Effect of Transforming Growth Factor-beta (TGF- β) on Mammary Development

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ABSTRACT

Mammary growth and development in heifers and cows is regulated by a complex interaction of multiple stimuli, including environmental, nutritional, and endocrine factors. The action of these various stimuli is mediated by a host of growth factors, including transforming growth factor-beta (TGF- β). TGF- β is a potent inhibitor of mammary epithelial cell growth and regulates extracellular matrix composition. TGF- β is expressed during bovine mammary development and its expression is particularly high at times of mammary growth and reorganization. The purpose of this paper is to review what we know about TGF- β and mammary growth and to explain how TGF- β affects the cell cycle in a normal epithelial cell line.

(**Key words:** mammary development, mammary growth, TGF-beta)

Abbreviation key: ECM = extracellular matrix, LAP = latency associated peptide, TGF- β = transforming growth factor-beta.

INTRODUCTION

Mammary growth and development in heifers and cows is regulated by a complex interaction of multiple stimuli, including environmental, nutritional, and endocrine factors. Much of the recent work on optimizing mammary development for dairy production has focused on the effect of photoperiod on mammary development and milk production (Dahl et al., 2000), the relationship of energy and protein in mammary development (Sjerssen, 1994; VanAmburgh et al., 1998), and the role of endocrine factors such as bST in stimulating mammary development and milk production (Sjerssen et al., 1999). It is clear that the action of these various stimuli is mediated by a host of growth factors. Transforming growth factor-beta (TGF- β) is a growth factor that is expressed during bovine mammary development and may prove to be especially important during a criti-

cal period of prepubertal mammary development in heifers.

TGF- β belongs to a superfamily of proteins that in general inhibit epithelial cell growth, stimulate mesenchymal cell proliferation, regulate extracellular matrix (ECM) deposition and degradation, control mesenchyme-epithelial interactions, and stimulate apoptosis. This family of proteins includes 3 mammalian isoforms known as TGF- β 1,2,3 and a number of growth factors that have been implicated in reproductive development in the bovine including activins and inhibins. TGF- β 1 is a 25 kDa polypeptide dimer that is found in a latent form and is secreted with a pre-pro region known as the latency associated peptide (LAP). Since LAP contains the signal sequence that causes secretion, the peptide must be secreted in association with LAP and then activated in the tissue by proteases (Massague and Chen, 2000).

TGF- β Expression During Mammary Development

TGF- β is expressed throughout normal mammary development, regulating epithelial-mesenchyme interactions during ductal branching morphogenesis, pregnancy, and involution. In general the expression of TGF- β 1 is high during mammary tissue morphogenesis, growth, and remodeling. Robinson et al. (1992) used Northern analysis and in situ hybridization to investigate the spatial and temporal distribution of TGF- β transcripts during mouse mammary development. In virgin mice, TGF- β 1 and TGF- β 3 gene expression was fairly abundant in end buds and mammary duct epithelium as well as with contiguous stromal cells. During pregnancy TGF- β 1 expression decreased and only trace amounts were found in lactating glands, but TGF- β 3 was heavily expressed in ducts and alveoli. Pregnancy was the only stage that TGF- β 2 transcripts were present (Robinson et al., 1992). Levels of TGF- β 1 mRNA increased dramatically during d 2 to 4 of mouse mammary involution and then steadily decreased (Strange et al., 1992).

Plath et al., (1997) examined the temporal expression of TGF- β 1 mRNA during mammatogenesis, lactogenesis, galactopoiesis and involution in the bovine mammary tissue using ribonuclease protection assays and reverse

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transcription-polymerase chain reaction (RT-PCR). They also studied the influence of estrogen, progesterone and prolactin on TGF- β expression in heifers after induced mammary development and lactogenesis. Very low levels of TGF- β 1 expression were detected during lactogenesis and galactopoiesis, increased levels were observed during mammary development of primigravid heifers, and the highest levels were detected during mammary development of virgin heifers and during involution. TGF- β 1 expression in the mammary gland was almost equal after induced mammary development and lactogenesis, and greater than during the physiological mammary development and lactogenesis. They concluded that the higher mRNA contents of TGF- β 1 during mammary development and involution may indicate that TGF- β acts as an autocrine or paracrine regulator during proliferation and reorganization of the bovine mammary gland (Plath et al., 1997).

Role of TGF- β in Regulating Mammary Gland Development and Extracellular Matrix Composition

TGF- β 1 is a potent inhibitor of mammary epithelial cell growth. Implantation of a pellet containing active TGF- β 1 into the mammary gland of prepubertal mice inhibited end bud formation and DNA synthesis in mammary epithelium (Silberstein and Daniel, 1987; Daniel et al., 1989). Both TGF- β 2 and TGF- β 3 mimic the effect of TGF- β 1 on the mammary gland (Robinson et al., 1991; 1992). Unlike the effect observed during early mammary development, local implants of activated TGF- β 1 did not alter mouse mammary development during pregnancy or after estrogen and progesterone treatment (Silberstein and Daniel, 1987; Daniel et al., 1989). However, TGF- β 1 treatment decreased casein secretion in mammary explants prepared from pregnant animals (Mieth et al., 1990; Robinson et al., 1992), but this inhibition did not occur when mammary acini or explants were prepared from lactating tissue (Sudlow et al., 1994). Jhappan et al. (1993) targeted a TGF- β 1 transgene to the mammary gland of pregnant mice by coupling it to the WAP promoter. Alveolar development and subsequent lactation were inhibited by expression of the TGF- β 1 transgene. In contrast, when the TGF- β 1 transgene was coupled to the MMTV promoter the mice not only developed normal mammary glands, but they also lactated normally (Pierce, 1995). These differences in mammary development and differentiation may reflect the timing of TGF- β 1 transgene expression as specified by the respective promoters.

TGF- β 1 also affects the composition of the extracellular matrix (ECM). Silberstein et al. (1990; 1992) showed using immunohistochemistry that TGF- β 1 is strongly expressed in the stromal tissue during mouse mammary ductal morphogenesis. Local implants of activated

TGF- β 1 stimulated the expression and deposition of ECM proteins and inhibited expression of ECM proteases (Silberstein et al., 1990). Inhibition of end bud growth by exogenous TGF- β 1 implants was associated with ectopic accumulation of collagen I mRNA and protein as well as accumulation of chondroitin sulfate (Daniel et al., 1989; Silberstein et al., 1990). TGF- β 1 treatment of human mammary epithelial cells in culture strongly induced mRNA and protein expression of ECM associated proteins and proteases including fibronectin, collagen IV, laminin, type IV collagenase, urokinase type plasminogen activator (uPA), and plasminogen activator inhibitor 1 (PAI-1) (Stampfer et al., 1993).

Expression and Function of TGF- β Receptors in Mammary Tissue

TGF- β like all growth factors elicits its response through its receptors. Type I and II receptors (TGF- β R1 and TGF- β R2) and betaglycan (also known as the type III receptor) bind the growth factor with high affinity and specificity. The type I and II receptors are proteins with carbohydrates attached and vary from 53 to 60 and from 70 to 100 kDa, respectively. Betaglycan is 200 to 300 kDa and is thought to be a nonsignaling receptor that helps TGF- β associate with TGF- β R2 (Massague, 1987). We have demonstrated using Western blot analyses that antibodies to human TGF- β receptors can be used to examine the expression of bovine receptors. Bovine TGF- β R1 and TGF- β R2 are slightly smaller in size than those observed in human and mouse cell lines (Plaut et al., 2001). These size differences may reflect differences in the amino acid composition of the receptor or differences in glycosylation patterns among species (TGF- β R1 is 42 kD and TGF- β R2 is 50 kD and 118 kD).

The type II receptor is the primary receptor associated with the binding of TGF- β 1 (Carcamo et al., 1995; Rodriguez et al., 1995). Mutations of TGF- β R2 are linked to loss of growth inhibition in many mouse and human mammary cell lines (Sun et al., 1995; Kalkhoven et al., 1996; Vivien et al., 1995). Gorska et al. (1998) blocked the activity of TGF- β R2 by developing a transgenic mouse with a kinase defective TGF- β R2 transgene coupled to the MMTV promoter. Precocious alveolar development and premature milk synthesis were observed. Joseph et al. (1999) used the same construct coupled to the metallothionein promoter to examine the effect of blocking TGF- β R2 expression in early mammary development. Lateral ductal branching increased, which disrupted normal spacing and caused the development of a disorganized ductal network. Interestingly, there was no effect on the morphology of the

Table 1. Percent specific binding of TGF- β 1 to dry versus lactating cow mammary tissue at different days prepartum.

Treatment	Days prepartum	Percent bound	Standard error
Dry	53	8.1	1.9
Lactating	53	8.7	2.2
Dry	35	9.4	1.0
Lactating	35	7.3	0.6
Dry	20	10.9	0.1
Lactating	20	13.2	1.6
Dry	7	9.4	0.2
Lactating	7	7.9	1.4

mammary gland when the dominant negative receptor was induced during pregnancy or involution. These studies suggest that TGF- β and its receptors play an important role in mammary development and supports the concept that TGF- β and the type II receptor are important in early mammary development.

We demonstrated that the number of TGF- β 1 binding sites vary with stage of mammary development in both bovine and mouse mammary tissue. In bovine tissue the number of TGF- β 1 binding sites were significantly higher in virgin glands than lactating glands (Plaut and Maple, 1995). Mammary membranes prepared from 3-, 5-, and 8-wk-old, pregnant, lactating, involuting and mature mice also exhibited differential expression of TGF- β 1 receptors (Munaim and Plaut, 1996). The specific binding for receptors was low during wk 3, increased at 5 wk, was maximum at 8 wk and decreased in pregnant, lactating and involuted glands. All three receptor types were present, with type III receptor being most abundant (Munaim and Plaut, 1996). These data indicate that receptors play a role in modulating the activity of TGF- β 1 in the mammary gland and support the conclusion that receptors vary with physiological state and hormonal status.

In another study we examined receptor binding during lactation and involution. Tissue samples were kindly provided by A. Capuco (Beltsville Agricultural Research Center, Beltsville, MD) and obtained from pregnant dairy cows that were 53, 35, 20, or 7 d prepartum. The cows either continued to lactate or were 7, 25, 40, or 53 d dry. A total of 27 cows were used representing 3 to 4 samples per group. Receptor binding was measured as described by Plaut and Maple (1995). There was no significant difference in receptor binding between the dry and lactating cows ($P > 0.05$; Table 1). However, there was a significant day effect with receptor binding being highest at day 20 prepartum ($P < 0.05$; Table 2). This may indicate that prior to calving lactating and nonlactating glands undergo similar physiological changes. It seems TGF- β 1 is playing a regulatory role in growth at 20 d prepartum. This coin-

Table 2. Percent specific binding of TGF- β 1 to cow mammary tissue at different days at different days prepartum.

Days prepartum	Percent bound	Standard error
53	8.3	1.0
35	8.4	0.9
20	12.0 ^a	1.1
7	8.6	0.8

^a $P < 0.05$.

cides with the time that the mammary gland is undergoing extensive remodeling for the next lactation. Data from A. Capuco shows that hydroxyproline levels, an amino acid primarily found in the ECM (Woessner et al., 1961), are highest at 20 d prepartum as compared to 53, 35, and 7 d prepartum (Capuco et al., 1997). Statistical analysis shows a strong correlation between hydroxyproline levels and receptor numbers ($r = 0.70$; McMullen and Plaut, 1995). This supports the hypothesis that TGF- β may play a role in regulating functional differentiation by affecting extracellular matrix components (Daniel et al., 1989). TGF- β may also play a role in regulating the onset of milk secretion by inhibiting casein synthesis (Daniel et al., 1989). TGF- β mRNA expression decreases once lactation begins and secretion of milk components occurs (Robinson et al., 1992). This coincides with our data that shows TGF- β receptor number decreases prior to lactogenesis (Table 2).

We used immunohistochemistry to determine where TGF- β type I and II receptors were localized in bovine mammary tissue. In this study formalin fixed, paraffin embedded tissues from heifers that were treated with rbST and fed for two different rates of gain and cows at many different stages of development and lactation were used. Antigens were unmasked from 5 μ tissue sections by heat treatment in 10 mM sodium citrate buffer, pH 6.0 (Tenaud et al., 1994). Slides were incubated with rabbit primary antibody to the TGF- β R1 or TGF- β R2 (4 μ g/ml; SC-398 or SC-400, respectively, Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The histostain kit (Histostain-SP kit, Zymed Laboratories, Inc) was used to develop the reaction. Amino-ethyl-carbozole (Histostain-SP kit), which stains red, was the chromogen. Tissues were counterstained with hematoxylin and sections were observed by light microscopy and photographed. The type I and II receptors were abundantly expressed and were localized along the ductal epithelium and around alveoli (Figure 1). Interestingly, there seemed to be a high expression of the receptor around milk fat globules (data not shown). While we expected to see abundant receptor in the fibroblasts, receptor levels were much lower (Figure 1). This is consistent with the literature on human breast tissue,

Figure 1. Localization of TGF- β R1 in 5 μ sections of bovine mammary tissue. Formalin fixed, paraffin embedded mammary tissue from heifers treated with rbST was used for immunohistochemistry. Slides were incubated with A) rabbit primary antibody to the TGF- β R1 (4 μ g/ml; SC- 398 Santa Cruz Biotechnology, Inc, Santa Cruz, CA) or B) 1% BSA-PBS alone to serve as control. The histostain kit (Histostain-SP kit, Zymed Laboratories, Inc) was used to develop the reaction. Amino-ethyl-carbozole (Histostain-SP kit), which stains red, was the chromogen. Tissues were counterstained with hematoxylin, and photographed at 200X.

which frequently does not report staining in the stromal compartment (Gobbi et al., 1999). There were no apparent treatment differences in animals fed for different rates of gain or treated with bST. However, this method would not be sensitive enough to detect subtle changes in receptor expression.

TGF- β Signal Transduction Pathway

In order to activate the TGF- β signaling system, TGF- β 1 dissociates from the type III betaglycan receptors and is activated by cleavage of LAP. TGF- β 1 can then bind to and activate the TGF- β R2 which in turn complexes with and activates TGF- β R1. Both receptors are serine-threonine kinases, which alter the phosphorylation state of the receptors themselves and the signal transduction factors known as the Smads (see review by Massague et al., 2000). Phosphorylation of TGF- β R1 activates a kinase that phosphorylates the receptor activated Smads, either Smad-2 or -3, which are anchored to the cell membrane by Smad anchor for receptor activation (SARA). Phosphorylation of the receptors releases Smad-2/3 from SARA. Release from SARA exposes the nuclear import signal and allows Smad 2/3 to bind to the co-Smad, Smad-4. This complex is translocated to the nucleus where it acts as a transcription factor. In the nucleus it can associate with other co-

Figure 2. Proposed mechanism of Smad activation of the TGF- β response pathway. In order to elicit a response, TGF- β 1 dissociates from the type III receptors (labeled III) and is activated by cleavage of the latency activate peptide (LAP). TGF- β 1 can then bind to the Type II TGF- β receptor (labeled II) which in turn binds to the type I receptor (labeled I) and phosphorylates the receptor. Phosphorylation of I invokes a kinase that phosphorylates the receptor activated Smads (either Smad 2 or 3) which are anchored in the membrane. They are anchored in the membrane by Smad anchor for receptor activation (SARA). Phosphorylation of the Smads releases it from SARA and exposes the nuclear import signal. This allows the Smad complex to bind to the co-Smad, Smad 4. The complex is translocated to the nucleus where it is an active transcriptional complex. In the nucleus it can associate with other coactivators and repressors as well as DNA binding cofactors to activate gene transcription. Smad7 can bind to the Smad complex and act as a repressor preventing the activation of the type II receptor thus inhibiting activity and acting as a feedback loop on TGF- β signaling. Depending on the combination of transcription factors that are bound to Smad, the activity may vary substantially.

activators and repressors as well as DNA binding cofactors to activate gene transcription (Figure 2).

TGF- β 's ability to phosphorylate its receptors and to activate its signal transduction pathways can be influenced by various signaling molecules and other interacting pathways. Smad7 can bind to the Smad complex and act as a repressor preventing the activation of the type II receptor. Blocking TGF- β R2 activation inhibits signal transduction and serves as a negative feedback loop on TGF- β activity. TGF- β activity is also regulated by the combination of transcription factors which interact with the Smad 2/3-Smad 4 complex (Massague and Wotton, 2000). Other signal transduction pathways that regulate TGF- β activity continue to be identified to account for the pleiotropic nature of the response to TGF- β (Yan et al., 1994; deCaestecker et al., 2000; Massague et al., 2000).

The Effect of TGF- β 1 on Cell Growth, the Cell Cycle and Apoptosis

TGF- β affects epithelial cell growth. Our work has shown that TGF- β inhibits primary mammary cells pre-

Figure 3. Effects of TGF- β 1 on the growth of NOG-8 cells. Cells were seeded at a density of 50,000 cells/ml with DMEM + 10% FBS. On the following day, cells were serum starved for 24 hrs to synchronize cells in the cell cycle. After serum starvation, media was changed to DMEM + 10% FBS and either 0 (◆) or 2.5 ng/ml (■) TGF- β was added. Cells were counted using a hemacytometer on d 2, 4, and 6.

pared from heifers (Purup et al., 1999). Response is dependent upon the dose of TGF- β with some stimulatory effects observed at low concentrations of TGF- β . Growth inhibition has also been observed in a bovine mammary epithelial cell line (Woodward et al., 1995). We used a normal mouse mammary epithelial cell line, NOG-8, to further characterize the effect of TGF- β on cell growth and its interaction with other growth factors in regulating cell growth. NOG-8 cells were plated at a density of 100,000 cells per well in 35 mm dishes with DMEM + 10% FBS + 1 μ g/ml insulin + 50 U/ml penicillin and 50 μ g/ml streptomycin, pH 7.2 to 7.3. On the following day, cells were serum starved for 24 hrs to synchronize cells in the cell cycle. After serum starvation, media was changed to DMEM + 10% FBS + 1 μ g/ml insulin + 50 U/ml penicillin/ 50 μ g/ml streptomycin. Either 0 or 2.5 ng/ml TGF- β 1 (R&D Systems, Minneapolis, MN) were added to each well with 0 or 20 ng/ml acidic fibroblast growth factor (aFGF), 20 ng/ml basic fibroblast growth factor (bFGF), 60 ng/ml epidermal growth factor (EGF), 10 ng/ml IGF-I, 10 ng/ml transforming growth factor alpha (TGF- α), or 100 ng/ml hydrocortisone. Cells were counted each day for 6 d using a hemacytometer. Media was changed on d 2 and 4. After a 6-d experimental period, the number of NOG-8 mammary epithelial cells treated with TGF- β 1 was $55 \pm 3.3\%$ relative to controls (Figure 3). TGF- β 's inhibitory action on NOG-8 cell growth was not negated when aFGF, bFGF, EGF, TGF- α , IGF or hydrocortisone was added to the media over the 6-d period (data not shown).

We used flow cytometry to determine if growth inhibition was due to cells being arrested in G0/G1 or whether they were dying at a greater rate. NOG-8 cells were

seeded at 780,000 cells per flask into 75 cm² flasks (Corning Incorporated, Corning, NY) with DMEM + 10% FBS and incubated overnight. Media was removed and replaced with serum free media for 24 h to synchronize the cells prior to treatment (Campisi et al., 1984). After 24 h cells were treated with DMEM + 10% FBS supplemented with 0 or 2.5 ng/ml TGF- β 1. Media was changed on d 2 and 4. At d 0, 2, 4, and 6 the cells were harvested, washed in phosphate buffered saline and counted on a hemacytometer. Stain solution [3% polyethylene glycol 6000, 50 μ g/ml propidium iodide (Sigma Chemicals, St. Louis, MO), 720 units/ml RNase (Worthington Biochemical Corporation, Lakewood, NJ), 0.1% Triton X in 4mM sodium citrate (pH to 7.8) and PBA (phosphate buffered saline + 0.1% sodium azide and 0.1% BSA)], was added to the cell suspension to equal a final concentration of 2 to 4 million cells/ml and incubated at 37°C for 30 min. 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 (Statistical Analysis System, Cary, NC). The data were expressed as percent of the total analyzed population (Figure 4).

Figure 4 shows that following 24 hours of serum starvation cells were synchronized with 73% of cells arrested in G0/G1 phase of the cell cycle. After 2 d of culture in DMEM + 10% FBS alone cells were cycling with approximately 20% less cells in G0/G1 and 15% more cells in S-phase relative to d 0 (Figure 4). When cells were cultured with DMEM + 10% FBS + TGF- β 1, they also began cycling, however at a slower rate, 68% of TGF- β 1 treated cells were in G0/G1 versus 52% of control cells ($P < 0.05$), and 12% of TGF- β 1 treated cells were in S-phase versus 23% of control cells ($P < 0.05$) indicating that TGF- β 1 does inhibit DNA synthesis. By d 6 the most striking difference in the treatments was the greater proportion of TGF- β 1 treated cells dying (26%) versus control cells (5%; $P < 0.0001$; Figure 4). To determine if cells were dying via apoptosis and the relative rate of apoptosis a TUNEL flow cytometry assay was used. Preliminary data indicated that cells treated with TGF- β 1 were undergoing apoptosis at a greater rate than controls. Thus, TGF- β 1 inhibits growth by preventing the transition to the S phase of the cell cycle and stimulates apoptosis.

TGF- β has been shown to induce apoptosis in other cell culture systems (Yu et al., 2002; Hagimoto et al., 2002). Yamamura et al. (2000) showed that both Smad proteins and the AP-1 complex participate in TGF- β 1 signaling for apoptosis. Overexpression of a dominant-negative Smad 3 mutant or Smad 7, both of which impair Smad-mediated signal transduction, inhibited TGF- β 1-dependent apoptosis (Yamamura et al., 2000).

Figure 4. Effect of TGF- β 1 on progression of NOG-8 cells through the cell cycle. NOG-8 cells were seeded at 780,000 cells per flask into 75 cm² flasks with DMEM + 10% FBS and then serum starved 24 h to synchronize the cells; after 24 h cells were treated with DMEM + 10% FBS supplemented with 0 or 2.5 ng/ml TGF- β 1. Media was changed on d 2 and 4. At d 0, 2, 4, and 6 the cells were harvested, washed in phosphate buffered saline and stained with 50 μ g/ml propidium iodide. Cells were sorted in a flow cytometer, and the proportion of cells in each phase of the cell cycle was evaluated using analysis of variance with treatment and day as main effects. Data are expressed as percent of the total analyzed population.

The JunDFosB form of the AP-1 complex is activated during TGF- β 1 induced apoptosis, and overexpression of a dominant-negative FosB mutant blocks apoptosis but not epithelial cell growth inhibition. Furthermore, JunDFosB binds to the 12-O-tetradecanoyl-13-acetate-responsive gene promoter element and recruits the Smad 3-Smad-4 complex to form a multicomponent complex (Yamamura et al., 2000). These data suggest that Smad proteins and the AP-1 complex synergize to mediate TGF- β 1 induced apoptosis.

Apoptosis in the mammary gland appears to coincide with changes in hormonal milieu and tissue remodeling, which occur during mammary development. Ductal morphogenesis in the virgin mouse mammary gland proceeds partially through apoptosis in the terminal end bud (Humphreys et al., 1996). During the human

menstrual cycle the peak of apoptosis in breast epithelial cells occurs when estrogen and progesterone levels are low just prior to the end of the women's cycle (Ferguson and Anderson, 1981). It has been well documented that mouse mammary involution is due in part to a high rate of apoptotic epithelial cell death (Walker et al., 1989; Strange et al., 1992; Guenette et al., 1993; Quarrie et al., 1995; Casey et al., 1996). Wilde et al. (1997) showed that bovine mammary cells also undergo apoptosis between lactations early in their dry period. Expression of TGF- β 1 in the mammary gland is also greatest at times of changes in hormonal milieu and tissue remodeling (Robinson et al., 1992; Plath et al., 1997) suggesting that TGF- β plays an important role in regulating cell turnover at times of mammary growth and reorganization. Furthermore, our data supports

Figure 5. Proposed mechanism of action of TGF- β on the cell cycle. TGF- β arrests cell in the G1 phase of the cell cycle by affecting many molecules. Of particular importance are the cyclin-dependent kinases (cdks) which, if inactivated, turn off the cell cycle and thus allow cells to be arrested in G1. TGF- β accomplishes this by binding to TGF- β inhibitory element (TIE) which decreases c-myc. This in turn increases the expression of p15Ink4b (p15) and p21Cip1 (p21). When p15 increases, it directly binds to the cdk4/6/cyclin D complex and displaces p27Kip1 (p27). This allows cyclin D to dissociate and inactivates the complex. In turn, p27, which is no longer bound to cdk4/6 binds to cdk2/cyclinE complex along with p21 and inactivates this cyclin-dependent kinase. Thus by increasing the level of one cdk inhibitor, p15, TGF- β can inhibit both cyclin D and E dependent kinases. TGF- β may also repress Cdc25A, which is a cdk acting phosphatase thus further blocking DNA synthesis.

the concept that TGF- β arrests cells in G0/G1 of the cell cycle thus preventing them from undergoing cell division, another important aspect of tissue morphogenesis.

Effect of TGF- β 1 on Cell Cycle Associated Gene Expression

TGF- β 1 affects the cell cycle in many ways. It can directly influence the cyclin dependent kinase inhibitors to prevent the cyclins and the cyclin dependent kinases from promoting G1 to S transition. Specifically, TGF- β decreases cell growth by increasing the expression of p15Ink4b (p15) and p21Cip1 (p21) (Sandhu et al., 1997). When p15 increases, it directly binds to the cdk4/6 complex and displaces p27Kip1 (p27) (Massague et al., 2000). In turn, p27, which is no longer bound to cdk4/6 binds to cdk2 and blocks its action (Figure 5). Thus by increasing the level of one cdk inhibitor, p15, TGF- β can inhibit both classes of G1 cdks. TGF- β may also repress Cdc25A which is a cdk acting phosphatase

Figure 6. Effect of TGF- β 1 on the morphology of NOG-8 cells. Cells were treated with A) 0, or B) 5 ng/ml TGF- β 1. Cells exposed to TGF- β 1 changed their morphology from a cobblestone morphology characteristic of epithelial cells (A) to a more elongated shape much like fibroblasts (B).

thus further blocking DNA synthesis (Massague et al., 2000).

Recently, investigators have identified a TIE (TGF- β inhibitory element) sequence in the promoter region of c-myc. When activated TIE has the ability to repress c-myc expression. Using a human breast cancer cell line, Chen et al. (2000) demonstrated that the Smad complex recognizes the TIE region. Therefore, it is hypothesized that repression of c-myc is mediated by interaction of the TIE elements with the activated Smads. This likely affects the ability of TGF- β to induce p15 and p21 (Chen et al., 2000). Other factors that have been implicated in the TGF- β 's regulation of the cell cycle include retinoblastoma protein (Rb) and Ras. TGF- β is known to prevent Rb phosphorylation, hypophosphorylated Rb in turn remains bound to E2F and thus cells are arrested in G1. While some of this effect may be mediated by increases in p21 levels, it is likely that TGF- β 1 has some direct effect on phosphorylation state. Activation of Ras appears to reverse TGF- β mediated growth inhibition (Oft et al., 1996).

To determine the effect of TGF- β 1 treatment on cell cycle related gene expression NOG-8 cells were seeded, synchronized by serum starvation, and treated with 0 or 2.5 ng/ml TGF- β 1 for 2, 4, or 6 d, as described for cell cycle analysis. Total RNA was isolated using RNeasy mini kit (Qiagen) and used as a template for 32 P-dCTP labeled cDNA probe synthesis (using the GEArray Q series procedure; SuperArray). Probes were hybridized to mouse cell cycle genes using Mouse Cell-cycle GEArray Q series (SuperArray Inc.). Image and data analyses were performed using the Storm Phosphorimager system. Analysis of variance was used to evaluate control genes. The control gene Pp1a was spot-

Table 3. TGF- β 1 induced fold change of cell cycle related gene expression relative to control treated NOG-8 cells.*

Gene name	function	d 2	d 4	d 6
Cdk5r ^a	homologous to Cdc2 expressed in apoptotic cells, upregulated to cause death.	...	...	↑5
Ccnd1 ^b	cyclin D1, down regulated to decrease cell growth	...	...	↓140
E2f4 ^c	transcription repressor, binds with p107 and Smads -3 and -4 to repress c-myc	↓14	...	...
cdc25b ^d	phosphatase, activates cyclin dependent kinases, activates cyclin a and b1	↓8	...	...
cdc20 ^e	activates anaphase promoting complex by degrading kip1p	...	...	↑7
cdc2a ^f	associated with apoptosis and hyperphosphorylation of Rb	↓4.5	...	...
mre11a ^g	endonuclease and exonuclease activity for DNA repair	↑8	...	↓18
Ubc ^h	ubiquitin associated with stress response, binds with mdm2 to promote cell repair	...	...	↑4
trp63 ⁱ	p53 homologue, prevents replication of damaged DNA leading to growth arrest and apoptosis	...	...	↑4.5
mdm2 ^j	binds to p53 and p63 and may rescue cells from apoptosis	...	↑4.5	↑5

*There was a minimum of a 4 fold difference in gene expression.

^aZhu et al., 2002.

^bBarnes and Gillett, 1998.

^cVairo et al., 1995.

^dForrest and Gabriella, 2001.

^eGordon and Roof, 2001.

^fChoi et al., 1999.

^gD'Amours and Jackson, 2002.

^hConaway et al., 2002.

ⁱDohn, et al., 2001.

^jKadakia et al., 2001.

ted in quadruplicate on each assay. The intraassay c.v. for each membrane was 14.2%. Since there was a significant increase in expression in Ppa1 in response to TGF- β 1, it was not compared across day or treatment. GAPDH, which was spotted in duplicate on each array, did not vary by day or treatment. The interassay c.v. was 12.8%, regardless, GAPDH was used to correct for blot variability. Values for each gene were divided by expression in the cells exposed to FBS alone (control) to obtain a difference in expression relative to controls. Our preliminary data indicate that TGF- β 1 affected the expression of many cell cycle related genes, we highlighted 10 genes that had a 4-fold or greater difference in expression from control (Table 3). The effect that TGF- β 1 had on the expression of these genes supports its role as a factor that induces cell cycle arrest and apoptosis.

Epithelial-Mesenchyme Transdifferentiation of NOG-8 Cells in Response to TGF- β

When TGF- β 1 is added to mammary epithelial cells in culture they undergo a transition from an epithelial to a mesenchymal cell type (EMT) (Miettinen et al., 1994). TGF- β induced EMT is reversible upon its removal from culture media. The change in cell morphology correlates with (a) decreased expression of the epithelial markers E-cadherin, ZO-1, and desmoplakin I and II; (b) increased expression of mesenchymal markers, such as fibronectin; and (c) a fibroblast-like reorganization of actin fibers (Miettinen et al., 1994).

To examine the effect of TGF- β 1, - β 2, or - β 3 treatment on cell morphology, mammary epithelial cells were treated with 0 or 5 ng/ml TGF- β . 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 epithelial-like cobblestone morphology to a fibroblast-like morphology (EMT) in greater than 75 % of the cells (Figure 6). We found the effect of TGF- β on EMT was dependent on the confluence of cells. At least 7500 cells/mm² were needed before EMT occurred. Removal of TGF- β from the medium caused the cells to revert back to cobblestone morphology within 2 d (data not shown). Cells underwent EMT in the presence of TGF- β , even when 20 ng/ml aFGF, 20 ng/ml bFGF, 60 ng/ml EGF, 10 ng/ml IGF-I, 10 ng/ml TGF- α , or 100 ng/ml hydrocortisone were also added to the culture media. None of these growth factors prompted transdifferentiation in the absence of TGF- β 1. To determine whether EMT was influenced by the extracellular matrix, NOG-8 cells were plated on various ECM. Cells plated on laminin, fibronectin, collagen IV, or plastic matrices underwent EMT in response to 5 ng/ml TGF- β 1, exhibiting the same morphology as EMT cells grown on plastic (data not shown).

To further define the phenotypic change cells undergo during EMT we examined fibronectin expression using immunohistochemistry. Cells were plated on 8-well chamber slides (Nunc, Naperville, IL) at 200,000 cells per well in the presence of 0 or 5 ng/ml TGF- β 1, and

then fixed in 3% paraformaldehyde. A histostain kit (Zymed, San Francisco, CA) was used for immunodetection of fibronectin using a 1:400 dilution of a rabbit anti-human fibronectin antibody (Sigma, St. Louis, MO). The reaction was coupled to Streptavidin-HRP and detected using the chromogen 3-Amino-9-ethyl carbazole (AEC). EMT cells expressed more fibronectin than did nontransdifferentiated cells (Figure 7a,b). Controls did not stain positively for fibronectin (Figure 7c). These data suggest that TGF- β induced EMT is an actual change of cell phenotype, which is evident in the increased expression of fibronectin, and not due to increased cell density as TGF- β 1 treated cultures have less cells per well than controls.

To determine whether TGF- β altered receptor binding during EMT, we measured TGF- β 1 binding to NOG-8 cells prior to and after exposure to TGF- β (Table 4). Receptor binding decreased following TGF- β induced EMT, however, the difference was not significant due to the large variability. When receptor subtypes were crosslinked and subject to PAGE, receptor population was redistributed in transdifferentiated cells compared to nontransdifferentiated cells (Figure 8). The proportion of cells expressing type II receptors increased from 43% to 64% and type I decreased from 38% to 11% ($P < 0.05$; Table 5; Figure 8). EMT associated redistribution of receptors occurred regardless of the ECM the cells were plated on (laminin, fibronectin, collagen or plastic). We are unclear of the in vivo significance of this change in receptor population since normally TGF- β R2 and - β R1 work in concert, with the binding domain present in TGF- β R2 and the signaling domain present in TGF- β R1. It is possible that the relative change in receptors may reflect changes in signaling pathways that have not yet been identified.

The manner in which TGF- β regulates EMT is still under investigation. Overexpression of a TGF- β R1 dominant negative mutant inhibits TGF- β induced EMT in a mammary epithelial cell line (Miettinen, et al., 1994). There is evidence that TGF- β induced Smad independent pathways regulate changes in ECM production. These same pathways likely mediate EMT in vitro. Neither expression of a dominant negative Smad3 or expression of Smad7 inhibit EMT. Part of the mechanism of EMT may be that TGF- β induces RhoA and its downstream target p160^{ROCK}. This, in turn, induces stress fiber formation, which induces the mesenchymal characteristics observed in response to TGF- β . Bhowmick et al. (2001) demonstrated that RhoA and p160 activation are essential for morphological changes from epithelial to fibroblast phenotype in a normal mouse mammary epithelial cell line. N-cadherin was increased and localized along the plasma membrane in response

Figure 7. Fibronectin expression in NOG-8 cells exposed to 0 or 5 ng/ml TGF- β 1. (A) A histostain kit was used for immunodetection of fibronectin using a 1:400 dilution of a rabbit anti-human fibronectin antibody in (A) NOG-8 cells treated with 5 ng/ml TGF- β 1, or (B) 0 ng/ml TGF- β 1. Isotype controls (C) exhibited no staining.

Table 4. Effect of extracellular matrix on binding of TGF- β 1 to NOG-8 cells.

Matrix cells plated on	Treatment	pm TGF- β 1 bound per cell	Standard error
plastic	control	398	353
	5 ng/ml TGF- β 1	242.5	262
collagen IV	control	420.5	143.5
	5 ng/ml TGF- β 1	307.5	105.5
fibronectin	control	1121.5	181.7
	5 ng/ml TGF- β 1	489.9	51.6
laminin	control	701	605.7
	5 ng/ml TGF- β 1	191.5	98.3

NOG-8 cells were plated and exposed to 0 or 5 ng/ml TGF- β 1. When morphological change was observed, receptor binding assays were performed and pm of TGF- β 1 bound per cell were calculated.

to p160 while RhoA was necessary to activate actin stress fibers and N-cadherin in response to TGF- β .

TGF- β induced EMT may in part regulate the extracellular matrix (ECM) to control cell growth and morphology. Our results showed the same pattern of response to TGF- β 1 regardless of the ECM the cells were plated on. Signal transduction pathways stimulated by extracellular matrix (ECM) components are critical to

cellular function (Ekblom, 1995). The ECM plays an important role in mammary gland development and differentiation (Lee et al., 1985; Bissell et al., 1987; Roskelley et al., 1994), and as mentioned above TGF- β plays a critical role in regulating the composition of the ECM (Daniel et al., 1989; Silberstein et al., 1990; Roberts et al., 1992; Stampfer et al., 1993).

The question still remains, if TGF- β induced EMT plays a role in regulating mammary gland development. It is clearly evident that TGF- β functions to regulate epithelial-mesenchyme interactions during mouse mammary ductal morphogenesis. In other tissues during embryonic organogenesis, TGF- β 1 is shown to regulate epithelial to mesenchyme transdifferentiation (Kaartinen et al., 1997). Keratinocytes that over express TGF- β 1 in transgenic mice take on a spindle shape morphology reminiscent of fibroblasts (Cui et al., 1996). In addition, mammary epithelial cells from transgenic mice that overexpress ras transdifferentiate into invasive fibroblastic type cells when exposed to TGF- β 1 (Oft et al., 1996).

CONCLUSION

TGF- β has potent effects on mammary growth and development. Our evidence and work of others indicate that it plays a role during mammogenesis, when the tissue is undergoing proliferation and during involution when extensive remodeling is occurring. It is likely that

Figure 8. The effect of epithelial to mesenchymal transdifferentiation (EMT) on TGF- β receptor population. NOG-8 cells were exposed to 0 (lanes 1,2) or 5 ng/ml TGF- β 1 (lanes 3,4), and receptor binding assays were performed followed by cross-linking and polyacrylamide gel electrophoresis. Cells were exposed to 1 μ g of TGF- β 1 during receptor binding assay to determine non-specific binding (lanes 2,4).

Table 5. Proportion of TGF- β bound to the Type I, II, and III receptors in NOG-8 cells before and after TGF- β 1 induced EMT.

Before TGF- β treatment

Type I = $37.5 \pm 7.0\%$ ^a

Type II = $43.2 \pm 10.3\%$ ^c

Type III = $19.3 \pm 6.3\%$

After TGF- β treatment

Type I = $10.6 \pm 12.2\%$ ^b

Type II = $64.5 \pm 15.8\%$ ^d

Type III = $24.9 \pm 21.7\%$

Different superscripts within a receptor type are different ($P < .05$).

TGF- β affects rate of cell division, rate of apoptosis, as well as composition of the ECM, which all contribute to the dynamic changes that occur during mammary development. However, additional studies on the *in vivo* role of TGF- β in ruminants must be done to substantiate the results found in mice and cell lines.

REFERENCES

- Barnes, D. M. and C. E. Gillett. 1998. Cyclin D1 in breast cancer. *Breast Cancer Res. Treat.* 52:1–15.
- Bhowmick, N. A., M. Ghiassi, A. Bakin, M. Aakre, C. A. Lindquist, M. E. Engel, C. L. Arteaga, and H. L. Moses. 2001. Transforming growth factor- β 1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol. Biol. Cell* 12:27–36.
- Bissell, M. J., and M. H. Barcellos-Hoff. 1987. The influence of extracellular matrix on gene expression: is structure the message? *J. Cell. Sci. Suppl.* 8:327–43.
- Carcamo, J., A. Zentella, and J. Massague. 1995. Disruption of transforming growth factor β signaling by a mutation that prevents transphosphorylation within the receptor complex. *Mol. Cell. Biol.* 15:1573–1581.
- Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. *J. Dairy Sci.* 80:477–487.
- Casey, T. M., C. Hongmin, K. Plaut, and J. F. Chiu. 1996. Involution of mouse mammary glands during whole organ culture occurs via apoptosis of epithelial tissue. *Cell Biol. Int.* 20:763–767.
- Chen, C. R., Y. Kang, and J. Massague. 2001. Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor β growth arrest program. *Proc. Natl. Acad. Sci.* 98:992–999.
- Choi, K. S., Y. W. Eom, Y. Kang, M. J. Ha, H. Rhee, J. W. Yoon, and S. J. Kim. 1999. Cdc2 and Cdk2 kinase activated by transforming growth factor- β 1 trigger apoptosis through the phosphorylation of retinoblastoma protein in FaO hepatoma cells. *J. Biol. Chem.* 274:31775–83.
- Conaway, R. C., C. S. Brower, and J. W. Conaway. 2002. Emerging roles of ubiquitin in transcription regulation. *Science* 296:1254–1258.
- Cui, W., D. J. Fowlis, S. Bryson, E. Duffie, H. Ireland, A. Balmain, and R. J. Akhurst. 1996. TGF β 1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* 86:531–542.
- Daniel, C. W., G. B. Silberstein, K. Van Horn, P. Strickland, and S. Robinson. 1989. TGF- β 1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. *Dev. Biol.* 135:20–30.
- Dahl, G. E., B. A. Buchanan, and H. A. Tucker. 2000. Photoperiodic effects on dairy cattle: a review. *J. Dairy Sci.* 83:885–93.
- D'Amours, D., and S. P. Jackson. 2002. The Mre11 complex: at the crossroads of dna repair and checkpoint signaling. *Nat. Rev. Mol. Cell. Biol.* 3:317–327.
- DeCaestecker, M. P., E. Piek, and A. B. Roberts. 2000. Role of transforming growth factor β signaling in cancer. *J. Natl. Cancer Inst.* 92:1388–1402.
- Dohn, M., S. Zhang, and X. Cheng. 2001. p63 α and δ p63 α can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. *Oncogene* 20:3193–3205.
- Eklom, P. 1995. Extracellular matrix in animal development. Role of extracellular matrix in animal development—an introduction. *Experientia* 51:851–2.
- Ferguson, D. J., and T. J. Anderson. 1981. Morphological evaluation of cell turnover in relation to the menstrual cycle in the “resting” human breast. *Br. J. Cancer* 44:177–81.
- Forrest, A. and B. Gabrielli. 2001. Cdc25B activity is regulated by 14-3-3. *Oncogene* 20:4393–4401.
- Gobbi, H., W. D. Dupont, J. F. Simpson, W. D. Plummer, P. A. Schuyler, S. J. Olson, C. L. Arteaga, and D. L. Page. 1999. Transforming growth factor- β and breast cancer risk in women with mammary epithelial cell hyperplasia. *J. Natl. Cancer Inst.* 91:2096–2100.
- Gordon, D. M. and D. M. Roof. 2001. Degradation of the kinesin kip1 at anaphase onset is mediated by the anaphase-promoting complex and Cdc20p. *Proc. Natl. Acad. Sci.* 98:12515–12520.
- Gorska, A. E., H. Joseph, R. Derynck, H. Moses, and R. Serra. 1998. Dominant-negative interference of the transforming growth factor β type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. *Cell Growth Diff.* 9:229–238.
- Guennette, R. S., H. B. Corbeil, J. Leger, K. Wong, V. Mez, M. Mooibroek, and M. Tenniswood. 1994. Induction of gene expression during involution of the lactating mammary gland of the rat. *J. Molec. Endocrinol.* 12:47–60.
- Hagimoto, N., K. Kuwano, I. Inoshima, M. Yoshimi, N. Nakamura, M. Fujita, T. Maeyama, and N. Hara. 2002. TGF- β 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. *J. Immunol.* 168:6470–8.
- Humphreys, R. C., M. Krajewska, S. Krnacik, R. Jaeger, H. Weiher, R. Krajewski, J. C. Reed, and J. M. Rosen. 1996. Apoptosis of terminal end bud of murine mammary gland: a mechanism of ductal morphogenesis. *Development* 122:4013–4022.
- Jhappan, C., A. G. Geiser, E. C. Kordon, D. Bagheri, L. Hennighausen, A. B. Roberts, G. H. Smith, and G. Merlino. 1993. Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *EMBO J.* 12:1835–45.
- Joseph, H., A. E. Gorska, P. Sohn, H. L. Moses, and R. Serra. 1999. Overexpression of a kinase-deficient transforming growth factor- β type II receptor in mouse mammary stroma results in increased epithelium branching. *Mol. Biol. Cell* 10:1221–1234.
- Kadakia, M., C. Slader, and S. J. Berberich. 2001. Regulation of p63 function by Mdm2 and MdmX. *DNA Cell. Biol.* 20: 321–330.
- Kalkhoven, E., B. A. Roelen, J. P. de Winter, C. Mummery, J. M. van den Eijnden-van Raalk, and P. T. van der Saag. 1996. Resistance to transforming growth factor β and activin due to reduced receptor expression in human breast tumor cell lines. *Cell Growth Diff.* 6:1151–1161.
- Kaartinen, V., X. M. Cui, N. Heisterkamp, J. Groffen, and C. F. Shuler. 1997. Transforming growth factor- β 3 regulates transdifferentiation of medial edge epithelium during palatal fusion and associated degradation of the basement membrane. *Dev. Dyn.* 209:255–60.
- Lee, E. Y., W. H. Lee C. S., Kaetzel, G. Parry, and M. J. Bissell. 1985. Interaction of mouse mammary epithelial cells with collagen substrata: regulation of casein gene expression and secretion. *Proc. Natl. Acad. Sci.* 82:1419–23.
- Massague, J. 1987. Identification of receptors for type- β transforming growth factor. *Methods in Enzymology* 146:174–195.
- Massague, J., S. W. Blain, and R. S. Lo. 2000. TGF- β signaling in growth control, cancer, and heritable disorders. *Cell* 103:295–309.
- Massague, J. and Y. G. Chen. 2000. Controlling TGF- β signaling. *Genes Dev.* 14:627–644.
- Massague, J. and D. Wotton. 2000. Transcriptional control by the TGF- β /Smad signaling system. *EMBO J.* 19:1745–1754.
- McMullen, J. and K. Plaut. 1995. Regulation of transforming growth factor- β 1 receptors in bovine mammary tissue. Partnerships in Science and technology: A Progress report. Vermont Technology Council and EpSCOR.
- Mieth, M., F. D. Boehmer, R. Ball, B. Groner, and R. Grosse. 1990. Transforming growth factor- β inhibits lactogenic hormone induction of β -casein expression in HC11 mouse mammary epithelial cells. *Growth Factors* 4:9–15.
- Miettinen, P., R. Ebner, A. Lopez, and R. Derynck. 1994. TGF- β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *Cell Biol.* 127:2021–2036.
- Munain, S. and K. Plaut. 1996. Developmental regulation of transforming growth factor-beta1 receptors in mouse mammary tissue. *FASEB* 10:A757.

- Oft, M., J. Peli, C. Rudaz, H. Schwarz, H. Beug H, and E. Reichmann. 1996. A mechanism of repression of TGF β /Smad signaling by oncogenic Ras. *Genes Dev.* 10:2642–2477.
- Pierce, D. F., A. E. Gorska, A. Chytil, K. S. Meise, D. L. Page, R. J. Coffey, and H. L. Moses. 1995. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc. Natl. Acad. Sci.* 92:4254–4258.
- Plath, A., R. Einspanier, F. Peters, F. Sinowatz, D. Schams. 1997. Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation. *J. Endocrinol.* 155:501–511.
- Plaut, K. and R. L. Maple. 1995. Characterization of binding of transforming growth factor-beta-1 to bovine mammary membranes. *J. Dairy Sci.* 78:1463–1469.
- Plaut, K., R. Maple, X. Cui, and S. Purup. 2001. Influence of feeding level and bST on TGF- β (TGF- β) and its receptor in mammary tissue of growing heifers. *J. Dairy Sci.* (abstract).
- Purup, S., M. Vestergaard, M. S. Weber, K. Plaut, R. M. Akers, and K. Sejrsen. 1999. Local regulation of pubertal mammary growth in heifers. Fourth International Workshop on the Biology of Lactation in Farm Animals. *J. Anim. Sci.* 78:36–45.
- Quarrie, L. H., C. V. Addey, and C. Wilde. 1996. Programmed cell death during mammary tissue involution induced by weaning, litter removal, and milk stasis. *J. Cell. Physiol.* 168:559–569.
- Robinson, S. D., G. B. Silberstein, A. B. Roberts, K. C. Flanders, and C. W. Daniel. 1991. Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. *Development*. 113:867–78.
- Robinson, S., G. Silberstein, C. W. Daniel. 1992. Evidence supporting a role for TGF- β isoforms in growth regulation and functional differentiation of the mouse mammary gland. Pages 43–52 in *Mechanisms regulating lactation and infant nutrient utilization*. Wiley-Liss, Inc., New York.
- Rodriguez, C., F. Chen, R. A. Weinberg, and H. F. Lodish. 1995. Cooperative binding of transforming growth factor (TGF)- β 2 to the types I and II TGF- β receptors. *J. Biol. Chem.* 270:15919–15922.
- Roskelley, C. D., P. Y. Desprez, and M. J. Bissell. 1994. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc. Natl. Acad. Sci.* 91:12378–12382.
- Sandu, C., J. Garbe, N. Bhattacharya, J. Daksis, C. H. Pan, P. Yaswen, J. Koh, J. M. Slingerland, and M. R. Stampfer. 1997. Transforming growth factor β stabilizes p15 INK4B Protein, Increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells. *Mol. Cell. Biol.* 17:2458–2467.
- Sejrsen, K. 1994. Relationships between nutrition, puberty and mammary development in cattle. *Proc. Nutr. Soc.* 53:103–11.
- Sejrsen, K., S. Purup, M. Vestergaard, M. S. Weber, and C. H. Knight. 1999. Growth hormone and mammary development. *Domest Anim Endocrinol.* 17:117–29.
- Silberstein, G. B., P. Strickland, S. Coleman, and C. W. Daniel. 1990. Epithelium-dependent extracellular matrix synthesis in transforming growth factor- β 1-growth-inhibited mouse mammary gland. *J. Cell Biol.* 110:2209–2219.
- Silberstein, G. B., K. C. Flanders, A. B. Roberts, and C. W. Daniel. 1992. Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor- β 1. *Dev. Biol.* 152:354–362.
- Silberstein, G. B. and C. W. Daniel. 1987. Reversible inhibition of mammary gland growth by transforming growth factor β . *Science* 237:291–293.
- Silberstein, G. B. and C. W. Daniel. 1987. Investigation of mouse mammary ductal growth regulation using slow release plastic implants. *J. Dairy Sci.* 70:1981–1990.
- Stampfer, M. R., P. Yaswen, M. Alhadeff, and J. Hosoda. 1993. TGF β induction of extracellular matrix associated proteins in normal and transformed human mammary epithelial cells in culture is independent of growth effects. *J. Cell Phys.* 155:210–221.
- Strange, R., F. Li, S. Saurer, A. Burkhardt, and R. Friis. 1992. Apoptotic cell death and tissue remodeling during mouse mammary gland involution. *Development.* 115:49–58.
- Sudlow, A. W., C. J. Wilde, and R. D. Burgoyne. 1994. Transforming growth factor- β 1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells. *Biochem. J.* 304:333–336.
- Sun, L., G. Wu, J. K. Willson, E. Zborowska, J. Yang, and I. Rajkarananayake. 1995. Expression of transforming growth factor β type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J. Biol. Chem.* 269:26449–26455.
- Tenaud, C., A. Negoescu, F. Labat-Moleor, Y. Legros, T. Soussi, and E. Brambilla. 1994. p53 immunolabeling in archival paraffin-embedded tissues: optimal protocol based on microwave heating for eight antibodies on lung carcinoma. *Mod. Pathol.* 8:853–859.
- Vairo, G., D. M. Livingston, and D. Ginsberg. 1995. 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.
- Van Amburgh, M. E., D. G. Fox, D. M. Galton, D. E. Bauman, and L. E. Chase. 1998. Evaluation of National Research Council and Cornell Net Carbohydrate and Protein Systems for predicting requirements of Holstein heifers. *J. Dairy Sci.* 81:509–26.
- Vivien, D., L. Attisano, J. L. Wrana, and J. Massague. 1996. Signaling activity of homologous and heterologous transforming growth factor- β receptor kinase complexes. *J. Biol. Chem.* 270:7134–7141.
- Walker, N. I., R. Bennett, and J. Kerr. 1989. Cell death by apoptosis during involution of the lactating breast in mice and rats. *Amer. J. Anat.* 185:19–32.
- Wilde, C. J., C. V. Addey, P. Li, and D. G. Fernig. 1997. Programmed cell death in bovine mammary tissue during lactation and involution. *Exp. Physiol.* 82:943–53.
- Woessner, J. F. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch. Biochem. Biophys.* 93:440–447.
- Woodward, T. L., N. Dumont, M. O'Connor-McCourt, J. D. Turner, and A. Philip. 1995. Characterization of transforming growth factor- β growth regulatory effects and receptors on bovine mammary cells. *J. Cell. Physiol.* 165:339–348.
- Yamamura, Y., X. Hua, S. Bergelson, and H. F. Lodish. 2000. Critical role of Smads and AP-1 complex in transforming growth factor-beta dependent apoptosis. *J. Biol. Chem.* 275:36295–302.
- Yan, Z., S. Winawer, and E. Friedman. 1994. Two different signal transduction pathways can be activated by transforming growth factor β 1 in epithelial cells. *J. Biol. Chem.* 269:13231–13237.
- Yu, L., M. C. Hebert, and Y. E. Zhang. 2002. TGF- β receptor-activated p38 MAP kinase mediates Smad-independent TGF- β responses. *EMBO J* 2002 Jul 15;21(14):3749–59.
- Zhu Y., L. Lin, S. Kim, D. Quaglini, R. A. Lockshin, and Z. Zakeri. 2002. Cyclin dependent kinase 5 and its interacting proteins in cell death induced in vivo by cyclophosphamide in developing mouse embryos. *Cell. Death Differ.* 9(4):421–30.