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Mammary gland morphogenesis is inhibited in transgenic mice that overexpress cell surface β1,4-galactosyltransferase

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SUMMARY

Mammary gland morphogenesis is facilitated by a precise sequence of cell-cell and cell-matrix interactions, which are mediated in part through a variety of cell surface receptors and their ligands (Boudreau, N., Myers, C. and Bissell, M. J. (1995). *Trends in Cell Biology* 5, 1-4). Cell surface β1,4-galactosyltransferase (GalTase) is one receptor that participates in a variety of cell-cell and cell-matrix interactions during fertilization and development, including mammary epithelial cell-matrix interactions (Barcellos-Hoff, M. H. (1992). *Exp. Cell Res.* 201, 225-234). To analyze GalTase function during mammary gland morphogenesis in vivo, we created transgenic animals that overexpress the long isoform of GalTase under the control of a heterologous promoter. As expected, mammary epithelial cells from transgenic animals had 2.3 times more GalTase activity on their cell surface than did wild-type cells. Homozygous transgenic females from multiple independent lines failed to lactate, whereas transgenic mice overexpressing the Golgi-localized short isoform of GalTase lactated normally. Glands from transgenic females overexpressing surface GalTase were characterized by abnormal and reduced ductal development with a concomitant reduction in alveolar expansion during pregnancy. The phenotype was not due to a defect in proliferation, since the mitotic index for transgenic and wild-type glands was similar. Morphological changes were accompanied by a dramatic reduction in the expression of milk-specific proteins. Immunohistochemical markers for epithelia and myoepithelia demonstrated that both cell types were present. To better understand how overexpression of surface GalTase impairs ductal morphogenesis, primary mammary epithelial cultures were established on basement membranes. Cultures derived from transgenic mammary glands were unable to form anastomosing networks of epithelial cells and failed to express milk-specific proteins, unlike wild-type mammary cultures that formed epithelial tubules and expressed milk proteins. Our results suggest that cell surface GalTase is an important mediator of mammary cell interaction with the extracellular matrix. Furthermore, perturbing surface GalTase levels inhibits the expression of mammary-specific gene products, implicating GalTase as a component of a receptor-mediated signal transduction pathway required for normal mammary gland differentiation.

Key words: mammary gland, galactosyltransferase, morphogenesis, transgenic mouse, mouse

INTRODUCTION

Mammary gland morphogenesis begins during fetal development, but the mammary primordia remains relatively undeveloped until puberty in females. In response to circulating ovarian hormones, the mammary epithelial duct system grows and branches throughout the mammary stroma, or mammary fat pad (Topper and Freeman, 1980). Growth and differentiation of the mammary epithelial and myoepithelial cells that comprise the branching duct take place at the ductal tips in structures known as terminal end buds (TEB; Daniel and Silberstein, 1987). Like other organ systems, development of the branching mammary ductal tree requires continuous epithelial-mesenchymal interactions and interactions with the extracellular matrix (Cunha et al., 1992; Daniel et al., 1995; Niranjan et al., 1995; Sakakura, 1991). Following puberty, the mammary epithelial duct system remains relatively quiescent until pregnancy.

During pregnancy, another series of hormonal responses induces a second stage of growth and differentiation in the mammary gland, culminating in the acquisition of lactation competence (Topper and Freeman, 1980). Alveolar budding occurs at multiple points along the epithelial branches. These buds consist of epithelial and myoepithelial cells surrounded by their basal lamina. The epithelial cells within the alveoli begin to synthesize and secrete large amounts of milk-specific gene products, while the myoepithelial cells acquire intrinsic contractility, facilitating the secretion of milk products. In addition to the specific hormonal milieu found during pregnancy, it has been shown that mammary epithelial cells require a basement membrane for proper differentiation (Barcellos-Hoff et al., 1989; Lin et al., 1995; Streuli et al., 1991). Synthesis of milk-

specific genes can be reconstituted in vitro in the presence of a laminin-rich basement membrane (Barcellos-Hoff et al., 1989; Li et al., 1987); specific domains of the laminin molecule (Streuli et al., 1995), as well as specific laminin receptors, have been implicated in this process (Howlett et al., 1995). Laminin is thought to mediate the expression of β-casein via the β_1 subunit of the integrin receptor by virtue of specific enhancer elements found within the β-casein gene (Streuli et al., 1995).

A variety of other cell surface receptors, their ligands, growth factors and metalloproteinases have been implicated in mammary gland morphogenesis (Wysolmerski et al., 1995; Yang et al., 1995). A role for many of these components has been demonstrated using transgenic technology to direct aberrant temporal or spatial expression of these gene products during acquisition of lactation competence (Kordon et al., 1995; Sympson et al., 1994; Witty et al., 1995). Thus, it is likely that a variety of receptor mechanisms act synergistically to mediate the proper growth, development and differentiation of the mammary gland during puberty and again during pregnancy.

Recent studies have implicated cell surface β1,4-galactosyltransferase (GalTase) as one mediator of mammary epithelial cell-matrix interactions (Barcellos-Hoff, 1992), analogous to GalTase function as a surface receptor for extracellular glycoside ligands during a variety of cellular interactions (Bayna et al., 1988; Begovac and Shur, 1990; Eckstein and Shur, 1989; Hathaway et al., 1989; Hathaway and Shur, 1992; Miller et al., 1992; Penno et al., 1989; Runyan et al., 1986). The involvement of surface GalTase in these events is supported by both in vitro and in vivo observations using a variety of reagents to perturb GalTase function, including blocking antibodies and competitive glycosides (reviewed in Shur, 1993). More recently, however, it has become possible to alter surface GalTase expression through transgenic technology and ask how this affects specific developmental events. This approach has proven successful in characterizing surface GalTase function during sperm-egg recognition (Youakim et al., 1994b).

In somatic cells, GalTase is expressed in two isoforms, termed long and short GalTase, due to differential transcription initiation from a single GalTase gene (Shaper et al., 1988). The two proteins differ in that the long form contains an additional 13 amino acids within the cytoplasmic domain not found in the short form. Several lines of evidence show that the long GalTase isoform represents the biologically relevant isoform on the cell surface where it mediates cellular interactions, whereas the short isoform functions as the traditional biosynthetic enzyme in the Golgi complex (Evans et al., unpublished; Lopez et al., 1991; Youakim et al., 1994a).

Current evidence suggests that cell surface GalTase exerts its affects on cellular interactions by associating with the cytoskeleton and specific signal transduction pathways. In this regard, a truncated form of long GalTase lacking the catalytic domain displaces the endogenous surface GalTase from its cytoskeleton association leading to a loss of GalTase-dependent cellular interactions (Evans et al., 1993). Furthermore, it has been reported that the cytoplasmic domain of GalTase is phosphorylated, suggesting a link to a kinase-mediated pathway (Strous et al., 1987). Finally, aggregation of GalTase on the sperm surface results in induction of the acrosome reaction due to an association of the long GalTase cytoplasmic domain with a pertussis toxin-sensitive heterotrimeric G-protein complex (Gong et al., 1995).

Herein, we created mice that overexpress surface GalTase under the control of a heterologous promoter and show that this aberrant expression inhibits mammary gland morphogenesis. At parturition, transgenic mothers are unable to lactate due to a morphogenetic defect first manifested during pubertal ductal development. The primary morphogenetic defect adversely affects subsequent mammary gland differentiation during pregnancy and expression of milk proteins is concomitantly reduced. In culture, mammary epithelial cells from transgenic females show abnormal behavior and defective morphogenesis on basal lamina matrices, as compared to wild-type cells. Collectively, our results support the notion that surface GalTase participates during the cellular interactions governing mammary gland morphogenesis, and proper regulation of surface GalTase expression is necessary to obtain normal mammary gland differentiation.

MATERIALS AND METHODS

Creation of transgenic mice

Transgenic mice overexpressing the long form of GalTase have been previously described (Youakim et al., 1994b). Briefly, the EV-142 expression vector containing the mouse metallothionein (*MT-1*) promoter and a fragment of the human growth hormone gene (*hGH*) containing the polyadenylation signal (Low et al., 1985) was fused with the coding region of the GalTase cDNA, engineered to include intron 4 as described (Youakim et al., 1994b). The linearized insert was microinjected into the male pronucleus of mouse zygotes as described (Youakim et al., 1994b) and the embryos were introduced into foster mothers. Offspring were tested for integration of the transgene by Southern analysis of DNA isolated from tail clips, using *hGH* sequences as probe. Founder mice were mated to wild-type mice to generate individual lines of transgenic mice. Two transgenic lines, designated *06* and *09*, were considered for further analysis based on high expression of the transgene in several adult tissues examined.

To generate homozygous transgenic animals, hemizygotes were mated, and the offspring were genotyped by PCR analysis to determine if they were transgenic or wild type. Transgenic animals were then bred to wild-type mice, and all offspring were genotyped. The presence of at least eight hemizygous and no wild-type offspring was considered proof of homozygosity. Homozygous animals were subsequently generated by matings between homozygous mice.

Since homozygous transgenic females are unable to nurse, their litters were fostered to wild-type females that had given birth on the same day.

Milk collection

At day 1.0 or day 3.0 following parturition, homozygous or wild-type females were separated from their pups for 4 hours. (Since homozygous females cannot support a litter, a suckling stimulus was maintained for 3 days by giving new wild-type pups to homozygous females every 12 hours). Mice were then injected intraperitoneally with oxytocin in PBS (0.3 IU, Sigma). After 10 minutes, mice were anesthetized and milk was withdrawn with a suction apparatus attached to 1 mm tubing. Milking was continued for 5-7 minutes.

Preparation of mammary tissue

Female mice were killed, and the skin, including the mammary tissue, was removed and pinned flat onto paraffin boards. For the detection of cell proliferation, 5-week-old virgins and day 15.0 pregnant mice were injected with BrdU (Sigma, 100 μg/g body weight in saline/0.007 N NaOH) 5 hours prior to killing. The entire skin was fixed overnight in Bouin's fluid. After washing in PBS, the mammary tissue was removed from the skin with a scalpel. Number 2, 3, or 4 glands were used for all experiments. Whole mounts were prepared

and stained with hematoxylin as described (Medina, 1973). For histological examination and for immunohistochemistry, the Bouin's-fixed tissues were dehydrated through a graded series of ethanol, cleared in Hemo-DE and embedded in paraplast. Paraffin sections were cut at 6 μm and stained with Heidenhain's trichrome for histological examination.

Immunohistochemistry

Paraffin sections were rehydrated to PBS and processed using one of the following protocols. Sections were immunostained to detect BrdU incorporation using a kit (Zymed) according to the manufacturer's instructions. Sections to be stained with anti-smooth muscle actin (Sigma) were incubated for 5 minutes in 3% H_2O_2, rinsed in PBS, incubated for 15 minutes in 0.1 M glycine and rinsed, then blocked with 5% BSA in PBS for 30 minutes (B-PBS). Sections to be stained with anti-cytokeratin antibodies (pooled AE1 and AE3 hybridoma supernatants, kindly provided by T.-T. Sun) were rinsed in dH_2O, then subjected to microwave 'antigen retrieval' for 12 minutes on full power, with a 1 minute cooling period after 7 minutes, in 0.01 M sodium citrate, pH 6.0 (Janssen et al., 1994; Shi et al., 1991). Sections were cooled for 20 minutes, rinsed 3 times in dH_2O, twice in PBS and incubated in B-PBS for 30 minutes. Sections were incubated in primary antibody for 1 hour in B-PBS (anti-smooth muscle actin at 1/250; AE1 and AE3 at 1/20 each), rinsed 3 times in PBS, and incubated 1 hour in biotinylated goat anti-mouse IgG antibody (1/250 dilution, Zymed) in B-PBS. After 3 rinses in PBS, sections were incubated for 10 minutes in avidin-HRP (1/100 dilution, Zymed), rinsed in dH_2O, and developed using DAB as chromogen. After a final rinse in dH_2O, sections were lightly counterstained in nuclear fast red (Zymed), dehydrated, cleared and coverslipped.

Western blotting

5-week-old virgin or day 15.0 pregnant mammary glands were isolated and homogenized using a Polytron (Branson, 15 seconds at setting 6, on ice) and protein was solubilized in 2X non-reducing sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol) for 2 hours at 75°C. Insoluble material was removed by centrifugation (5 minutes, 13,000 ***g***), and protein concentration was determined using a modified BCA assay (Pierce). An equal volume of 280 mM β-mercaptoethanol, 0.001% bromophenol blue was added, 50 μg of protein was resolved on 10% reducing SDS-polyacrylamide gels, and electrophoretically transferred to nitrocellulose for 4 hours at room temperature. The nitrocellulose was blocked overnight in 20 mM Tris, 137 mM NaCl, pH 7.6, 0.1% Tween-20 (T-TBS) containing 5% non-fat milk (for GalTase immunodetection) or 2% BSA (for milk protein immunodetection). Membranes were incubated for 3 hours at room temperature in primary antibody in T-TBS containing the appropriate blocking reagent. Anti-GalTase polyclonal antibody, raised against a recombinant GalTase fusion protein (Nguyen et al., 1994), was used at a 1/500 dilution, and anti-milk polyclonal antibody, kindly provided by M. Bissell, was used at a dilution of 1/1000 (Talhouk et al., 1992). After washing exhaustively in T-TBS, membranes were incubated for 1 hour at room temperature in HRP-conjugated donkey anti-rabbit IgG (Amersham), diluted 1/5,000 in T-TBS plus blocking reagent. After washing in T-TBS, membranes were developed using ECL chemiluminescence detection (Amersham) and exposed to film. Equal loading of proteins was confirmed by Coomassie blue staining of adjacent lanes (not shown).

RNase protection assay

Total RNA was isolated from 5-week-old virgin (puberty), 10-week-old virgin (resting), day 15.0 pregnant, or day 1.0 lactating mammary glands using Tri-Reagent (Molecular Research Center) according to the manufacturer's instructions. A ^{32}P-labeled riboprobe corresponding to nucleotides –239 through +287 of GalTase was synthesized as previously described (Youakim et al., 1994b). 5 μg of total RNA was hybridized to an excess of labeled probe overnight at 59°C. Unhybridized probe was degraded with an RNase cocktail and protected probe was precipitated according to the manufacturer's instructions (Ambion). Protected fragments were separated on a 5% acrylamide/8 M urea gel and visualized by autoradiography. Control experiments were done to establish that both GalTase and actin probes were in excess and that increasing RNA concentration produced a linear increase in signal. Control samples included probe combined with yeast RNA, with or without RNase digestion.

Northern analysis of milk proteins

Total RNA was isolated from day 12.0 pregnant and day 1.0 lactating mammary glands as described above. 5 μg of total RNA was separated on a 1.2% agarose/formaldehyde gel and transferred to nitrocellulose. RNA was probed with a ^{32}P-labeled 550 bp *Pst*I fragment of whey acidic protein (WAP) cDNA or a 370/430 bp *Pst*I fragment of β-casein cDNA generated by random priming (both probes kindly provided by J. Rosen). Probes were hybridized to immobilized RNA, membranes were washed as previously described (Lopez and Shur, 1988) and exposed to X-ray film.

Reverse transcription-PCR

Total RNA was isolated from day 16.0 pregnant mammary glands and from mammary cultures using Tri-Reagent as described. The RNA was treated with RNase-free DNase (5 U, Ambion) for 30 minutes at 37°C to remove any contaminating DNA, repurified and quantitated. 1 μg of total RNA was reverse transcribed with Mo-MuLV reverse transcriptase (200 U, BRL) primed with random hexamers (0.125 U, Boehringer Mannheim) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, with 5 U RNase inhibitor and 0.5 mM of each dNTP for 50 minutes at 42°C. The reaction mixture was heat-inactivated at 70°C for 15 minutes, brought to 100 μl with dH_2O and stored at –20°C. Control reactions in which reverse transcriptase was omitted were included for each sample.

Primers (synthesized by Genosys) were designed to span at least one intron (as an additional control for genomic DNA contamination). Primers used were as follows: (1) endogenous long GalTase sense, 5′-CCGTAGCCCACCCCCTCTTA-3′, and antisense, 5′-ACGGAATGGGATGATGATGG-3′, to give a 594 bp product: (2) transgenic GalTase sense, 5′-TTGGGGTTGGGGAGGAGAAG-3′, and antisense, 5′-TTATTAGGACAAGGCTGGTG-3′, to give a 424 bp product: (3) β-casein sense, 5′-GCCTTGCCAGTCTTGCTAAT-3′, and antisense, 5′-GGAATGTTGTGGAGTGGCAG-3′, to give a 251 bp product (does not span an intron): (4) WAP sense, 5′-AAAAGCCAGCCCCATTGAGG-3′, and antisense, 5′-AGGGTTATCACTGGCACTGG-3′, to give a 294 bp product: (5) α-lactalbumin (α-LA) sense, 5′-TCGTTCCTTTGTTCCTGGTG-3′, and antisense, 5′-GGGCTTGTAGGCTTTCCAGT-3′, to give a 377 bp product.

PCR reactions included 2.5 μl of cDNA from the reverse transcriptase reaction, 5 U Taq (Promega), 1.5 mM $MgCl_2$, 0.4 μM each primer, 200 μM each nucleotide, in a total volume of 100 μl. Formamide (5%) was included in reactions containing primers for endogenous long GalTase. PCR conditions were as follows: 94°C, 5 minutes, (one cycle); 94°C, 30 seconds, 58°C, 30 seconds, 72°C, 1 minute (cycle number varied – see below); 72°C, 5 minutes, (one cycle) in the presence of 2 μCi [^{32}P]-labeled dCTP (3000 Ci/mmol, DuPont, New England Nuclear). Pilot experiments were performed to determine optimum Mg^{2+} concentration, optimum cycle number, and additive requirements for each primer set. Optimum cycle number fell within a range in which a linear relationship between the quantity of starting RNA and the final PCR product was maintained (Gause and Adamovicz, 1994). Quantitation of the results was performed by phosphoimage analysis (Molecular Dynamics) and was based on incorporation at 20 cycles (β-casein), 24 cycles (WAP and α-LA) and 30 cycles (endogenous and transgenic GalTase), and was corrected by comparison to PCR reactions using control primer sets (mouse β-actin, Clontech). No PCR product was detected in samples in which reverse transcriptase was omitted.

Primary cultures

Culture conditions used were essentially modified from Darcy et al.

(1995) and Lee et al. (1984). Mammary glands were isolated from 16.0 day pregnant females and minced in Hank's balanced salt solution (HBSS). Tissue was digested for 90 minutes at 37°C in a shaker in 10 ml of HBSS containing 0.3% (w/v) collagenase (Gibco) and dispase (2.5 U/ml, Collaborative Biomedical Products). Every 30 minutes the tissue was passed through a pipet to break up large fragments. Following digestion, the tissue was passed successively through 500 μm mesh cloth (Nitex, Tetko), then 60 μm mesh cloth (Nitex). The 500 μm retentate was discarded, and the 60 μm retentate (larger cell clusters, or spheroids) and eluate (single cells and small cell clusters) were retained: the two populations were cultured individually. Cells and spheroids were washed twice with DME/F12 containing 5% BCS by gentle centrifugation. Finally, the 60 μm eluate (single cells and small clusters) was resuspended and centrifuged at 620 revs/minute for 3 minutes, and the supernatant containing largely fibroblasts was discarded. Cells were seeded at approximately 2500/mm^2 on matrix-coated plates. Plates were prepared by adding chilled Matrigel (Collaborative Biotechnologies) to chilled plates (50 ml/cm^2) and polymerizing for 30 minutes at 37°C. Cells were seeded in DME/F12 containing 5% BCS, gentamycin (50 μg/ml), fetuin (1 mg/ml), transferrin (4 μg/ml), insulin (5 μg/ml, all from Gibco), hydrocortisone (1 μg/ml, Sigma), and ovine prolactin (3 μg/ml; Hormone Distribution Program, NIDDK). After 24 hours, the media was changed and the BCS and fetuin were omitted. Media was changed daily throughout the culture period. Photographs were taken every 24 hours on a Nikon Diaphot-TMD inverted microscope (Nikon). For histological analysis, spheroids were fixed at 6 days in culture, embedded in paraffin as described above, sectioned at 6 μm, and stained with hematoxylin and eosin.

Surface GalTase enzyme assays

GalTase enzyme activity was assayed on mammary epithelial cells cultured as single cells and small clusters for 6 days. To facilitate harvesting of intact cells, cells were cultured in the presence of Matrigel added as an overlay in the media (200 μg/ml), rather than as an underlying matrix (Streuli et al., 1995). Cells were washed once with Ca^{2+}- and Mg^{2+}-free balanced salt solution and were released with ice-cold EDTA. Cells were centrifuged and washed twice with DME/1% BSA, resuspended in medium B containing 0.4% BSA and a protease inhibitor cocktail as described (Youakim et al., 1994b). Enzyme assays were conducted in a total volume of 50 μl containing 100,000 cells as described (Youakim et al., 1994b).

RESULTS

Production of transgenic mice

The preliminary characterization of the transgenic mice used in this study has been described (Youakim et al., 1994b). Homozygous females from both the *06* and the *09* lines used in this study exhibited a similar phenotype, as did females resulting from intercrosses between the lines (i.e., *06/09* females). Homozygous females from both transgenic lines (i.e., *06/06*; *09/09*) were analyzed for each experiment described. A third transgenic line, *16*, also exhibited a lactation-deficient phenotype, but this line has not been further characterized. The transgene exhibits a high basal level of expression in a variety of tissues, including mammary gland (Youakim et al., 1994b and unpublished observations). Treatment with zinc did not enhance the phenotype as judged by morphological criteria or by RNase protection analysis (not shown); therefore, all experiments were done in the absence of zinc induction.

Characterization of the lactation-deficient phenotype

We initially noted that homozygous transgenic females from either the *06* or the *09* line were unable to support a litter. Further analysis confirmed that this phenotype was independent of the genotype of the pups; e.g., wild-type pups did not survive when placed with homozygous transgenic females. Mortality of litters was 100%, and mortality was not due to lack of maternal behavior since homozygous females exhibited normal nesting behavior and suckled their young. Rather, the pups were unable to obtain milk.

To determine if transgenic females produced any milk, we attempted to obtain milk from six transgenic females and two wild-type females. Compared to an average of 118 μl of milk per wild-type female, we obtained no milk from four transgenic females, less than 2 μl from one transgenic female, and less than 10 μl from another transgenic female. A 10-fold increase in the concentration of oxytocin failed to increase milk yield from the transgenic females. In conclusion, overexpression of surface GalTase produces a lactation-deficient phenotype. To elucidate the basis for this defect, we focused our attention on an analysis of mammary gland development.

Transgene expression

RNA was isolated from pubertal virgin (5 week), resting virgin (10 week), midgestation pregnant, and lactating wild-type and transgenic mammary glands (lactating stage mammary glands in this study refers to the day of parturition, since the lack of milk in transgenic females resulted in significant involution of the mammary gland within 72 hours). RNase protection analysis was used to determine the levels of transgene expression, since this technique allows a distinction between transgenic, endogenous long, and endogenous short GalTase transcripts. Analysis of protected probe fragments demonstrates transgene expression at all stages of mammary gland development (Fig. 1A). The expression of the endogenous long GalTase transcript remained relatively constant relative to actin expression, which was used as an internal standard for RNA loading.

Overexpression of GalTase protein in transgenic glands was confirmed by Western blotting and by assaying GalTase activity on intact cells. Levels of GalTase protein were markedly elevated in transgenic resting virgin mammary glands relative to wild-type glands (Fig. 1C). Western analysis is unsuitable for determining transgene protein levels in pregnant or lactating mammary glands, since the antibody detects all forms of GalTase including the endogenous short GalTase, transcripts of which are reduced in response to transgene expression during pregnancy and lactation (discussed below). Therefore, overexpression of GalTase protein was confirmed by assaying surface GalTase activity on intact cultured mammary epithelial cells. Cell surface GalTase activity was 2.3-fold higher on transgenic mammary epithelial cells (derived from mid-gestation pregnant glands) compared to wild-type cells (1939 cts/minute/μg protein/hour versus 855 cts/minute/μg protein/hour).

During lactation, GalTase is a component of the lactose synthetase complex, which synthesizes lactose (milk sugar). As expected, the expression of the endogenous short GalTase transcript increased dramatically during pregnancy and lactation in wild-type glands (Lopez et al., 1991; Fig. 1A). Interestingly, the levels of the endogenous short GalTase transcript did not increase in transgenic mammary glands (Fig. 1A). As shown below, this result is indicative of lactation incompetence and defective mammary gland development.

Overexpressing the short GalTase isoform does not produce a lactation-deficient phenotype

We also created transgenic mice that overexpress the short, biosynthetic isoform of GalTase. Expression of the short GalTase transgene in these mice was confirmed by enzyme assay in several adult tissues (not shown), as well as by RNase protection analysis of RNA isolated from pregnant mammary glands (Fig. 1A). Levels of transgene expression in mice overexpressing the short GalTase isoform were comparable to or higher than expression levels of the long GalTase transgene. Western immunoblotting confirmed that the short GalTase transgenic protein was overexpressed (not shown). These mice have been bred to homozygosity and seven individuals representing three generations show no evidence of lactation deficiency. Furthermore, in contrast to transgenic mice overexpressing the long GalTase isoform, mice overexpressing the short GalTase transgene had normal levels of the endogenous short GalTase transcript (Fig. 1A). Finally, 5-week-old virgin short GalTase transgenic mammary glands were morphologically normal (not shown). Thus, the lactation-deficient phenotype associated with overexpression of GalTase is unique to the long GalTase isoform, which is the biologically active form on the plasma membrane (Shur, 1993).

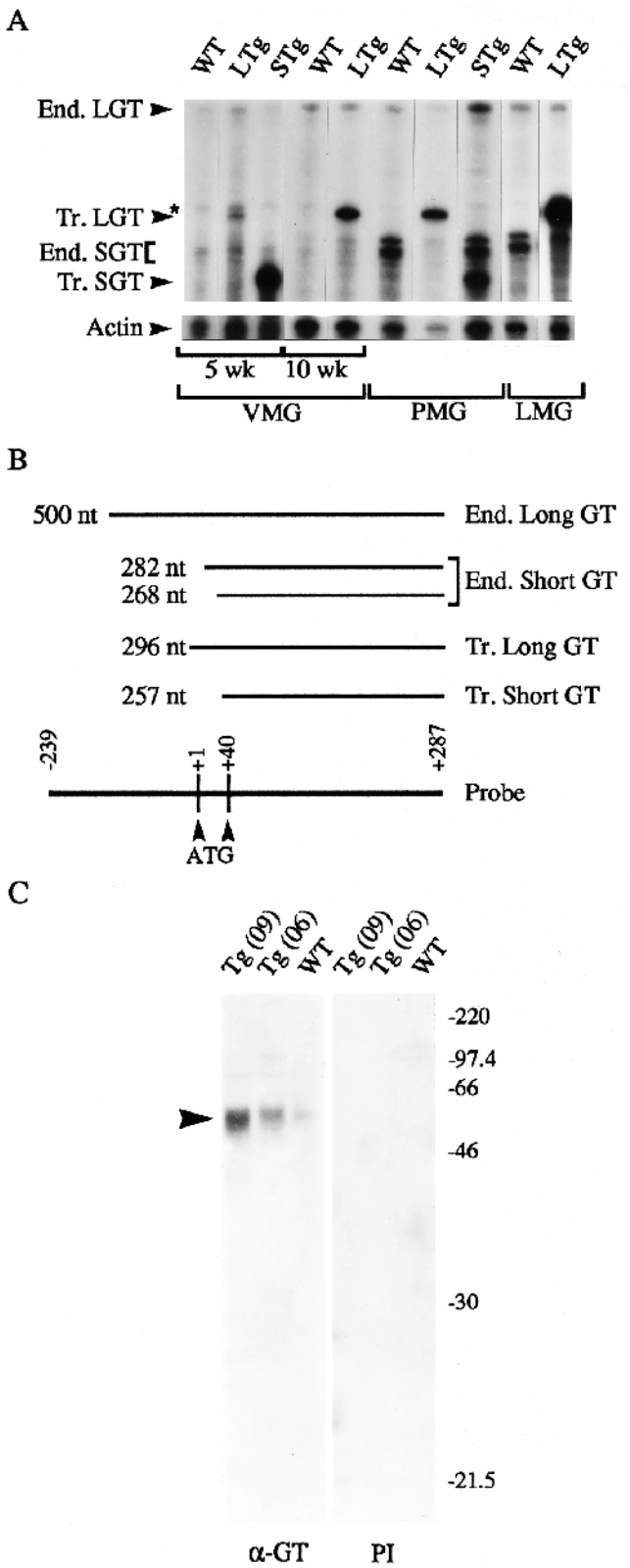

Fig. 1. Expression of transgenic GalTase message and protein. (A) RNase protection analysis was performed on RNA isolated from 5-week- (5 wk) and 10-week (10 wk)-old virgin (VMG), day 15.0 pregnant (PMG), and day 1.0 lactating (LMG) mammary glands. RNA from wild-type animals (WT) and animals expressing either the long (LTg) or short (STg) GalTase transgene were compared. 5 μg of total RNA was hybridized to a ^{32}P-labeled riboprobe as described in Materials and Methods. Following digestion of single-stranded RNA, the protected probe was resolved on an acrylamide/urea gel. Protected fragments represent the endogenous long GalTase transcript (End. LGT; 500 nt), two endogenous short GalTase transcripts (End. SGT; 282 nt and 268 nt), a transgene-specific long GalTase transcript (Tr. LGT; 296 nt), and a transgene-specific short GalTase transcript (Tr. SGT; 257 nt). A minor endogenous long GalTase transcript (301 nt; asterisk) was also apparent. A riboprobe specific to actin transcripts was included in all hybridization reactions to serve as a loading control. The assay was repeated a minimum of six times for each stage of development, including samples from both the *06* and the *09* lines, with similar results. (B) Diagrammatic representation of the probe and major protected fragments. Undigested probe runs as a single band of 563 nt (not shown). (C) Proteins isolated from 10-week-old virgin mammary glands were electrophoresed on polyacrylamide gels, electroblotted to nitrocellulose and probed with anti-GalTase antisera (α-GT) or preimmune sera (PI) as described in Materials and Methods. A single band of approximate molecular mass 56×10^3 M_r (arrowhead) was detected in wild type (WT) and both long GalTase transgenic lines (Tg (*06*) and Tg (*09*)). Increased levels of GalTase protein were detected in the transgenic mammary glands relative to wild type. Equal loading of protein was determined by Coomassie Blue staining of adjacent lanes (not shown). The approximate position of molecular mass markers ($M_r\times10^{-3}$) are shown on the right. The assay was repeated three times with similar results.

Morphological analysis of transgenic mammary gland development

Virgin mammary glands

Overexpression of surface GalTase resulted in an obvious morphological phenotype first evident in the pubertal (i.e., 5 week) virgin mammary gland. Examination of defatted whole-mount glands showed reduced development of the mammary epithelial ductal tree in transgenic glands, as compared to age-matched wild-type glands (Fig. 2A-C). At this stage, transgenic glands were significantly smaller than wild-type glands, with

severely reduced duct development and smaller TEBs. The ductal structure as well as the morphology of the TEB appeared relatively normal when examined histologically (not shown).

At the resting virgin stage (i.e., 10 week), the epithelial ducts in wild-type glands extended to the limit of the mammary fat pad, but not in transgenic glands (Fig. 2D-G). The integrity of the larger ducts appeared compromised in transgenic glands, manifested as a dilated, irregular lumen. The extent of secondary branching was also significantly reduced. Finally, the morphology of the duct termini was enlarged in transgenic glands, resembling more closely the TEB of pubertal, actively growing ducts. In contrast, the resting wild-type virgin gland exhibited quiescent terminal duct structures characteristic of this stage.

In order to determine if the changes in morphology associated with the transgenic mammary gland were simply due to delayed development, virgin mammary glands were examined histologically. Whereas the ducts and TEBs of 5-week-old transgenic glands appeared histologically normal, the terminal duct structures of 10-week-old virgin transgenic glands did not resemble wild-type TEB at the actively proliferating pubertal stage, or terminal ducts of the resting virgin stage (Fig. 3). The wild-type TEB at puberty showed a zone of active proliferation and differentiation, being multilayered and in close association with the underlying stroma and basement membrane (Fig. 3A); however, the terminal structure seen in the 10-week-old transgenic mammary gland consisted of one to two layers of cells, only loosely associated with the basement membrane (Fig. 3C). The transgenic terminal duct structure was also clearly distinct from the wild-type resting virgin terminal duct structure, which was indistinguishable from the ductal epithelium (Fig. 3B). Thus, the characteristic phenotype of the terminal duct structure seen in transgenic virgin mammary glands is a unique feature of the transgenic phenotype, and can be attributed to overexpression of surface GalTase.

Pregnant mammary glands

During pregnancy, alveolar proliferation takes place and milk protein gene expression is induced. This is easily visualized by histological analysis (Fig. 4). The dense alveolar development seen by day 16.0 of pregnancy was apparent in wild-type glands, (Fig. 4A), as was the characteristic secretory phenotype of individual alveolar epithelial cells. In contrast, transgenic mammary glands taken at day 16.0 of pregnancy showed dramatically reduced alveolar development (Fig. 4B), and alveolar epithelial cells showed no evidence of secretory behavior.

Lactating mammary glands

On the day of parturition, wild-type mammary glands are characterized by the secretion of mammary-specific gene products into the alveolar lumens. Alveolar epithelia contained numerous secretory vesicles, and the lumens of the ducts were enlarged and filled with milk (Fig. 4C). In contrast, alveolar development was still reduced in the transgenic gland at parturition, as was the synthesis and release of milk (Fig. 4D). The alveolar epithelial cells in the transgenic gland acquired more of a secretory phenotype than that seen at midgestation

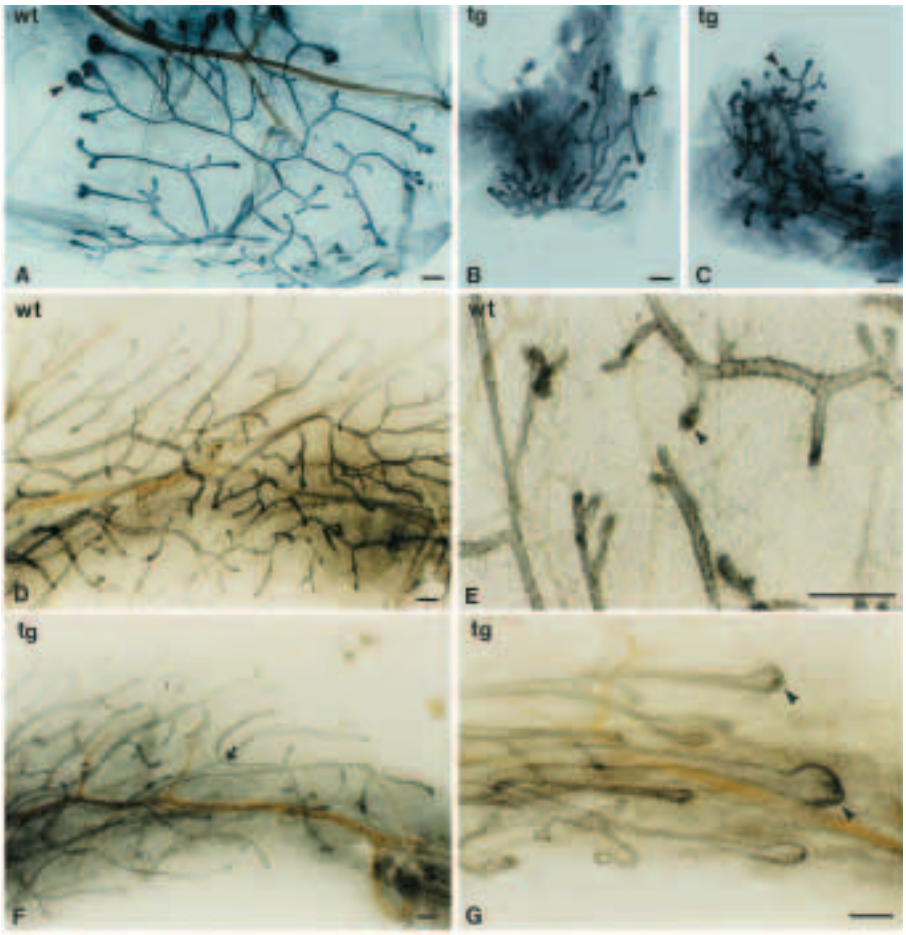

Fig. 2. Whole-mount preparations of wild-type (wt) and transgenic (tg) virgin mammary glands. Mammary glands were isolated from 5-week-old (A-C) or 10-week-old virgin females (D-G), fixed, extracted with acetone and stained with hematoxylin. (A) At 5 weeks of age, wild-type mammary glands showed extensive growth and branching, and possessed numerous large TEB (arrowhead). (B,C) Transgenic 5-week-old mammary glands from two different animals showed reduced growth and development compared to wild type. TEB (arrowheads) were numerous, but were smaller than wild type. (D,E) At 10 weeks of age, wild-type virgin mammary glands showed extensive branching of the ducts, which extended to the limit of the mammary fat pad (just out of the field in D). Terminal duct structures (E, arrowhead), were quiescent and characteristically small. (F,G) At 10 weeks of age, transgenic virgin mammary glands showed irregularly shaped ducts, with larger lumens in some areas (F, arrow). Ductal growth and branching was less extensive than in the age-matched wild-type gland. Terminal duct structures (G, arrowheads) in the transgenic gland were enlarged compared to wild type. Bars, 1.0 mm.

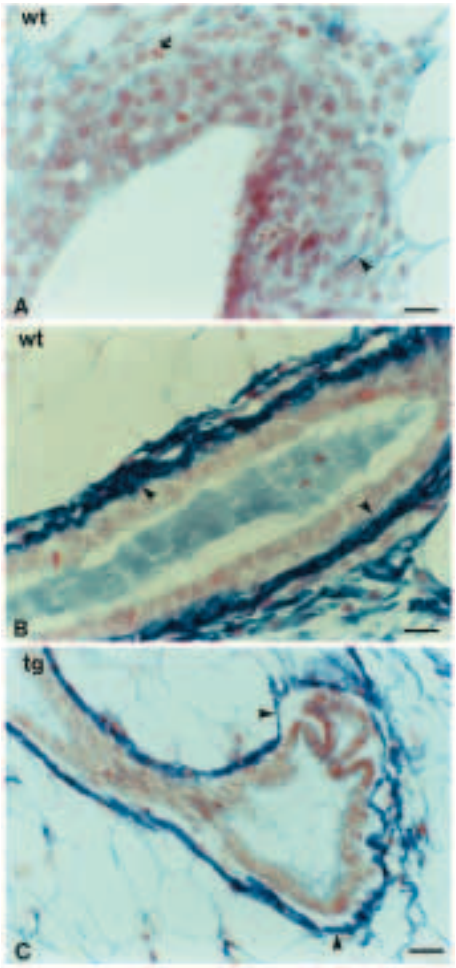

Fig. 3. Transgenic mammary gland terminal duct structures are abnormal. Terminal duct structures of 5-week-old virgin wild-type (wt), 10-week-old virgin wild-type, and 10-week-old virgin transgenic (tg) mammary glands were compared after embedding in paraffin and sectioning. (A) At 5 weeks of age, wild-type TEBs showed a characteristic proliferative morphology, with frequent mitotic figures (arrow). TEBs were multilayered and a close association with the underlying basement membrane was evident (arrowhead). (B) At 10 weeks of age, wild-type terminal duct structures were morphologically indistinguishable from non-terminal regions of the duct. A collagen-rich basement membrane has accumulated around the entire duct structure (deep blue). Ductal epithelial cells remained in close contact with the basement membrane (arrowheads). (C) In contrast, terminal duct structures from the 10-week-old virgin transgenic gland did not resemble wild-type terminal structures at either puberty (A) or quiescence (B). They consisted of one to two cell layers and no mitotic figures were visible. A collagen-rich basement membrane has accumulated, but a disruption of the cell-basement membrane associations was apparent (arrowheads). Bars, 10 μm.

pregnancy, however, the secreted material appeared to contain larger lipid droplets when compared to wild type.

In conclusion, histological analysis of the transgenic mammary glands suggests that overexpression of surface GalTase reduces mammary gland ductal development and differentiation beginning at puberty, resulting in a reduced framework with which to produce alveoli in preparation for the synthesis of milk. Furthermore, those alveoli that do develop are relatively unable to synthesize and secrete milk.

Transgenic mammary glands exhibit normal proliferation

It is possible that reduced mammary gland development in transgenic mice resulted from reduced cell proliferation. Indeed, there is evidence that surface GalTase modulates cell growth in culture, possibly by interacting with the EGF receptor (Hinton et al., 1995). Therefore, we examined cell proliferation in TEBs at puberty and in alveoli at midgestation pregnancy, two stages of active cell proliferation. Mice were treated with BrdU 5 hours prior to killing and mitotic index was determined on sections of mammary gland treated immunohistochemically to visualize BrdU incorporation. The number of BrdU-positive cells in TEBs at 5 weeks of age, or in alveolar epithelia at midgestation pregnancy, was not significantly different comparing wild-type and transgenic glands (Fig. 5). Therefore, reduced cell proliferation cannot account for the reduced glandular development.

Immunohistochemical analysis of mammary cell markers

We considered the possibility that overexpression of surface GalTase adversely affected the terminal differentiation of one of the ductal cell types necessary for normal mammary gland function. During TEB growth, basal stem cells give rise to the ductal epithelial cells as well as the myoepithelial cells that encircle the epithelial cells and aid in milk secretion by virtue of their intrinsic contractile ability. Immunohistochemical markers were used to identify each of these cells types to determine if normal terminal differentiation was occurring in the transgenic glands. Monoclonal antibodies AE1 and AE3 label specific cytokeratins found in many epithelial cell types including mammary epithelia (Asch and Asch, 1987), and anti-smooth muscle actin antibodies specifically label the myoepithelial cells within the mammary gland (Gugliotta et al., 1988). As seen in Fig. 6, both differentiated cell types were present in the transgenic mammary gland. Shown are immunohistochemical stains of resting virgin glands; glands from midgestation pregnancy and day 1.0 of lactation revealed similar results (not shown). Notably, staining with antibodies specific for each of these cell types highlights the abnormal morphology of these cells in transgenic animals. Compared to wild-type ductal epithelial cells that have a columnar morphology (Fig. 6A), the epithelial cells of the transgenic gland were significantly more rounded to cuboidal (Fig. 6B) and the layer of myoepithelial cells appeared more sparse (compare Fig. 6C and D).

Transgenic mammary glands synthesize reduced levels of milk-specific gene products

The morphological analysis of the transgenic mammary gland suggested that milk production was significantly reduced. To determine directly if this was the case, northern analysis was performed on RNA isolated from wild-type and transgenic day 12.0 pregnant and day 1.0 lactating mammary glands. Probes corresponded to two milk-specific gene products, β-casein, whose synthesis is induced by day 8.0 of pregnancy (Hobbs et al., 1982), and WAP, whose synthesis is induced by day 14.0 of pregnancy (Pittius et al., 1988). A significant level of both β-casein and WAP mRNA was detected in the wild-type mammary glands (Fig. 7A); however, the amount of both β-casein and WAP mRNA was significantly reduced in the transgenic gland. This reduction in milk protein synthesis was

confirmed at the protein level by Western immunoblot analysis using a polyclonal antibody raised against mouse milk (Talhouk et al., 1992). The antibody detects multiple casein species and all of these casein proteins were significantly reduced in midgestation pregnant transgenic glands (Fig. 7B).

To better quantify the expression of milk-specific genes, we performed quantitative RT-PCR analysis on day 16.0 pregnant mammary glands using primer sets specific for β-casein, WAP, and α-LA (Table 1). Pilot experiments were done to optimize PCR conditions for each primer set and to determine the appropriate cycle number for linear accumulation of PCR product, such that relative message levels for a given gene product could be compared between wild-type and transgenic samples (see Materials and Methods). Quantitation of the RT-PCR and normalization to internal β-actin controls revealed that the reduction in β-casein gene expression was approximately 2-fold, whereas WAP and α-LA expression were both reduced by 10- to 20-fold.

Transgenic mammary epithelial cells show abnormal interaction with the extracellular matrix in vitro

Thus far our results demonstrate that overexpression of surface GalTase disrupts normal mammary gland morphogenesis, resulting in reduced duct and alveolar development, abnormal epithelial cell histology and reduced milk protein gene expression, culminating in complete lactation failure. Based on previous studies of cell surface GalTase function in other in vitro and in vivo systems, it seems logical to suggest that overexpression of GalTase might interfere with normal cell-cell and/or cell-matrix interactions.

To gain some insight into this problem, primary cultures of mammary epithelial cells were established from wild-type and transgenic day 16.0 pregnant mammary glands. Culture conditions were used that favor the maintenance of the differentiated state, as assayed by the synthesis of milk protein gene products (Barcellos-Hoff et al., 1989). This is accomplished by supplying an exogenous laminin-rich basement membrane (Matrigel) and supplementing the media with lactogenic hormones. Cells were cultured either as single cells and small clusters, or as spheroids. Under these conditions, mammary epithelial cell interactions and differentiation have been well defined (Barcellos-Hoff et al., 1989; Darcy et al., 1995).

Differences were observed between wild-type and transgenic mammary epithelial cells within the first 24-48 hours of culture on Matrigel. Wild-type cells cultured as either single cells or small cell clusters typically attach and migrate on the matrix to form anastomosing networks. Later, these networks contract to form clusters that ultimately form small spheroids embedded within the matrix. When spheroids are isolated from pregnant mammary glands and cultured, they maintain their spheroid morphology and eventually become embedded within the matrix by 'organizing' the matrix around themselves, removing it from the adjacent culture surface. While the wild-type mammary epithelial cells behaved in this predictable and defined fashion in culture (Fig. 8A,C,E), the transgenic mammary epithelial cells behaved abnormally. Cells from transgenic females cultured singly or in small clusters attached normally to the matrix, but the migration and formation of anastomosing networks was delayed by 24-48 hours (Fig. 8B,D). Differences between wild-type and transgenic cells were less apparent by 72-96 hours in culture (not shown). In addition, transgenic mammary epithelial cells cultured as spheroids were less efficient at organizing the Matrigel matrix around themselves, as evidenced by the persistence of the matrix monolayer adjacent to the transgenic spheroids throughout the culture period (Fig. 8F).

Selected spheroid cultures were fixed and sectioned after 6 days in culture to visualize their histology. As expected, the wild-type spheroids were surrounded by Matrigel and there was clear evidence of vectorial secretion of milk products, although by this time in culture there was also some collapse of spheroids accompanied by evidence of cell death in the spheroid lumen (Fig. 8G). In contrast, the transgenic mammary

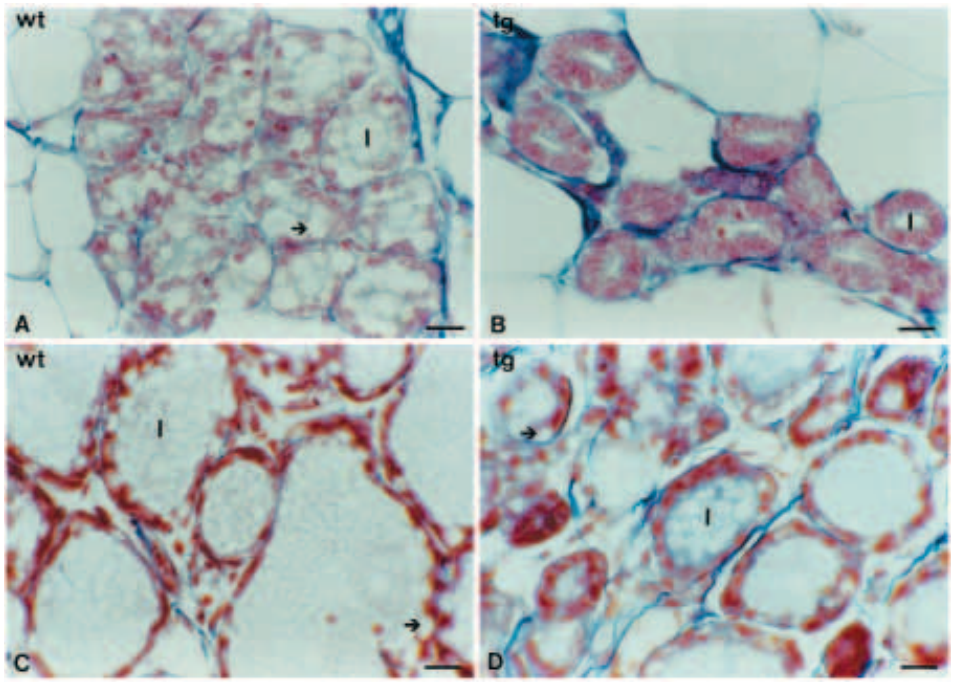

Fig. 4. Histological analysis of wild-type (wt) and transgenic (tg) pregnant and lactating mammary glands. (A) At day 16.0 of pregnancy, the wild-type mammary gland displayed a morphology characteristic of its secretory phenotype, with numerous secretory vacuoles (arrow) within alveolar epithelial cells, and a slightly enlarged alveolar lumen. (B) In contrast, the transgenic day 16.0 pregnant gland appeared quiescent, with no signs of secretion. (C) On the first day after parturition, the wild-type lactating gland was characterized by significantly enlarged alveolar lumens that contain milk proteins and lipid droplets synthesized and secreted by the alveolar epithelium (arrow). (D) The transgenic day 1.0 lactating gland showed limited alveolar epithelial secretion (arrow) and milk accumulation in the alveolar lumen. l, alveolar lumen. Bars, 10 μm.

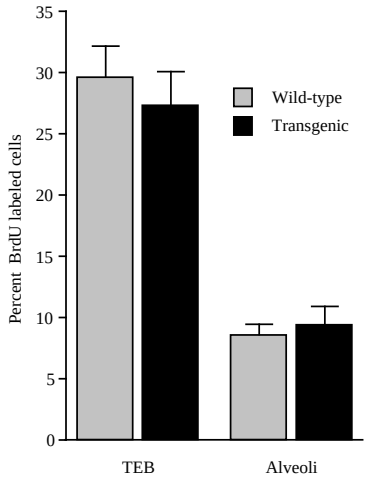

Fig. 5. Transgenic mammary epithelial cells proliferate normally. 5-week-old virgin and day 15.0 pregnant females were injected with BrdU prior to killing, and sectioned mammary glands were immunolabed with anti-BrdU antibody to detect proliferating cells. The relative number of BrdU-incorporating cells was determined in TEB of 5-week-old glands and in alveoli of pregnant glands. No significant difference was seen between wild-type and transgenic samples. Determinations were made by counting 100 alveoli from each of two wild-type, and each of three transgenic pregnant glands (average cell number/alveolus = 17.5), or four TEBs from two wild-type or ten TEBs from three transgenic virgin glands (average cell number/TEB = 145). Bars, ±s.e.m.

epithelial cells showed a reduced secretory phenotype, though they appeared to be appropriately polarized. Transgenic spheroids were often incompletely surrounded by the Matrigel (Fig. 8H). Interestingly, in transgenic cultures there were abundant regions containing cells with a mesenchymal morphology. These cell types were not apparent in wild-type spheroid cultures. Mesenchymal cell clusters were surrounded by Matrigel, and were frequently associated with smaller clusters of polarized epithelial cells. These observations raise the possibility that overexpression of surface GalTase results in a matrix-dependent conversion of epithelial cells to a mesenchyme-like cell type.

After 6 days in culture, RNA was isolated from wild-type and transgenic mammary epithelia cultured as single cells. Quantitative RT-PCR analysis confirmed that transgene expression was maintained throughout the culture period (Fig. 9A) and that cultured transgenic mammary cells continued to express reduced levels of milk-specific gene products (Fig. 9B).

DISCUSSION

The results presented here show that altering the expression of cell surface GalTase inhibits the morphogenesis of mammary epithelial cells into the functional ducts and alveoli characteristic of the mature mammary gland. Overexpressing surface GalTase leads to abnormalities in duct morphology, reduced ductal growth and reduced branching in virgin mice. This results in diminished alveolar development during pregnancy and those alveoli that do develop have a reduced capacity to synthesize and secrete milk proteins. Ultimately, this leads to a failure in lactation upon parturition. The defective mammary gland morphogenesis resulting from overexpression of surface GalTase is consistent with previous in vitro studies suggesting that GalTase facilitates the ability of mammary epithelial cells to migrate and form anastomosing networks on laminin-rich basal lamina matrices (Barcellos-Hoff, 1992).

Table 1. Quantitation of milk protein gene expression in day 16.0 pregnant mammary glands by RT-PCR*

Genotype	α-LA	WAP	β-Casein
+/+	1,615†‡	7,031	11,296
06/06	103 (6%)§	355 (5%)	5,189 (46%)
09/09	175 (11%)	212 (3%)	4,676 (41%)

*The assay was performed three times, with similar results.
†All PCR reactions were corrected by comparison to control reactions using an actin primer set.
‡Arbitrary units, based on quantitation by phosphoimage analysis of ^{32}P-labeled PCR products, resolved by acrylamide gel electrophoresis.
§Percent relative to wild type for each gene product.

A variety of observations are consistent with the hypothesis that defective mammary gland development results from overexpression of surface GalTase. Three independent lines of transgenic mice are characterized by the same lactation-deficient phenotype. Two lines, *06* and *09*, were studied in detail, while a third line, *16*, also failed to lactate, but was not examined further. All three lines express high levels of the long GalTase transgene in a variety of embryonic, fetal and adult tissues, as judged by RNase protection assays, Western immunoblotting and assays of GalTase enzyme activity (Youakim et al., 1994b and unpublished observations). In this regard, mammary epithelial cells from transgenic animals have increased levels of surface GalTase expression when compared to wild-type cells. When mammary glands were metabolically labeled with [^{3}H]galactose, the profile of [^{3}H]galactosylated glycoproteins, as revealed by SDS-PAGE fluorography, was similar between wild-type and transgenic glands (unpublished observations) suggesting that glycosylation patterns are not grossly affected in the transgenic glands, similar to that reported in other cell types (Youakim and Shur, 1993). However, most compelling is the observation that transgenic mice overexpressing the short GalTase transgene have no observable phenotype and lactate normally. Short GalTase represents the purely biosynthetic isoform in the Golgi complex and has no effect on cellular interactions when overexpressed in transfected cells (Evans et al., 1993; Youakim and Shur, 1993).

Morphological analysis of the mammary glands reveals that the initial defect in long GalTase transgenic animals occurred during the early stages of gland development and differentiation. The establishment of the mammary ductal tree occurs during puberty in response to a surge in the levels of circulating ovarian hormones. Following puberty, this mammary duct framework remains relatively quiescent until pregnancy. Overexpression of surface GalTase leads to defective morphogenesis of the mammary ductal tree, characterized by reduced size and branching and by irregular and dilated ducts. The cytoarchitecture of the ductal epithelial cells is altered, in that they appear smaller and more rounded than the columnar and tightly apposed cells in wild-type ducts. The myoepithelial cell layer also appears to be reduced in thickness. However, overex-

pressing surface GalTase does not affect the determination of cells in the mammary duct, since both epithelial and myoepithelial cells are present, as judged by immunohistochemical markers for these two cell types.

The morphology of terminal duct structures in mature virgin females is also abnormal, appearing enlarged compared to wild-type terminal ducts. This abnormal morphology does not simply reflect a delay in development, since transgenic terminal duct structures from mature virgin females do not resemble the TEBs of pubertal wild-type virgin mice. Finally, histological examination of mature virgin glands reveals a striking loss of epithelial cell interactions with the extracellular matrix, as compared to wild type. The morphology of the transgenic gland is consistent with overexpression of surface GalTase interfering with epithelial cell interactions with the adjacent basal lamina.

The reduced size of the mammary ductal tree must be accompanied by a reduction in cell number, since histological examination does not suggest a denser cell arrangement in transgenic glands. Furthermore, surface GalTase signals have been associated with cell proliferation in culture (Hinton et al., 1995). However, our evidence argues against reduced proliferation in transgenic mammary glands. BrdU incorporation in wild-type and transgenic glands was similar in both TEBs of 5-week-old virgin glands and the alveoli of midgestation pregnant glands. An alternative possibility to account for the overall reduced mammary gland size is an increase in cell death, possibly through an apoptotic mechanism. Recently, evidence suggests that such a mechanism is responsible for the involution of the mammary gland at weaning and that apoptosis results from the loss of cell-basement membrane interactions due to proteolytic degradation of the basement membrane (Boudreau et al., 1995; Lund et al., 1996). In light of the apparent disturbance in cell-basement membrane interactions observed in mice overexpressing surface GalTase, the level of apoptosis in transgenic mice will be examined in future studies.

During pregnancy, a second wave of mammary development occurs, again in response to circulating hormones. At this time, alveoli bud from the mammary ducts and the synthesis and secretion of milk proteins begins. Alveolar development is reduced in GalTase transgenic animals due to the reduced size of the ductal tree. This is best observed by examining sections of pregnant mammary glands at low magnification, where the amount of stroma relative to alveoli is significantly greater in transgenic glands than in wild type. However, alveolar reduction alone can not account for the almost complete lactation failure seen in transgenic animals. Instead, we also noted morphological defects in the alveoli of transgenic animals, characterized by a lack or reduction in secretory activity and little accumulation of secretory products in the ductal lumens. Similar observations were made at day 1.0 of lactation, including a reduction in secretory activity and lumenal accumulation of secretory products in transgenic animals compared to wild type. Concomitantly, the expression of the milk proteins α-LA, WAP and β-casein was significantly reduced in transgenic animals. The reduction in milk-specific gene expression varied among the gene products examined. This may reflect the difference in the onset of expression of these gene products; β-casein expression occurs early in pregnancy (Hobbs et al., 1982), whereas WAP and α-LA genes are activated much later (Pittius et al., 1988; Vilotte and Soulier, 1992). It is also possible that differences in the regulation of these genes render them more or less sensitive to perturbations in cellular interactions; however, it should be noted that both β-casein and WAP require cell-matrix interactions for proper expression (Chen and Bissell, 1989; Schmidhauser et al., 1990).

Despite the morphological defects associated with overex-

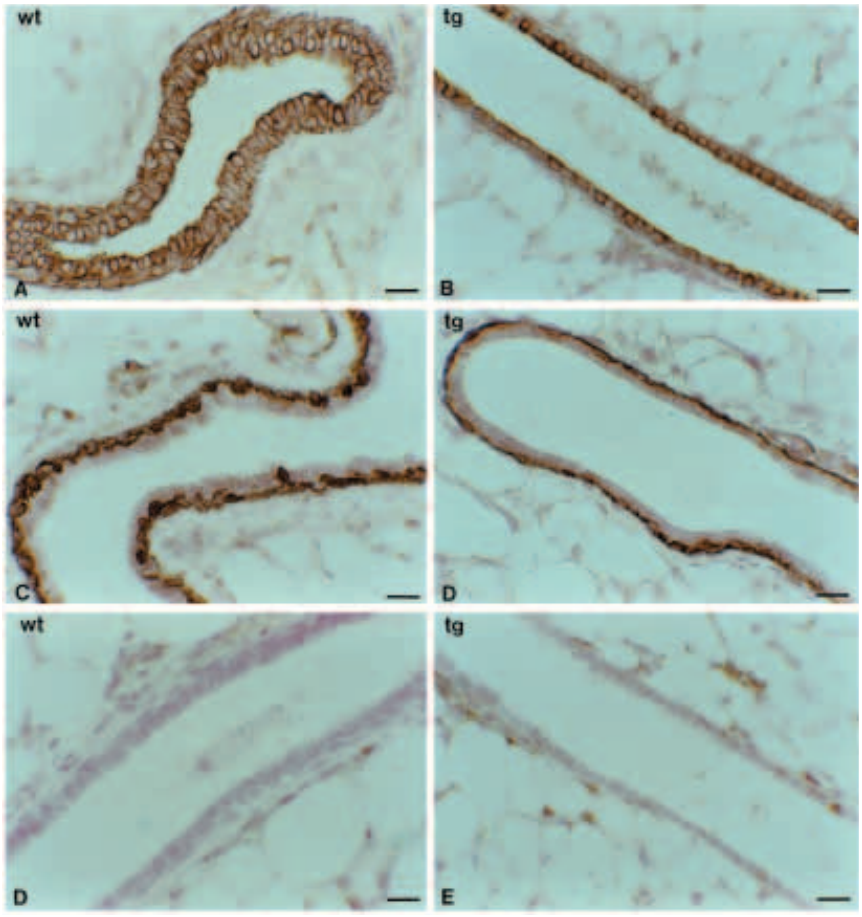

Fig. 6. Both mammary epithelial cells and myoepithelial cells are present in transgenic mammary glands. Sections of virgin mammary glands were immunostained with antibodies against cytokeratins (A,B) or against smooth muscle actin (C,D) to distinguish epithelial and myoepithelial cells, respectively. Both cell types were clearly present in both wild-type (wt, A,C) and transgenic (tg, B,D) glands. The abnormal morphology characteristic of the transgenic mammary epithelial cells was accentuated by anti-cytokeratin immunolabeling (compare A and B), and the layer of myoepithelial cells in transgenic ducts appeared thinner in anti-smooth muscle actin-stained sections (compare C and D). Control preparations (E,F) were incubated with nonimmune IgG and secondary antibody. Bars, 10 μm.

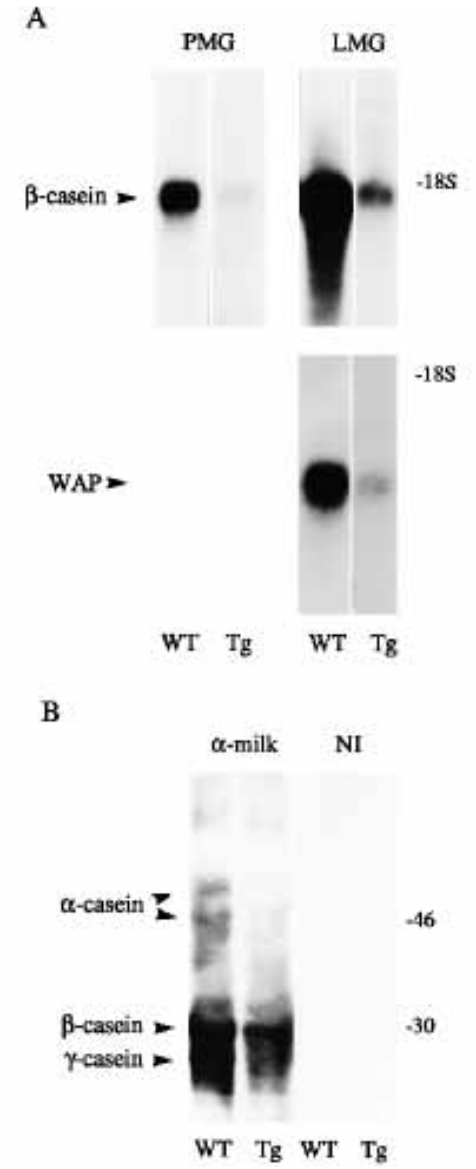

Fig. 7. Transgenic mammary glands express reduced levels of milk-specific genes. (A) 5 μg of total RNA isolated from day 12.0 pregnant (PMG) or day 1.0 lactating mammary glands (LMG) was electrophoresed, transferred to nitrocellulose, and probed with ^{32}P-labeled β-casein or WAP cDNA fragments as described in Materials and Methods. An intense band corresponding to the β-casein or WAP transcript was detected in wild-type samples (WT). A significantly less intense band was detected in transgenic samples (Tg). At day 12.0 of pregnancy, WAP transcript levels are too low to be detected by northern analysis. Equal loading of RNA was determined by ethidium bromide detection of 28S and 18S ribosomal RNA. The position of 18S ribosomal RNA is indicated on the right. (B) Proteins were isolated from day 15.0 pregnant mammary glands, electrophoresed on polyacrylamide gels, electroblotted to nitrocellulose, and probed with an antibody raised against mouse milk (α-milk). As indicated on the left, the antibody recognizes predominantly the major isoforms of the casein gene family (α, β, and γ). A significant reduction in casein protein was detected in transgenic mammary glands (Tg) compared to wild type (WT). NI, nonimmune control blot. Equal loading of protein was determined by Coomassie Blue staining of adjacent lanes (not shown). The approximate position of molecular mass markers ($M_r \times 10^{-3}$) are shown on the right.

pression of surface GalTase, alveolar development and secretory activity are not completely eliminated; by day 1.0 of lactation, there is evidence of secretory products within alveolar lumens. By histological examination, however, the appearance of the secretions is different from that seen in wild-type glands. The possibility exists that the alteration in milk protein gene expression results in both reduced and abnormal secretory products. Conceivably, viscosity of the milk could be altered, making it impossible to remove. Such a phenotype has been observed in α-LA-deficient mice (Stinnakre et al., 1994). The phenotype itself eliminates the possibility of examining this issue further, since we are unable to obtain milk in sufficient quantities for assay.

The behavior of mammary epithelial cells on basal lamina, cultured as single cells, small cell clusters or spheroids, supports the notion that surface GalTase contributes to the normal mammary epithelial cell phenotype by interacting with the basal lamina. The behavior of normal mammary epithelial cells has been well characterized in culture (Barcellos-Hoff et al., 1989; Darcy et al., 1995) and, in our assays, wild-type mammary epithelial cells behaved as expected. In contrast, transgenic cells cultured as single cells or small clusters did not associate normally with the matrix, in that they failed to migrate and form anastomosing networks as quickly as did wild-type cells. Transgenic mammary gland spheroids behaved abnormally as well, in some cases failing to properly organize the Matrigel matrix around themselves. In other cases, mesenchymal cell foci were observed upon histological examination, often associated with clusters of polarized epithelial cells. This suggests the possibility that transgenic mammary epithelial cells transform to a mesenchymal phenotype when cultured with a basement membrane. An example of a similar transformation occurs when mammary epithelial cell lines lose cell surface syndecan-1 expression (Kato et al., 1995). An alternative explanation for these results is the possibility that overexpression of surface GalTase confers a survival or growth advantage on mammary stromal cells. Further study will be required to distinguish between these possibilities.

The cumulative in vivo and in vitro observations are consistent with the hypothesis that surface GalTase participates during normal mammary epithelial cell interactions with the basal lamina, and that overexpression of GalTase interferes with normal ductal morphogenesis. Increasing surface GalTase expression could interfere with cell-matrix interactions in one of several ways. First, overexpressing surface GalTase could make the cells overly adhesive to the basal lamina matrix and/or could compete with a rate-limiting number of cytoskeletal attachment sites, either of which can reduce cell migration on basal lamina as reported for fibroblasts that overexpress surface GalTase (Appeddu and Shur, 1994). Alternatively, excess GalTase could result in the overstimulation of a downstream intracellular signal cascade, similar to that seen in sperm that overexpress GalTase. These sperm are hypersensitive to their egg coat ligand resulting from hyperactivation of GalTase-associated G-protein signaling (Gong et al., 1995). It is also likely that perturbation of one surface receptor affects the expression or function of other parallel mechanisms. For example, eliminating syndecan expression on mammary epithelial cells results in morphological changes (discussed above) accompanied by a reduction in E-cadherin expression and a reorganization of β1 integrin (Kato et al., 1995). In this regard, preliminary results suggest that a variety of cell surface

receptors are altered in the mammary glands of long GalTase transgenic mice (unpublished observations).

Cell surface GalTase has been shown to be a receptor for laminin in a variety of cell types, including fibroblasts, embryonic mesenchymal cells, neural crest cells and PC12 cells (Eckstein and Shur, 1989; Hathaway and Shur, 1992; Runyan et al., 1986). GalTase is specifically involved in the migration and spreading of these cells on the laminin matrix, but does not mediate their initial adhesion to laminin nor does it function during migration on fibronectin (Runyan et al., 1988). It is interesting that the GalTase-binding site in laminin has been identified as oligosaccharides within the A chain of laminin, contained within the globular C-terminal region (Begovac et al., 1991). An overlapping region of laminin is thought to be involved in transmitting differentiation signals via the β1 integrin receptor to mammary epithelial cells (Streuli et al., 1995). Since many receptors are known to bind to this laminin domain, and since this region of laminin is not sufficient to induce mammary differentiation alone (Streuli et al., 1995), it is likely that surface GalTase serves as one component of a complex system of receptor-mediated signals that enable mammary epithelial cells to undergo differentiation. Similarly, while our results are consistent with surface GalTase mediating an interaction with the basal lamina, we cannot rule out other possible ligands for GalTase. For example, on F9 cells, surface GalTase is capable of binding laminin as well as E-cadherin and lysosomal associated membrane proteins (Maillet and Shur, 1993). Therefore, the possibility exists that mammary epithelial cell GalTase is mediating its affects via interactions with other cell-surface or matrix-associated glycoprotein(s). The observation that GalTase interacts with E-cadherin is especially intriguing,

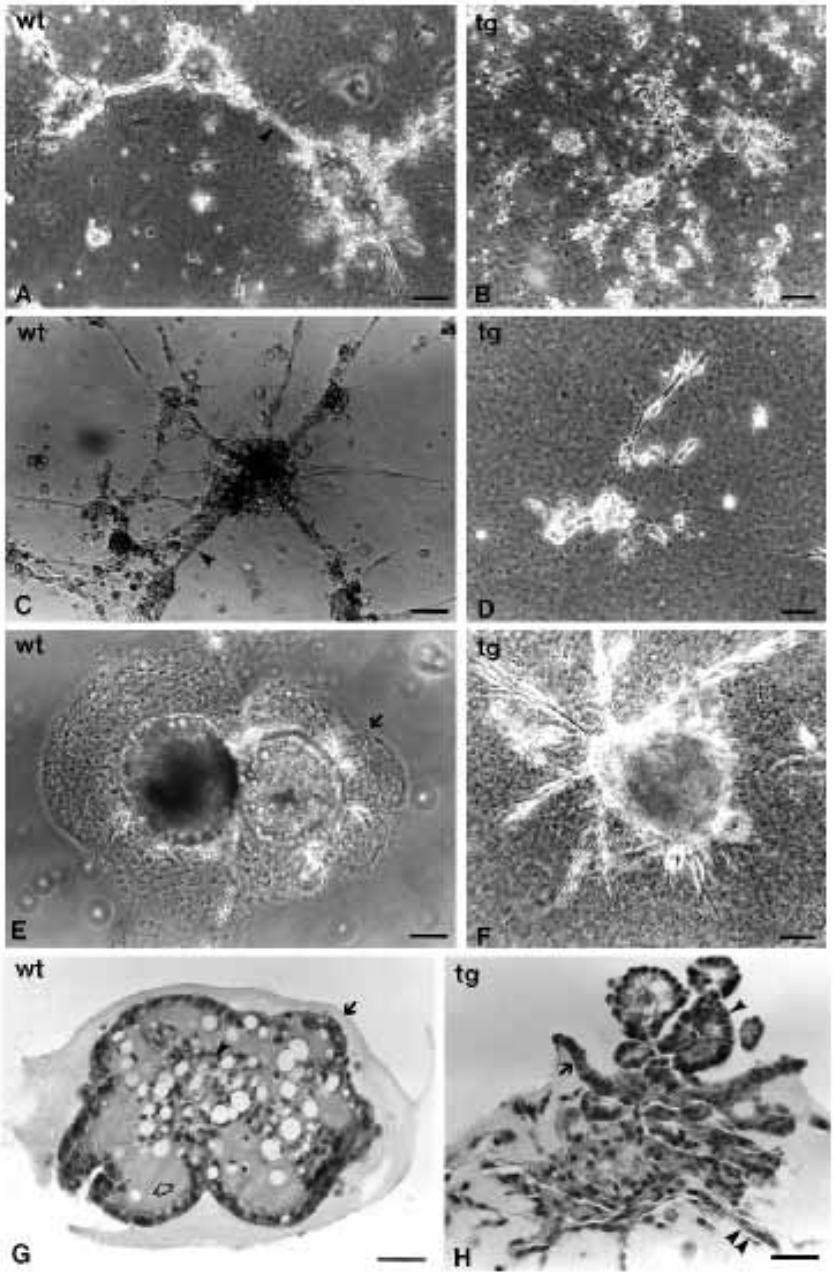

Fig. 8. Transgenic mammary epithelial cells are altered in their ability to interact with a basement membrane in culture. Day 16.0 pregnant mammary epithelial cells were prepared for culture as described in Materials and Methods. Cells were isolated either as single cells and small clusters, or as larger clusters termed spheroids. Cells were cultured in defined media on Matrigel basement membranes for a total of 6 days, and were photographed each day. (A,C) At 24-48 hours in culture, wild-type single cells and small clusters (wt) have attached, migrated and begun to form anastomosing networks and cell cords with each other (arrowheads). (B,D) In contrast, transgenic cells (tg) have attached, but showed few signs of anastomosing networks or cell cords. (E,F) All spheroids remained rounded on the basement membrane throughout the culture period and wild-type spheroids (E, wt) appeared to reorganize the basement membrane in their vicinity, as evidenced by the loss of the matrix from the plate surface (arrow). Transgenic spheroids (F, tg) did not appear to reorganize the basement membrane. (G,H) Spheroids were fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin after 6 days in culture. Wild-type spheroids (G, wt) maintained an obvious polarity, with evidence of milk accumulation in the lumen (open arrow). A number of pycnotic cells have also accumulated in the lumen (arrowhead). The Matrigel matix has surrounded the spheroid (arrow). Transgenic spheroids (H, tg) have established extensive contacts with the Matrigel (arrow), but did not become surrounded by it. Transgenic epithelial cells appeared to maintain a polarity (arrowhead), however there was little evidence of lumenal milk secretion or accumulation. Transgenic cultures were characterized by the presence of mesenchymal cells that entered and migrated within the Matrigel, possibly of epithelial origin (double arrowhead). Observations are based on mammary cultures isolated from three wild-type and four transgenic animals. (A-F) Bars, 50 μm; (G,H) bars, 25 μm.

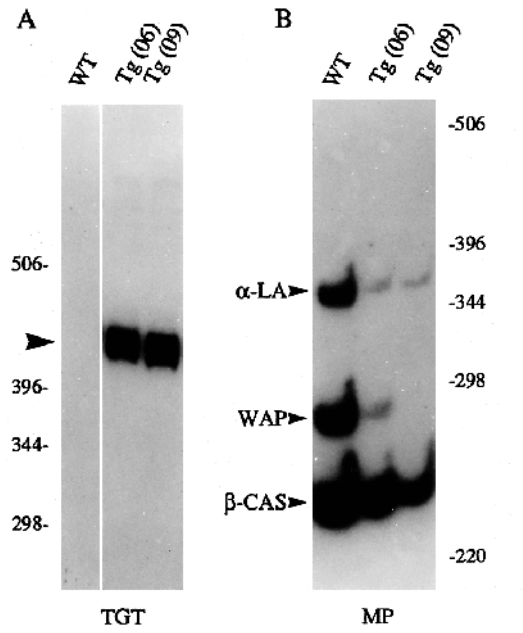

Fig. 9. Cultured mammary epithelial cells from transgenic females expressed reduced levels of milk-specific gene products. After 6 days in culture, total RNA was isolated from wild-type (WT) and transgenic (Tg (06) and Tg (09)) cells. cDNA was prepared as described in Materials and Methods, and was used in RT-PCR analysis. (A) The long GalTase transgene maintained a high level of expression in cultured transgenic mammary epithelial cells (TGT). A single band of 424 nt (arrowhead) was detected in transgenic cultures representing the GalTase transgene. (B) Milk protein gene expression (MP) remained suppressed in transgenic cells. Primer sets for α-LA, WAP and β-casein (β-CAS) detected similar differences between wild-type and transgenic milk protein gene expression to that seen in intact mammary glands (Fig. 7; Table 1). PCR product sizes are; α-LA, 377 nt; WAP, 294 nt; β-casein, 251 nt. The approximate position of molecular mass markers, in nucleotides, are shown on the left in A and on the right in B. The assay was repeated three times, with similar results.

since E-cadherin is expressed on the basolateral surfaces of mammary epithelial cells, where it mediates intercellular adhesions (Daniel et al., 1995). Finally, the substrate specificity of surface GalTase has been shown to change coincident with changes in cellular differentiation, raising the possibility that surface GalTase on mammary epithelial cells interacts with multiple ligands on the cell surface as well as in the extracellular matrix (Hathaway et al., 1989; Miller et al., 1992; Romagnano and Babiarz, 1990). In any event, it is clear that mammary gland morphogenesis involves a cascade of cellular interactions, both during primary ductal development and during alveolar differentiation. Since altering several cell surface receptors and extracellular matrix components perturbs mammary gland morphogenesis, an understanding of the interplay between cell-cell and cell-matrix interactions, and the signal transduction mechanisms that result, will be a prerequisite to understanding the morphogenesis of this organ.

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