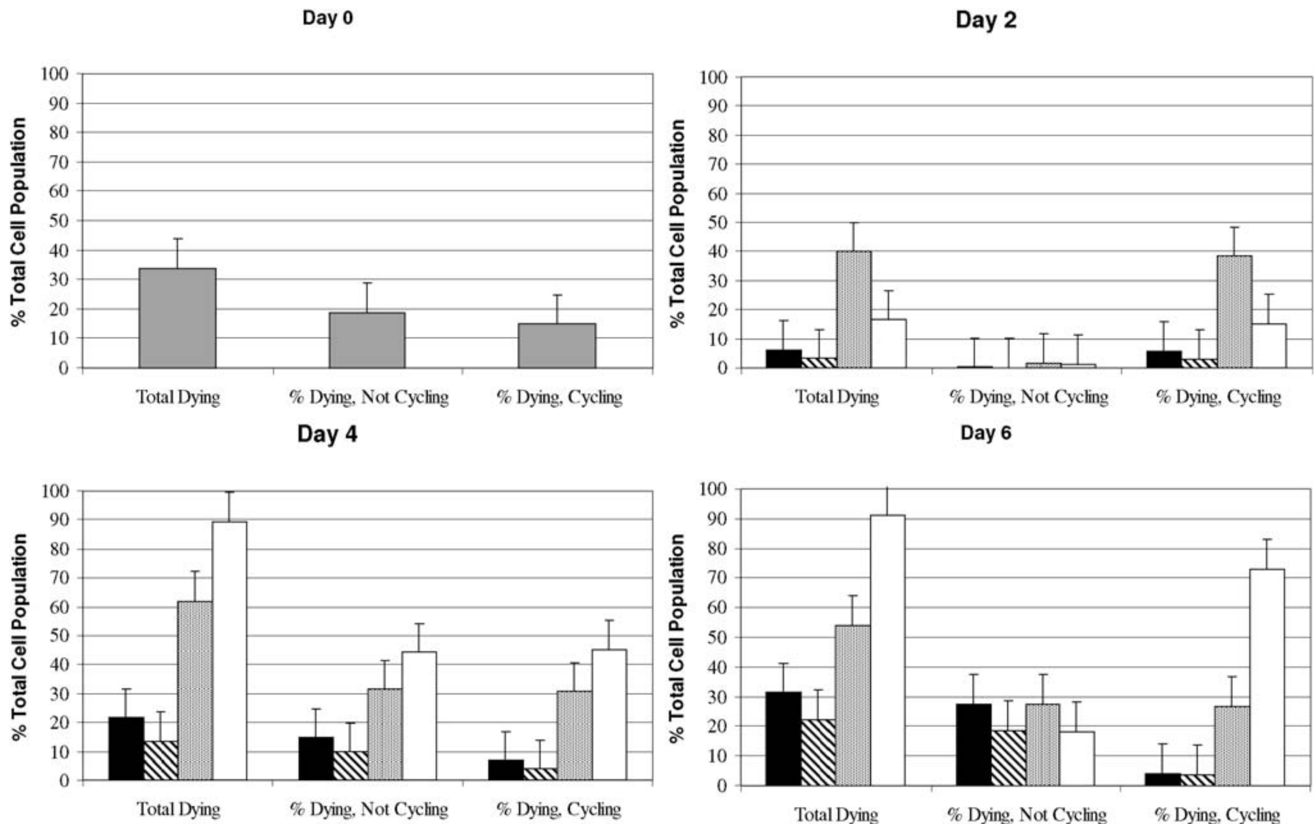


Figure 3. The dying population was examined using a TUNEL flow cytometry assay combined with a BrdU pulse-chase experiment. The pulse-chase experiment was used to determine whether cells were cycling prior to cell death. Flow analysis was used to determine (i) the total dying population, which was the proportion of the entire analyzed population (P1) positively stained with Tricolor using the TUNEL assay; (ii) the percentage of cells dying and not cycling, which was the proportion of the P1 cells only labeled with Tricolor; and (iii) the percentage of cells dying and cycling, which was the proportion of the P1 cells double labeled with Tricolor and fluorescein isothiocyanate. Gray bars, serum starved 24 hrs; black bars, control; striped bars, 10 ng/ml TGF-α; dotted bars, 2.5 ng/ml TGF-β; white bars, 10 ng/ml TGF-α and 2.5 ng/ml TGF-β1. The experiment was done in duplicate; data are represented as mean ± SE; statistical analysis indicated an overall treatment by day effect on the percentage of dying cells ($P < 0.05$).

versus 90% in the combined treatment. Only 20% to 30% of the cells were dying in either TGF-α or control cells. The percentage of dead cells that were cycling decreased in all treatments except the combined growth factor treatment, in which the cycling cell population increased 26%. In the control and TGF-α treatments, most of the cells were not cycling before death. In the TGF-β treatment, the percentages of the cycling and noncycling cells in the dying population were approximately equal. For the combined treatment, the percentage of the dying population that was cycling before cell death was approximately four times greater than the percentage of cell population that was not cycling before cell death (Fig. 3).

Effect of Growth Factors on Cell Morphology and TGF-β Receptor Binding. Cultures were examined daily using a light microscope to monitor change in cellular morphology. Control cultures and cultures treated with TGF-α maintained a cobblestone morphology typical of epithelial cells throughout the 6-day experimental period (Fig. 4). Cultures treated with TGF-β1 alone as well as combined cultures were phenotypically transformed from a cobblestone morphology to a spindle-shaped morphology typical of fibroblast cells (EMT) on Days 2 and 4, respectively (Fig. 4). Interestingly, on Day 6 the cultures treated with TGF-α plus TGF-β1 often began to detach from the bottom of cell culture plates, making them difficult to study. Since TGF-α did not stimulate transdifferentiation in the absence of TGF-β1, further investigations on EMT were done with TGF-β1 alone. In order to further characterize EMT, immunocytochemistry was used to evaluate the level of fibronectin expression in culturesm, as increased expression of mesenchymal markers is a hallmark of EMT (30). Fibronectin expression increased in cells that underwent EMT following 2 days of TGF-β1 treatment (Fig. 5), supporting our microscopy observations of EMT.

EMT is also characterized by reorganization of the actin cytoskeleton. Epithelial cells interact with a specialized ECM, known as the basement membrane (BM), through integrins, which are the major ECM receptors (47). Integrins connect the BM to the intracellular actin cytoskeleton and *via* recruitment of focal adhesion kinases, which in turn initiate signaling cascades involved in regulating cell shape, migration, and differentiation (48), suggesting that the substrata that epithelial cells are plated on may affect their

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

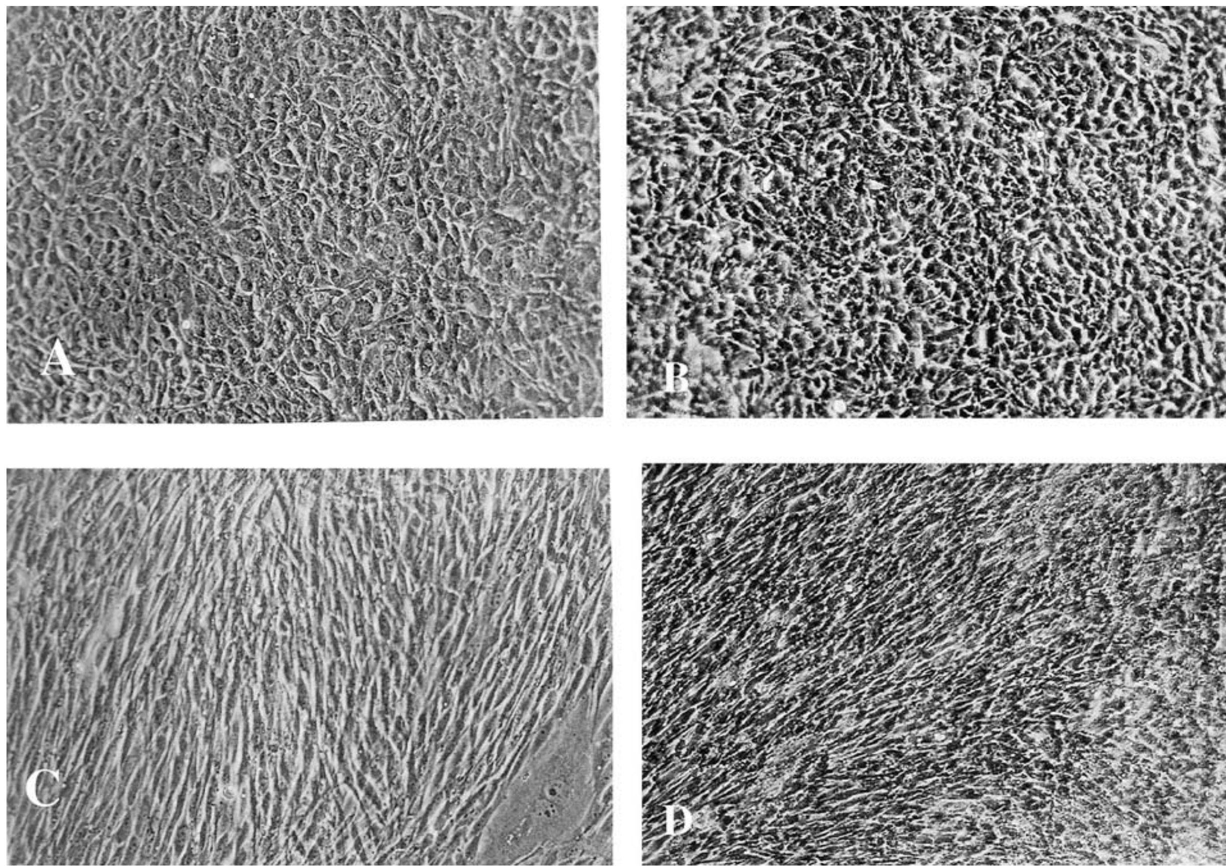


Figure 4. Cells were observed daily with a light microscope using phase contrast to examine any changes in morphology. Control cultures (A) and cultures treated with 10 ng/ml TGF-α (B) maintained a cobblestone morphology typical of epithelial cultures throughout the 6-day experimental period. However, EMT became evident on Day 2 in cultures treated with 2.5 ng/ml TGF-β (C) and on Day 4 in cultures treated with 10 ng/ml TGF-α plus 2.5 ng/ml TGF-β (D). Magnification: ×100.

ability to undergo EMT. However, NOG-8 cells treated with TGF-β underwent EMT on all three substrata tested: laminin, fibronectin, and collagen IV (data not shown).

TGF-β–induced EMT has been shown to be stimulated through pathways that are distinct from TGF-β–stimulated growth inhibition and apoptosis in epithelial cells (2, 30, 49–51). Since TGF-β signaling cascades are initiated through cell surface receptors, and alterations in receptor numbers and/or stoichiometry may affect the stimulation or inhibition of distinct signaling cascades, we measured whether EMT was accompanied by changes in TGF-β receptor binding or distribution of subtypes. TGF-β1 receptor binding was greatest in control compared with TGF-β1–treated cultures on all matrices tested. Binding was greatest in control cultures plated on fibronectin, but these differences were not significant due to large variability in response (Table 1). Polyacrylamide gel electrophoresis of the cross-linked samples indicated that the receptor population was redistributed in TGF-β–induced EMT cultures compared with nontransdifferentiated control cultures (Table 2 and Fig. 6). The proportion of cells plated on plastic expressing TGF-βR2 increased from 43% to 64%, and TGF-βR1 decreased from 38% to 11% ($P < 0.05$). Further, EMT-associated redistribution of receptors occurred, regardless of the matrix on which the cells were plated (Table 2).

Growth Factor Effect on Cell Cycle Gene Expression. The effect of growth factor treatment on the expression of cell cycle–related genes was examined using a cDNA miniarray with mouse cell cycle genes. There was a significant interaction between treatment and day in many of the genes ($P < 0.05$; Table 3). Differences in patterns of gene expression between the treatments were illustrated with arrows, indicating at least a 4-fold increase or decrease in gene expression relative to control cultures on each day (Table 3). From these data we selected three genes that represented one of the main subgroups of the miniarray–cell cycle regulatory (Cdc25b), differentiation (Trp63), and cell death (Mre11a)–to validate changes using RTq-PCR (Fig. 7). Ccd25b was induced by TGF-α on Day 2, dropped down to control levels on Day 4, and was suppressed relative to control on Day 6. TGF-β and the combined treatments had no effect relative to control cultures on Cdc25b expression on Days 2 and 4, but on Day 6 the expression was depressed relative to control. TGF-α induced the expression of Trp63 relative to the

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

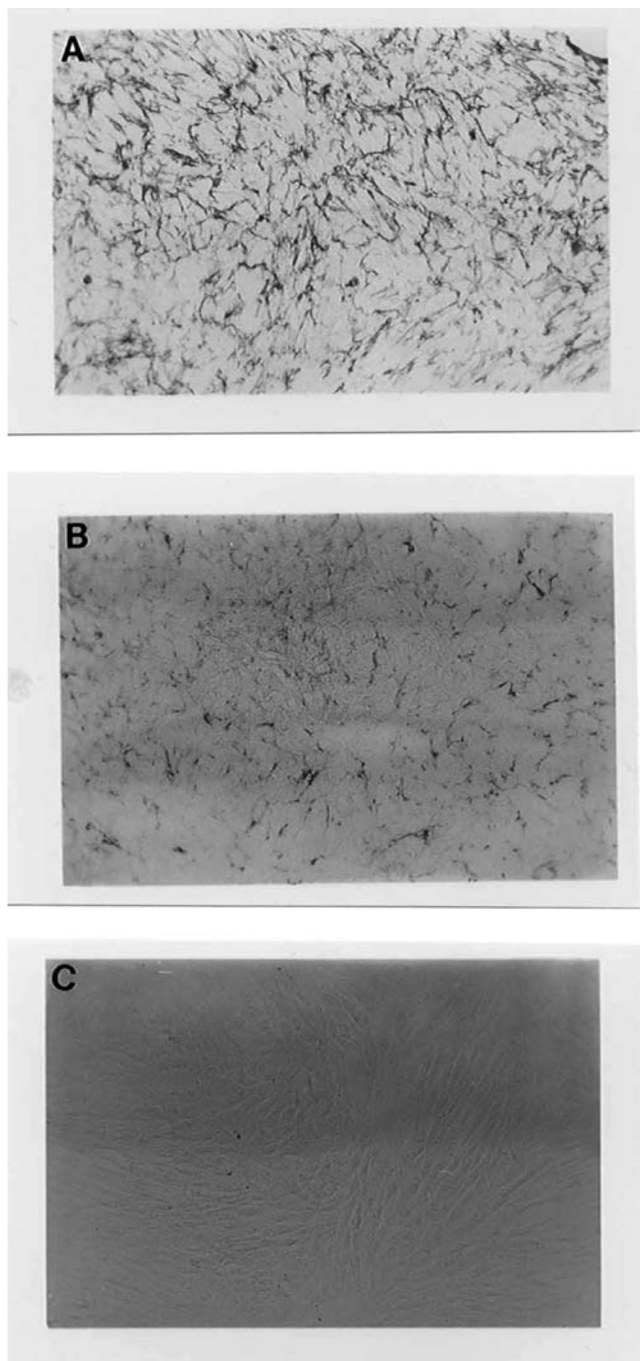


Figure 5. Fibronectin expression was evaluated with immunocytochemistry when cultures reached 90% confluence. Expression of fibronectin was greater in cultures induced to undergo EMT in the presence of 2.5 ng/ml TGF-β1 (A) versus nontransdifferentiated control cultures (B). A mouse IgG control (C) was run with each experiment and shows no staining. Magnification: ×100.

control on Days 2 and 4 but was similar to control levels on Day 6. On the other hand, Trp63 expression was suppressed in TGF-β cultures on Days 2 and 6 relative to control cultures and was suppressed in the combined treatment relative to controls throughout the 6-day period. The expression level of Mre11a was similar in all cultures on

Table 1. Effect of Extracellular Matrix on Binding of TGF-β1 to NOG-8 Cells[a]

Matrix	TGF-β1 (ng/ml)	TGF-β1 bound/cell, nm	SE
Plastic	0	398	353
	2.5	242	262
Collagen IV	0	421	144
	2.5	308	105
Fibronectin	0	1121	182
	2.5	490	52
Laminin	0	701	606
	2.5	192	98

[a] NOG-8 cells were plated and exposed to 0 or 2.5 ng/ml TGF-β1. When EMT was observed in TGF-β–treated cultures, receptor binding assays were performed. Receptor binding was not significantly different between TGF-β1– and control-treated cultures for any matrix.

Day 2, but on Day 4 Mre11a was suppressed relative to controls in TGF-β and combined treatments, and then on Day 6 the expression of Mre11a was suppressed relative to controls in all three growth factor treatments.

Discussion

Effects of Growth Factor Treatment on Cell Growth and Morphology. TGF-α–treated mammary epithelial cell cultures had the greatest sustained growth rate. These cultures had more cells cycling and relatively few dying cells throughout the 6-day experimental period. These data are consistent with previous literature showing that TGF-α is a mitogen in mammary epithelial cultures (12, 14). Cultures treated with TGF-α maintained cobblestone morphology typical of normal epithelial cells throughout the experimental period, indicating that they did not undergo EMT or transformation under these culture conditions.

The slower growth rate in TGF-β–treated cells was due to both inhibition of DNA synthesis and induction of cell death or apoptosis, as evident in the slower rate of entry into the S phase of the cell cycle (Fig. 2) and the greater percentage of cells dying throughout the 6 days (Fig. 3). Interestingly, most of the cells induced to undergo cell death on Day 2 had been in S phase 18–20 hrs earlier. As the experimental days progressed, cells from both the non-cycling and cycling populations were stimulated equally to undergo cell death.

As expected, cultures treated with TGF-β1 underwent EMT, as evident in the change in phenotypic morphology and increased expression of fibronectin (Figs. 3 and 4). Further, we found that TGF-β1 induced EMT on all types of matrices tested, suggesting that TGF-β–induced EMT overrides ECM-focal adhesion signaling. TGF-β–induced EMT was also accompanied by a redistribution of TGF-β receptor populations. The number of TGF-βR1s decreased as TGF-βR2s and betaglycan (also known as the type III receptor) increased. The significance of this change in

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

Table 2. Effect of ECM and TGF-β1 on Distribution of TGF-β Receptor Types[a]

Matrix	TGF-β1 (ng/ml)	Type I	Type II	Type III
Plastic	0	39.4	40.7	19.9
	2.5	21.2	71.3	7.5
Collagen IV	0	46.3	33.7	20.0
	2.5	21.0	62.8	16.2
Fibronectin	0	33.2	40.5	26.4
	2.5	0	43.4	56.6
Laminin	0	31.1	57.9	11.0
	2.5	0	80.4	19.1

[a] NOG-8 cells were plated and exposed to 0 or 2.5 ng/ml TGF-β1 until EMT occurred in TGF-β–treated cultures. Binding assays were then performed by exposing cells to ^{125}I-TGF-β1 for 30 mins at 4°C with or without 10 ng/ml TGF-β1. Following binding assays, samples were resuspended in sample buffer, boiled for 2 mins, cooled and loaded in an 8% polyacrylamide gel, and run at 10 mA for stacking gel and 20 mA for separating gel. Gels were fixed, amplified, dried, and exposed to X-ray film, and receptor population was measured by densitometric analysis and expressed as percentage of total receptor population.

receptor population is unclear, since TGF-βR2 and TGF-βR1 normally work in concert, with a 1:1 stochiometry (25, 52, 53). It is possible that epithelial cells use divergent pathways for EMT and growth inhibition (30, 50, 51), and the change in receptor distribution reflects changes in signaling pathways. Thus, the change in distribution of the receptor population upon EMT is quite intriguing and needs further investigation, as it may provide clues as to why TGF-β switches from a tumor suppressor to a progression factor. The role of TGF-β in cancer progression and metastasis is believed to be due to the dissociation of its growth inhibitory effects from its effects on the ECM (54) as well as the inappropriate induction of EMT in tumors (33).

As expected, we observed a dichotomy of behavior when cultures were treated with TGF-α and TGF-β concurrently. These cultures were marked by high rates of proliferation, death, and cellular transformation. The change from a growth phase to a death phase in the cultures treated with TGF-α plus TGF-β on Day 4 was due to a 5-fold induction of cell death. These cells also had the greatest proportion of cells in S phase on Day 4, suggesting that TGF-α continued to stimulate cell growth while TGF-β was inducing cell death. Thus, even though a greater percentage of cells were cycling, even more were dying, reflecting the change from the growth to death phase and pointing to the importance of not just relying on measuring DNA synthesis as a marker of cell growth. Both the cycling and noncycling pools of cells contributed equally to the dying population, and it is important to note that this pattern of enhanced rates of cycling cells coupled with high rates of cell death is typical of what is seen in tumors. TGF-β is clearly the molecular switch regulating the change in growth behavior in the combined cultures, since TGF-β stimulated cell death while TGF-α suppressed cell death relative to controls.

Similar to TGF-β–treated cultures, the combined treatment went through EMT, although it was on Day 4 in TGF-α plus TGF-β–treated cultures versus Day 2 in cultures treated with TGF-β alone. There is evidence that EMT may be involved in the dedifferentiation program that leads to malignant carcinoma and cell detachment from primary tumors (33, 55). In this cell culture system, the combined treatment frequently became detached in sheets from cell culture dishes at Day 6. This may be indicative of anchorage-independent growth. We know that this detachment was not due to "overcrowding" of these cultures, as both the control treated and TGF-α–treated cultures contained many more cells than the combined treatment. It also is not a phenomenon of low cell number, since TGF-β–treated cells did not detach. Since TGF-β1–induced EMT did not cause cells to detach from cell culture plates, the transformation we are seeing in these cultures appears to be due to the combined effects of the TGFs, which may mimic tumor growth behavior rather than cancel each other out.

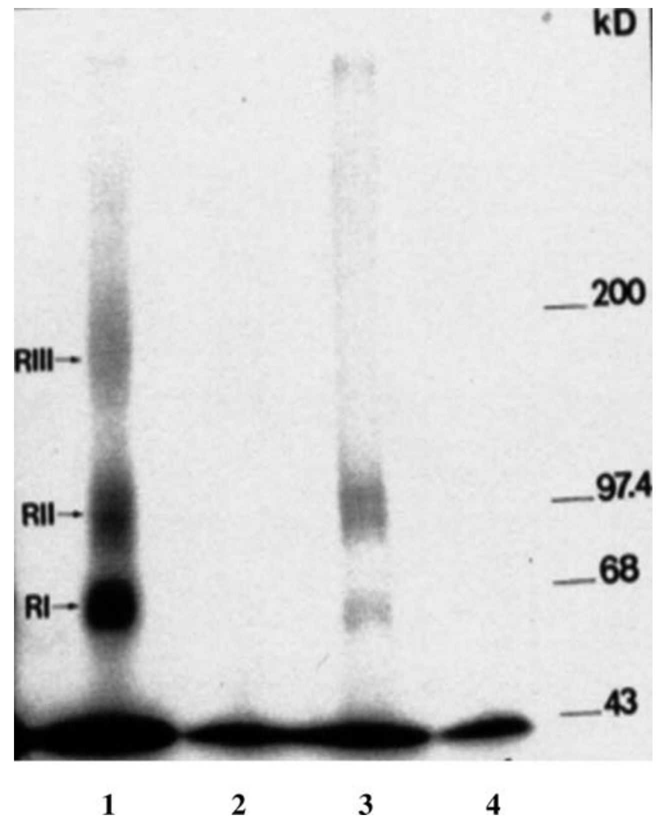


Figure 6. The effect of TGF-β treatment and EMT on distribution of TGF-β receptor subtypes was examined by plating NOG-8 cells on cell culture plastic at 10,000 cells/cm^2 with DMEM and 10% FBS supplemented with 0 (lanes 1 and 2) or 2.5 ng/ml TGF-β1 (lanes 3 and 4). When cells underwent EMT after 2 days in TGF-β–treated cultures, competitive receptor binding assays were performed by incubating samples with ^{125}I-TGF-β1 + 0 ng/ml TGF-β1 (lanes 1 and 3) or ^{125}I-TGF-β1 + 10 ng/ml TGF-β1 (lanes 2 and 4). Following binding assays, samples were prepared for electrophoresis and loaded in an 8% polyacrylamide gel. A shift in receptor type was noted from control cultures (lane 1) to cultures treated with 2.5 ng/ml TGF-β1 (lane 3), as there was an increase in type II receptors relative to type I.

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

Table 3. Miniarray-Detected Changes in Cell Cycle–Related Gene Expression Patterns Expressed Relative to Control-Treated NOG-8 Cells on Days 2, 4, and 6[a]

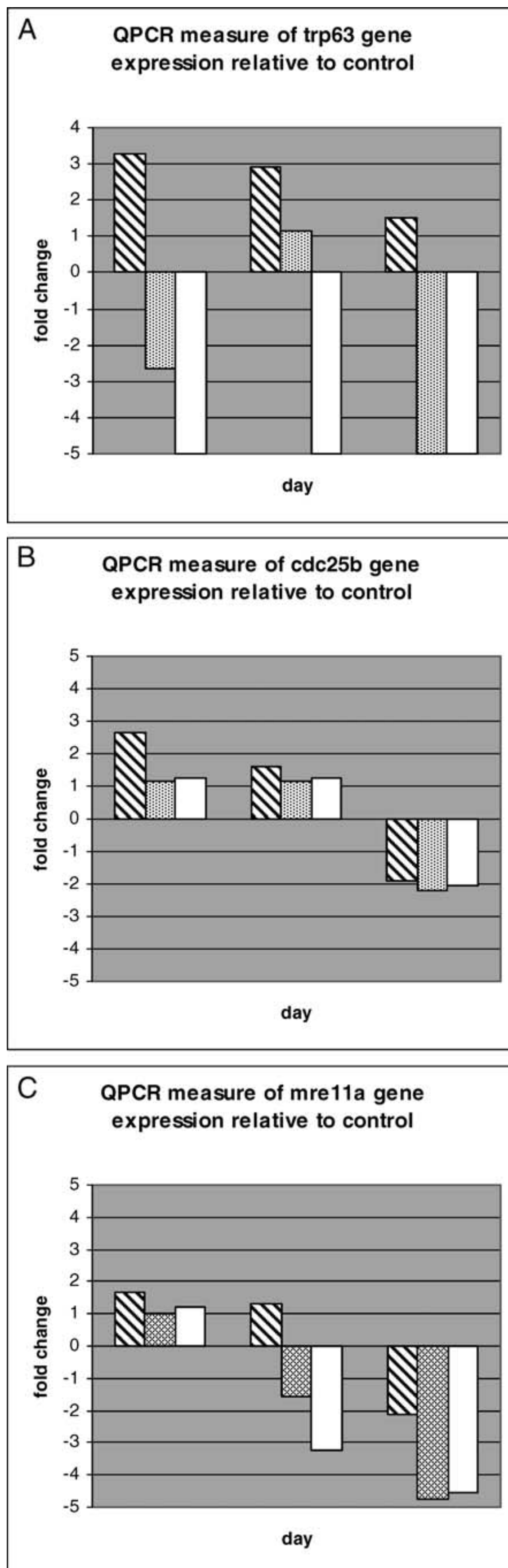
Gene name	Function	TGF-α			TGF-β			TGF-α +TGF-β		
		Day 2	Day 4	Day 6	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6
Cdk5r[b]	Homologous to Cdc2 expressed in apoptotic cells, upregulated to cause death	—	—	—	↓	↑	↑	↑	—	—
Ccnd1[c]	Cyclin D1, upregulated to stimulate cell cycle progression	—	—	—	↑	↑	↑	↑	↑	↑
E2f4[d]	Transcription repressor, binds with p107 and Smads 3 and 4 to repress c-*myc*	↑	↑	↓	↑	—	—	↑	↑	↑
cdc25b[e,f]	Phosphatase, activates cyclin-dependent kinases, activates cyclin a and b1 for G_2 to S	↑	—	↓	—	—	↓	—	—	↓
cdc20[g]	Activates anaphase-promoting complex by degrading kip1p	—	↑	—	↑	—	—	↑	↑	—
cdc2a[h]	Associated with apoptosis and hyperphosphorylation of Rb	—	↑	↑	—	↑	↑	↑	↑	↑
mrella[f,i]	Endonuclease and exonuclease activity for DNA repair	—	—	↓	—	↓	↓	—	↓	↓
Ubc[j]	Ubiquitin associated with stress response, binds with mdm2 to promote cell repair	↑	—	↑	—	—	↑	↓	—	↑
trp63[f,k]	p53 homolog, needed for stem cell maintenance	↑	↑	—	↓	—	↓	↓	↓	↓
mdm2[l]	Binds to p53 and p63 and may rescue cells from apoptosis	—	↑	—	↑	↑	↑	↑	↑	↑
bcl2[m]	Suppresses apoptosis	↑	↑	↑	↑	↑	—	↑	↑	—
bax[m]	Dimerizes with bcl2; when overexpressed induces apoptosis	—	↑	↑	—	↑	↑	—	↑	↑

[a] Mouse cell cycle GEArray Q series (SuperArray Inc.) cDNA miniarrays were used to measure gene expression in control cultures and cultures treated with TGF-α, TGF-β1, or TGF-α plus TGF-β for 2, 4, or 6 days following serum starvation. Miniarray analysis experiments were performed two times, with duplicate arrays run for each treatment in each experiment. Blot variability was corrected by normalizing GAPDH expression among blots, whereas gene expression was normalized to the control (10% FBS with no growth factors) on each day. There was a significant interaction between treatment and day for many of the genes, including those listed in the table ($P < 0.05$). Arrows indicate at least a 4-fold decrease (↓) or increase (↑) in gene expression relative to controls within each day. —, indicates that there was a less than 4-fold difference in gene expression with RTq-PCR.
[b] Zhu *et al.* (71).
[c] Barnes and Gillett (72).
[d] Vairo *et al.* (73)
[e] Forrest and Gabrielli (74).
[f] Indicates that the pattern of change in gene expression was validated.
[g] Gordon and Roof (75).
[h] Choi *et al.* (76).
[i] D'Amours and Jackson (77).
[j] Conaway *et al.* (78).
[k] Dohn *et al.* (79).
[l] Kadakia *et al.* (80).
[m] Oltvai *et al.* (81).

Effect of Growth Factor Treatment on Cell Cycle Regulatory Gene Expression. There was a significant interaction between treatment and day on the expression of many of the cell cycle regulatory genes that were measured with cDNA miniarrays. Overall, TGF-β appeared to dominate over TGF-α in transcriptional regulation of cell cycle regulatory proteins (Table 3), as gene expression patterns of combined treatments were more similar to TGF-β–treated cultures than they were to TGF-α.

RTq-PCR was used to validate the expression pattern of a subset of these genes, Cdc25b, Trp63 and Mre11a, that had not yet been reported in the literature to be regulated by the TGFs. Cdc25b is a member of the Cdc25 family of phosphatases, which activate cyclin-dependent kinases to enable progression through the cell cycle; specifically, Cdc25b activates the G_2- and S-phase cyclin-cdk complexes, cyclin A-cdk2 and cyclin B, respectively (56, 57). The expression pattern of Ccd25b in TGF-α cultures is consistent with its role in cell cycle progression. Growth curves demonstrated a greater rate of growth in TGF-α–treated cultures concurrent with the greater level of Cdc25b expression relative to control cells. As growth rate of TGF-α cultures slowed and then entered into a lag phase, Cdc25b expression levels dropped to control levels and below. In TGF-β and the combined treated cultures, the expression of Cdc25b was not different from control cultures on Days 2 and 4 of the experimental period, but it was suppressed relative to controls on Day 6. This pattern of expression is

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014



consistent with the role of Cdc25b and the growth pattern of the TGF-β–treated cultures, in that between Days 4 and 6 the cells enter a lag phase of growth. However, the expression pattern of Cdc25b in the combined treatment is somewhat contrary to the cell cycle analysis data. On Day 4, a significantly greater proportion of cells were in S phase than all other treatments; however, the expression of Cdc25b was similar to control levels, suggesting that levels of Cdc25b mRNA may not be indicative of its activity.

Trp63 expression was greater in TGF-α–treated cultures relative to controls on Days 2 and 4, whereas it was suppressed relative to controls in TGF-β–treated cultures on Days 2 and 6, and it was suppressed all three experimental days relative to controls in the combined treatment. Trp63 appears to be essential for maintenance of a stem cell population, as $p63^{-/-}$ mice have no hair follicles, no teeth, and no mammary, lacrimal, or salivary glands (58, 59). Further, trp63 stains the basal undifferentiated cells of the mammary gland and other organs, including skin and prostate, and has been suggested to be a marker of undifferentiated basal mammary "stem" cells (58, 59). Interestingly, overexpression of TGF-α in transgenic mice specifically changes proliferative rates in cells characterized as mammary stem cells (60). Together, these data suggest that TGF-α not only stimulates proliferation in stem cells but also stimulates genes that act to maintain the stem cell population. Further, it is likely that the tumor-suppressive role of TGF-β may in part override TGF-α–stimulated gene expression, as TGF-β treatment suppressed Trp63 expression in the combined treated cultures, which was antagonistic to the action of TGF-α.

The Mre11 protein is a subunit of a multisubunit nuclease that is composed of Mre11, Rad50, and Nbs1/Xrs2 (MRN; Ref. 61). MRN functions to repair DNA double-stranded breaks by homologous recombination at the G_1 checkpoint and at the G_1/S checkpoint (62). Mre11a expression in TGF-α–treated cultures was not different from controls during the first 4 days of the experimental period, but it was suppressed on Day 6 relative to control cultures. The suppression of Mre11a expression relative to controls indicates that fewer cells are being repaired to reenter the cell cycle in TGF-α–treated cultures, which reflects the fact that the cells are at a higher density than controls and are clearly in a lag phase. The suppression of Mre11a in TGF-β and the combined treated cultures on Days 4 and 6 is indicative of the greater percentage of cells dying in these cultures and suggests that Mre11a expression

←
Figure 7. RT-qPCR was used to validate the patterns in the change of expression of three cell cycle–related genes: (A) trp63; (B) cdc25b; and (C) mre11a over the 6 days in culture in cells cultured with 10 ng/ml TGF-α (striped bars), 2.5 ng/ml TGF-β (cross-hatched bars), or 10 ng/ml TGF-α and 2.5 ng/ml TGF-β (open bars). Changes in expression of genes were expressed relative to the controls for each day.

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

is turned off or suppressed in dying cells, and fewer cells are being repaired to reenter the cell cycle.

Conclusion. There is a critical balance between apoptosis, or programmed cell death, and proliferation during tissue morphogenesis and maintenance. An imbalance between cell proliferation and apoptosis may contribute to tumorigenesis and tumor progression. The rate of tumor growth depends in part on an excess of proliferation over apoptosis (63), and many anticancer agents produce their therapeutic effect by decreasing proliferation and increasing apoptosis (64–66). Interestingly, tumors exhibit both abnormally high rates of cell proliferation and apoptotic cell death. In breast cancer, high apoptotic counts are positively associated with proliferation index as well as high histologic grade, a high risk of lymph node metastasis, and shortened disease-free state (20, 63, 67–70). Late-stage aggressive breast tumors are also characterized by lack of differentiation and epithelial plasticity, EMT. Therefore, in several ways, the treatment of normal epithelial cell cultures concurrently with TGF-α and TGF-β1 mimicked tumor growth, as these factors induced enhanced rates of cell proliferation and cell death as well as cellular transformation. Thus, this culture system may serve as an excellent model to study what occurs during the progression of breast cancer, and may be used to help define targets for anticancer therapy.

1. Todaro GJ, De Larco JE. Growth factors produced by sarcoma virus-transformed cells. Cancer Res 38:4147–4154, 1978.
2. Anzano MA, Roberts AB, Smith JM, Sporn MB, De Larco JE. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. Proc Natl Acad Sci U S A 80:6264–6268, 1983.
3. Roberts AB, Frolik CA, Anzano MA, Sporn MB. Transforming growth factors from neoplastic and nonneoplastic tissues. Fed Proc 42:2621–2626, 1983.
4. Amundadottir LT, Nass SJ, Berchem GJ, Johnson MD, Dickson RB. Cooperation of TGF alpha and c-Myc in mouse mammary tumorigenesis: coordinated stimulation of growth and suppression of apoptosis. Oncogene 13:757–765, 1996.
5. Daroqui MC, Puricelli LI, Urtreger AJ, Elizalde PV, Lanuza GM, Bal de Kier Joffe E. Involvement of TGF-beta(s)/T(beta)Rs system in tumor progression of murine mammary adenocarcinomas. Breast Cancer Res Treat 80:287–301, 2003.
6. de Jong JS, van Diest PJ, van der Valk P, Baak JP. Expression of growth factors, growth factor receptors and apoptosis related proteins in invasive breast cancer: relation to apoptotic rate. Breast Cancer Res Treat 66:201–208, 2001.
7. Gorska AE, Jensen RA, Shyr Y, Aakre ME, Bhowmick NA, Moses HL. Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-beta receptor exhibit impaired mammary development and enhanced mammary tumor formation. Am J Pathol 163:1539–1549, 2003.
8. Muraoka RS, Koh Y, Roebuck LR, Sanders ME, Brantley-Sieders D, Gorska AE, Moses HL, Arteaga CL. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. Mol Cell Biol 23:8691–8703, 2003.
9. Pilichowska M, Kimura N, Fujiwara H, Nagura H. Immunohistochemical study of TGF-alpha, TGF-beta1, EGFR, and IGF-1 expression in human breast carcinoma. Mod Pathol 10:969–975, 1997.
10. Rose-Hellekant TA, Sandgren EP. Transforming growth factor alpha- and c-myc-induced mammary carcinogenesis in transgenic mice. Oncogene 19:1092–1096, 2000.
11. Wakefield LM, Piek E, Bottinger EP. TGF-beta signaling in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia 6:67–82, 2001.
12. Smith GH, Sharp R, Kordon EC, Jhappan C, Merlino G. Transforming growth factor-alpha promotes mammary tumorigenesis through selective survival and growth of secretory epithelial cells. Am J Pathol 147: 1081–1096, 1995.
13. Smith JA, Barraclough R, Fernig DG, Rudland PS. Identification of alpha transforming growth factor as a possible local trophic agent for the mammary gland. J Cell Physiol 141:362–370, 1989.
14. Zajchowski DA, Sager R. Induction of estrogen-regulated genes differs in immortal and tumorigenic human mammary epithelial cells expressing a recombinant estrogen receptor. Mol Endocrinol 5:1613–1623, 1991.
15. McGeady ML, Kerby S, Shankar V, Ciardiello F, Salomon D, Seidman M. Infection with a TGF-alpha retroviral vector transforms normal mouse mammary epithelial cells but not normal rat fibroblasts. Oncogene 4:1375–1382, 1989.
16. Shankar V, Ciardiello F, Kim N, Derynck R, Liscia DS, Merlo G, Langton BC, Sheer D, Callahan R, Bassin RH. Transformation of an established mouse mammary epithelial cell line following transfection with a human transforming growth factor alpha cDNA. Mol Carcinog 2:1–11, 1989.
17. Matsui Y, Halter SA, Holt JT, Hogan BL, Coffey RJ. Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell 61:1147–1155, 1990.
18. Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH, Merlino GT. TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61:1137–1146, 1990.
19. Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL, Lee DC. Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell 61:1121–1135, 1990.
20. de Jong JS, van Diest PJ, Baak JP. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. Histopathology 36:306–312, 2000.
21. Umekita Y, Enokizono N, Sagara Y, Kuriwaki K, Takasaki T, Yoshida A, Yoshida H. Immunohistochemical studies on oncogene products (EGF-R, c-erbB-2) and growth factors (EGF, TGF-alpha) in human breast cancer: their relationship to oestrogen receptor status, histological grade, mitotic index and nodal status. Virchows Arch A Pathol Anat Histopathol 420:345–351, 1992.
22. Albanell J, Baselga J. Trastuzumab, a humanized anti-HER2 monoclonal antibody, for the treatment of breast cancer. Drugs Today (Barc) 35:931–946, 1999.
23. Albanell J, Baselga J. The ErbB receptors as targets for breast cancer therapy. J Mammary Gland Biol Neoplasia 4:337–351, 1999.
24. Yeon CH, Pegram MD. Anti-erbB-2 antibody trastuzumab in the treatment of HER2-amplified breast cancer. Invest New Drugs 23:391–409, 2005.
25. Massague J, Cheifetz S, Ignotz RA, Boyd FT. Multiple type-beta transforming growth factors and their receptors. J Cell Physiol Suppl(Suppl 5):43–47, 1987.
26. Pierce DF Jr., Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, Daniel CW, Hogan BL, Moses HL. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. Genes Dev 7:2308–2317, 1993.
27. Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen L, Roberts AB, Smith GH, Merlino G. Targeting expression of a

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J 12:1835–1845, 1993.
28. Pierce DF Jr., Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ Jr., Moses HL. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci U S A 92:4254–4258, 1995.
29. Forrester E, Chytil A, Bierie B, Aakre M, Gorska AE, Sharif-Afshar AR, Muller WJ, Moses HL. Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. Cancer Res 65:2296–2302, 2005.
30. Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol 127:2021–2036, 1994.
31. Shook D, Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev 120:1351–1383, 2003.
32. Gilles C, Polette M, Piette J, Delvigne AC, Thompson EW, Foidart JM, Birembaut P. Vimentin expression in cervical carcinomas: association with invasive and migratory potential. J Pathol 180:175–180, 1996.
33. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2:442–454, 2002.
34. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. Clin Cancer Res 11:937s–943s, 2005.
35. Chen CR, Kang Y, Massague J. Defective repression of c-myc in breast cancer cells: a loss at the core of the transforming growth factor beta growth arrest program. Proc Natl Acad Sci U S A 98:992–999, 2001.
36. Xie L, Law BK, Aakre ME, Edgerton M, Shyr Y, Bhowmick NA, Moses HL. Transforming growth factor beta-regulated gene expression in a mouse mammary gland epithelial cell line. Breast Cancer Res 5: R187–R198, 2003.
37. Hynes NE, Jaggi R, Kozma SC, Ball R, Muellener D, Wetherall NT, Davis BW, Groner B. New acceptor cell for transfected genomic DNA: oncogene transfer into a mouse mammary epithelial cell line. Mol Cell Biol 5:268–272, 1985.
38. Yosef H, Tamhane AC. Multiple Comparison Procedures. New York: John Wiley & Sons, 1987.
39. Van't Hof J. Cell Cycle Analysis. New York: Academic Press, 1973.
40. Freshney R. Culture of animal cells: A manual of basic technique. New York: Wiley-Liss, 1994.
41. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 27:1–20, 1997.
42. Sgonc R, Boeck G, Dietrich H, Gruber J, Recheis H, Wick G. Simultaneous determination of cell surface antigens and apoptosis. Trends Genet 10:41–42, 1994.
43. Plaut K, Maple RL. Characterization of binding of transforming growth factor-beta 1 to bovine mammary membranes. J Dairy Sci 78:1463–1469, 1995.
44. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25: 169–193, 2000.
45. Okada M, Ohmura E, Kamiya Y, Murakami H, Onoda N, Iwashita M, Wakai K, Tsushima T, Shizume K. Transforming growth factor (TGF)-alpha in human milk. Life Sci 48:1151–1156, 1991.
46. Xu R, Doan QC, Regester GO. Detection and characterisation of transforming growth factor-beta in porcine colostrum. Biol Neonate 75: 59–64, 1999.
47. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 110:673–687, 2002.
48. Giancotti FG, Ruoslahti E. Integrin Signaling. Science 285:1028–1033, 1999.
49. Sokol JP, Neil JR, Schiemann BJ, Schiemann WP. The use of cystatin C to inhibit epithelial-mesenchymal transition and morphological transformation stimulated by transforming growth factor-beta. Breast Cancer Res 7:R844–R853, 2005.
50. Sokol JP, Schiemann WP. Cystatin C antagonizes transforming growth factor beta signaling in normal and cancer cells. Mol Cancer Res 2: 183–195, 2004.
51. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol 8:1243–1252, 1998.
52. Carcamo J, Zentella A, Massague J. Disruption of transforming growth factor beta signaling by a mutation that prevents transphosphorylation within the receptor complex. Mol Cell Biol 15:1573–1581, 1995.
53. Rodriguez C, Chen F, Weinberg RA, Lodish HF. Cooperative binding of transforming growth factor (TGF)-beta 2 to the types I and II TGF-beta receptors. J Biol Chem 270:15919–15922, 1995.
54. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: tumor promoting effects of transforming growth factor-beta. Breast Cancer Res 2:125–132, 2000.
55. Grunert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol 4:657–665, 2003.
56. Chen MS, Hurov J, White LS, Woodford-Thomas T, Piwnica-Worms H. Absence of apparent phenotype in mice lacking Cdc25C protein phosphatase. Mol Cell Biol 21:3853–3861, 2001.
57. Chua SS, Ma Z, Ngan E, Tsai SY. Cdc25B as a steroid receptor coactivator. Vitam Horm 68:231–256, 2004.
58. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398:708–713, 1999.
59. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C, McKeon F. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398:714–718, 1999.
60. Chepko G, Slack R, Carbott D, Khan S, Steadman L, Dickson RB. Differential alteration of stem and other cell populations in ducts and lobules of TGFalpha and c-Myc transgenic mouse mammary epithelium. Tissue Cell 37:393–412, 2005.
61. Assenmacher N, Hopfner KP. MRE11/RAD50/NBS1: complex activities. Chromosoma 113:157–166, 2004.
62. Lavin MF. The Mre11 complex and ATM: a two-way functional interaction in recognising and signaling DNA double strand breaks. DNA Repair (Amst) 3:1515–1520, 2004.
63. Liu S, Edgerton SM, Moore DH 2nd, Thor AD. Measures of cell turnover (proliferation and apoptosis) and their association with survival in breast cancer. Clin Cancer Res 7:1716–1723, 2001.
64. Archer CD, Parton M, Smith IE, Ellis PA, Salter J, Ashley S, Gui G, Sacks N, Ebbs SR, Allum W, Nasiri N, Dowsett M. Early changes in apoptosis and proliferation following primary chemotherapy for breast cancer. Br J Cancer 89:1035–1041, 2003.
65. Campiglio M, Locatelli A, Olgiati C, Normanno N, Somenzi G, Vigano L, Fumagalli M, Menard S, Gianni L. Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level. J Cell Physiol 198:259–268, 2004.
66. Chang J, Ormerod M, Powles TJ, Allred DC, Ashley SE, Dowsett M. Apoptosis and proliferation as predictors of chemotherapy response in patients with breast carcinoma. Cancer 89:2145–2152, 2000.
67. de Jong JS, van Diest PJ, Baak JP. Number of apoptotic cells as a prognostic marker in invasive breast cancer. Br J Cancer 82:368–373, 2000.
68. Mori I, Yang Q, Kakudo K. Predictive and prognostic markers for invasive breast cancer. Pathol Int 52:186–194, 2002.
69. Srinivas P, Abraham E, Ahamed I, Madhavan M, Vijayalakshmi NR,

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014

Nair MK, Balaram P. Apoptotic index: use in predicting recurrence in breast cancer patients. J Exp Clin Cancer Res 21:233–238, 2002.
70. Vakkala M, Lahteenmaki K, Raunio H, Paakko P, Soini Y. Apoptosis during breast carcinoma progression. Clin Cancer Res 5:319–324, 1999.
71. Zhu X, Di Y, Hu C, Wang Z. Expression of positive and negative regulators of cell cycle during wound healing. Chin Med J (Engl) 115: 326–330, 2002.
72. Barnes DM, Gillett CE. Cyclin D1 in breast cancer. Breast Cancer Res Treat 52:1–15, 1998.
73. Vairo G, Livingston DM, Ginsberg D. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev 9:869–881, 1995.
74. Forrest A, Gabrielli B. Cdc25B activity is regulated by 14-3-3. Oncogene 20:4393–4401, 2001.
75. Gordon DM, Roof DM. Degradation of the kinesin Kip1p at anaphase onset is mediated by the anaphase-promoting complex and Cdc20p. Proc Natl Acad Sci U S A 98:12515–12520, 2001.
76. Choi KS, Eom YW, Kang Y, Ha MJ, Rhee H, Yoon JW, Kim SJ. Cdc2 and Cdk2 kinase activated by transforming growth factor-beta1 trigger apoptosis through the phosphorylation of retinoblastoma protein in FaO hepatoma cells. J Biol Chem 274:31775–31783, 1999.
77. D'Amours D, Jackson SP. The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. Nat Rev Mol Cell Biol 3:317–327, 2002.
78. Conaway RC, Brower CS, Conaway JW. Emerging roles of ubiquitin in transcription regulation. Science 296:1254–1258, 2002.
79. Dohn M, Zhang S, Chen X. p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. Oncogene 20:3193–3205, 2001.
80. Kadakia M, Slader C, Berberich SJ. Regulation of p63 function by Mdm2 and MdmX. DNA Cell Biol 20:321–330, 2001.
81. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619, 1993.

Downloaded from ebm.sagepub.com at SAGE Publications on October 30, 2014