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Title

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Targeted Expression of Stromelysin-1 in Mammary Gland Provides Evidence for a Role of Proteinases in Branching Morphogenesis and the Requirement for an Intact Basement Membrane for Tissue-specific Gene Expression

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Abstract

The extracellular matrix (ECM) is an important regulator of the differentiated phenotype of mammary epithelial cells in culture. Despite the fact that ECM-degrading enzymes have been implicated in morphogenesis and tissue remodeling, there is little evidence for a direct role for such regulation in vivo. We generated transgenic mice that express autoactivated isoforms of the matrix metalloproteinase stromelysin-1, under the control of the whey acidic protein gene promoter, to examine the effect of inappropriate expression of this enzyme. Stromelysin-1 is implicated as the primary player in the loss of basement membrane and loss of function in the mammary gland during involution. The transgene was expressed at low levels in maramary glands of virgin female mice, leading to an unexpected phenotype: The primary ducts had supernumerary branches and showed precocious development of alveoli that expressed /3-casein at levels similar to that of an early- to midpregnant gland. Lactating glands showed high levels of transgene expression, with accumulation at the basement membrane, and a decrease in laminin and collagen IV, resulting in a loss of basement membrane integrity; this was accompanied by a dramatic alteration of alveolar morphology, with decreased size and shrunken lumina containing little/3-casein. During pregnancy, expression of endogenous whey acidic protein and r-casein was reduced in transgenic glands, confirming the observed dependence of milk protein transcription on ECM in mammary epithelial cells in culture. These data provide direct evidence that stromelysin-1 activity can be morphogenic for mammary epithelial cells, inducing hyperproliferation and differentiation in virgin animals, and that its lytic activity can, indeed, disrupt membrane integrity and reduce mammary-specific function. We conclude that the balance of ECM-degrading enzymes with their inhibitors, and the associated regulation of ECM structure, is crucial for tissue-specific gene expression and morphogenesis in vivo.

The differentiated state is plastic, requiring continuous and active control for both its acquisition and its maintenance (Bisseli, 1981; Blau, 1992). In vertebrate cells in culture, there is an increasing body of evidence that the extracenular matrix (ECM) t plays a seminal role both in inducing and in maintaining the differentiated state, particularly in epithelia (Gmbstein, 1954; BisseU et al., 1982; Damsky and Werb, 1992; Adams and Watt, 1993; Hay, 1993; Juliano and Haskill, 1993), but the question of how tissue-specific gene expression is induced or maintained in vivo has not been elucidated (Schmidt et al., 1993).

Synthesis and secretion of milk proteins by cultured mammary epithelial ceils has been shown to result from a hierarchical series of events at the cellular level that involve hormones, ceU-cell interactions, and ceI1-ECM interactions (for review see Lin and Bissell, 1993). In particular, the expression of milk proteins is strongly regulated by interactions with the basement membrane. It appears that integrins may be used by mammary epithelial cells in culture for recognition of ECM and regulation of tissue-specific function (Streuli et al., 1991). Furthermore, novel enhancers that re-spond specifically to ECM have been discovered in tissuespecific genes (Schmidhauser et al., 1992).

ECM remodeling of the basement membrane has been suggested as a mechanism of altering morphogenic tissue interaction (for review see Bernfield et al., 1984). The key players in such a process are likely to be the ECM-degrading metallopmteinases (for review see Alexander and Werb, 1991; Birkedal-Hansen, 1993). In particular, the basement membrane-degrading matrix metalloproteinases (MMPs), including stromelysin-l, are implicated as primary determinants that regulate the loss of nmnmmry function during involution (Talhouk et al., 1991), because the implantation of slow-release pellets containing the tissue inhibitor of metalloproteinases (TIMP)-I delays involution (Talhouk et al., 1992).

To establish the validity of the concept that ECM regulates gene expression in vivo and to elucidate the molecular mechanisms involved, we generated transgenic mice that inappropriately express an autoactivating rat stromelysin-1 gene targeted to mammary gland under the control of the gene promoter for whey acidic protein (WAP), a milk protein that accumulates beginning in midpregnancy (Hennighausen et al., 1991). We chose rat stromelysin-1 because, first, this proteinase has a wide range of ECM substrates, including fibronectin, lamlnin, type IV collagen, and proteoglycans (Chin et al., 1985); second, it is implicated in physiological control of mammary gland function during involution (Talhouk et al., 1992); and third, autoactivating mutant constructs for the proenzyme form of stromelysin-1 have been generated and well characterized (Sanchez-Lopez et al., 1988). We chose the WAP promoter because it has been used extensively to target transgene expression to the mammary gland during midpregnancy and lactation with high fidelity and specificity (Pittius et al., 1988), thus avoiding artifactual results.

Our experimental rationale was to alter the ratio of ECM-degrading proteinases to inhibitors in favor of the proteinases in the presence of the full lactational stimulus. During pregnancy and lactation, proteinase inhibitors are more abundant than MMPs, and the gland is functionally differentiated (Talhouk et al., 1992), when the transgene should be expressed. Because the structure and biochemistry of mouse mammary gland are well understood, interpretation of mammary phenotype is straightforward, allowing subtle changes to be readily detected. The transgenic mice we have produced make it possible for us to address the effect of inappropriate

expression of these enzymes in the whole animal, where all the compensatory mechanisms are intact. We have discovered that these enzymes are morphogenic in an interactive mesenchymal-epithelial developmental system and are thus important players in the signaling events that occur between these cell types.

Materials and Methods

Mammary Tissue

Mammary tissue was obtained from normal and transgeulc CD-I mice (Charles River, Wilmin~on, MA) at various stages of development, as described previously (Talhouk et al., 1991, 1992). For all morphological analyses, left and right inguinal (number 4) mammary glands were used. All experiments were performed under protocols approved by the Animal Welfare and Research Committee, I.atwrence Berkeley Laboratory, and the Committee on Avimal Research, University of California, San Francisco.

Generation of Transgenic Mice

The CAI0:SL:MI or CA10:SL:M2 vector (see Fig. 1 A), designed to direct expression of antoactive stmmelysin-1 to the mammary gland of pregnant and lactating mice, was assembled from portions of a 7.2-kb WAP mouse genomic DNA clone. A 2.4-kb EcoRI-Kpnl fragment containing the WAP gene promoter was subcloned into the Bluescript vector (Stratagem, La Jolla, CA) together with a linker region containing internal HindIII and SaII sites. A 2.8-kb 3' untranslated region SalI fragment, which included a portion of the last coding exon, the 3' intron, and the noncoding Y exon (with the polyadenylation signal) was then litigated 3' of the SalI linker, site to form the CA10 vector. Previous studies with the WAP promoter suggest that thee regions of the gene are required for proper expression in vivo (Dale et al., 1992). Specifically mutated eDNAs for rat stsomelysin-1 (transin-1) (containing a $Val^{92} \rightarrow Gly^{92}$ or $Pro^{93} \rightarrow Val^{93}$ transition, M1 and M2, respectively), encoding an autoactivating enzyme, were subcloned as 1.8-kb inserts into the CA10 vector at the HindIII cloning site to form the CA10:SL:M1 or M2 construct. The mouse WAP genomie DNA clone was kindly provided by L. Hennighausen (Pitfius ct al., 1988), and the two mutant rat stromelysin-I eDNAs were kindly provided by R. Sanehez-Lopez and R. Breathnaeh (Sanchez-Lopez et al., 1988).

Transgenic mice were generated in the outbred CD-1 strain as described by Hogan et al. (1986). The CA10:SL:M1 and CA10:SL:M2 plasmids were purified on a Waters GenPak FAX column (Millipore, Bedford, MA), resuspended in Tris-EDTA buffer at 1-2 ng/ml, and microinjected into 1-cell fertilized eggs. The injected embryos were transferred into oviducts of recipient pseudopregnant female CD-I mice, and the offspring were analyzed for the presence of the transgene. Of the 258 eggs injected with the MI construct, 129 were transferred to recipient pseudopregnant CD-I mice, and 4 of 14 offspring contained the transgene. Likewise, of 460 eggs injected with the M2 construct, 290 were transferred, and 8 of 27 offspring contained the transgene.

DNA Analysis

To determine the relative abundance, integrity, and presence of the WAPstromelysin-1 transgene in the offspring, DNA was isolated from tail cuts, digested with EcoRI to generate the 1.8-kb insert, and analyzed by DNA blot analysis on nylon membranes (Hybond N+, Amersham Corp., Arlington Heights, IL) as described by Reddy et al. (1991). The membranes were hybridized overnight at 68°C to a random-primed ^{32}P-rediolaboled 1.8-kb rat stromelysin-1 DNA isolated from an EcoRI digest of CA10:SL:M2. The blots were washed twice for 15 rain each in 2× SSC 0× SSC ffi 0.15 M NaCl, 0.015 M sodium citrate)/0.2% SDS followed by a 30-min wash in 0.1× SSC/0.1% SDS at 68°C before exposing to XAR-5 film (Eastman Kodak Co., Rochester, NY) for fluorography at -80°C.

RNA Preparation and Analysis

Total RNA was prepared from mammary tissue or from enzymatically dissociated mouse mammary epithelial cells as described previously (Lee et al., 1985). RNA samples were resolved on 1% agarose formaldehyde gels under denaturing conditions, transferred to nylon membranes (Hybond-N), and hybridized to ^{32}P-dCTP random-primed radiolabeled mouse DNA probes. Blots with l or 2 µg total RNA were used for detection of β-casein and WAP mRNA expression, cDNAs for β-casein (Riehards et al., 1981) and WAP (Campbell et al., 1984) were kindly provided by J. Rosen, Bnylor College of Medicine, Baylor, TX, and L. Hennighausen, National Institutes of Health, Bethesda, MD, respectively. Prehybridization, hybridization, and posthybridization washes were carried out according to the method of Talhouk et al. (1992).

Reverse Transcription-Polymerase Chain Reaction

To examine the expression of the transgene, total RNA from normal and transgenic mice was purified as described previously (Talhouk et al., 1992), equal amounts of total RNA (4/µg) were treated with DNase (amplification grade; Bethesda Research Laboratories), and 1 µg reverse transcribed. Two sets of nonspecific rat/mouse stromelysin-1 primers with similar target sequences of 201 bp and 207 bp were then used to amplify 120 ng of the resulting cDNA by PCR: 5' GACAAATTCTGAGGTTTGATGAGA, 3' ACCAGCTGTTGCTCTI'CAATATGTG and 5' CTGGAGGTTrGATGAGAAGA, 3' AAACCAGCTGTTGCTCTICA, respectively. Unlike the rat stromelysin-l, mouse stromelysin-1 does not contain a Baml-H recognition sequence in this region. Equal volumes of the resulting PCR reaction products were then digested with BamI-H restriction enzyme to yield two fragments of 169-bp and 38-bp, respectively, from the rat stromelysin-1 transgene. Specific DNA fragments were resolved on a 4% agarose gel and visualized by ethidium bromide staining.

Histology and Immunocytochemistry

Whole-mount staining of the mammary gland (Medina, 1973) was performed by fixing the flattened mammary gland tissue overnight in Caraoy's solution (75 % ethanol; 25 % glacial acetic acid). After brief dehydration in 70% ethanol, the gland was stained overnight in carmine alum (0.2% carmille dye [wtJvol] and 0.5% aluminum potassium sulfate [wt/vol]). Once

dehydrated in increasing concentrations of ethanol, the gland was defatted in toluene and placed in methyl salicylate for loag-term storage. Examination and photography of the whole-mount stained glands were performed on a Wild Leitz dissecting microscope. To count end buds and determine branching frequency, calibrated slides of whole-mount glands were projected and measured.

Immunofluorescence staining for type IV collagen, laminin, and β-casein was performed on frozen sections. Mammary tissue was embedded in Tissue-Tek OCT compound (Miles Diagnostic Division, Elkhart, IN) and frozen in an ethanol/dry ice bath. Sections (4 μm) were cut with a Leitz cryotome, placed on gelatin-coated slides, and stained by immunottuorescence (Harlow and Lane, 1988) as described by Talhouk et al. 0992). Polyclonal rabbit antisera to mouse laminirl (E.Y. Labs, San Marco, CA) and type IV collagen were used at a 1:300 dilution and detected by staining with biotinylated anti-rabbit IgG (Amersbam Corp.) followed by Texas redstreptavidin, which were used at 1:50 dilution and 1:2,000 dilution, respectively, for 30 rain each at ambient temperature. For detection of β-casein, mouse monoclonal antibody to rat E-casein (gift of C. Kaetzel, Case Westera Reserve University, Cleveland, OH) was FITC-conjugeted (Harlow and Lane, 1988) and used at a dilution of 1:50. Zeiss epifluoresceoce optics and Tri-X-400 film (Eastman Kodak Co.) were used.

For stromelysin-1 immunocytochemistry, and for hematoxylin and eosin staining: paraffin-embedded sections were used. Mammary glands were fixed in 4 % paraformaldehyde, dehydrated in 30% ethanol followed by 70 % ethanol, and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin or stained for stromelysin-1 as described by Talhouk et al. 0992). Monoclonal mouse anti-human stromelysin-1 antibody (clone SL 188.2, used at 5 μg/ml in blocking buffer), a gift from S. M. Wtlhelm, Miles Research Center, West Haven, CT (Wilhelm et al., 1992), was previously shown to react with mouse stromelysin-1 (Talhouk et al., 1992). Its reaction with rat stromelysin-1 was confirmed by immunoblot analysis of conditioned medium after expressing a CMV promoter-drivan expression vector containing SL:M2 in Comma D cells (unpublished observation). Alkaline pbosphatase-conjugated streptavidin (1:100) was used to localize stromelysin-I by alkaline phosphatase substrate (ABC kit, Vector Labs., Burlingame, CA). Endogenous alkaline phospbatase activity was blocked by levamisole (Sigma Chem. Co., St. Louis, MO).

Protein Blotting

For preparation of samples, freshly isolated mammary tissue was immediately frozen in liquid nitrogen. The tissue was then pulverized to a fine powder, weighed, and either stored at -70"C or suspended 1:5 (wt/vol) in extraction buffer (1% Triton X-100, 500 mM Tris-HCl, pH 7.6, 200 mM NaCI, and 10 mM $CaCl_2$). The suspension was frozen on dry ice, thawed four times, and then centrifuged at 12,000 g for 30 rain at 4°C; the supernatant and pellet fractions were removed and stored at -70°C. Samples from the Triton-insoluble pellet fractions, containing the basement membrane components, were resolved on 12 % SDS-polyacrylamide gels under denaturing conditions for Coomassie blue staining to ensure equal loading of the samples, and on 7% SDS-polyacrylamide gels for transfer to Immobilon-P membranes (Millipore Continental Water Systems, Bedford, MA) with a dry blot apparatus (American Bionetics, Hayward, CA). Membranes were incubated overnight in a wash buffer (100 mM Tris-HC1, pH 7.5, 150 mM

NaCI, 0.3% Tween 20) containing 2% fatty acid-free BSA (Sigma Chem. Co.). The membranes were then incubated for 1 h with polyclonal rabbit anti-mouse laminin or type IV collagen antibodies (diluted 1:300 in blocking buffer) and washed three times, for 20 rain each, to remove unbound proteins. Bound antibody was detected by addition of alkaline phospbataseconjugated anti-rabbit IgG (Caltag Labs., South San Francisco, CA), followed by BCIP/NBT (bromo-chloro-indolyl phosphate/nitroblue tetrazolium; Sigma). All washings and incubations were done at ambient temperature. Extracts of ECM from the mouse Engelbreth-Holm-Swarm sarcoma were also blotted in parallel with tissue extract samples as controls for basement membrane components. Biotinylated size markers were detected with the use of alkaline phosphatase-conjugated streptavidin (Zymed Labs., San Francisco, CA).

Results

The WAP Promoter Targets the Stromelysin-1 Transgene to Mammary Gland

We constructed two plasmids, CA10:SL:M1 and CA10:SL:M2, containing 2.4 kb of the WAP promoter and 2.8 kb of the 3' untranslated region linked to the mutant stromelysin-1 cDNAs (Fig. 1 A). These regions of the WAP gene have been shown to be absolutely necessary for proper regulation and maximum expression of the endogenous mouse WAP gene (Dale et ai., 1992). The two rat stromelysin-1 cDNA constructs used were mutated in the highly conserved $P^{88}RCGVPDV^{95}$ segment near the amino-terminus of the proenzyme sequence (Sanchez-Lopez et ai., 1988; Park et al., 1991). As a result, these stromelysin-1 enzyme activities are independent of the presence of activators. The M1 and M2 mutations contain a $Val^{92} \rightarrow Gly^{92}$ or a $Pro^{93} \rightarrow Val^{93}$ transition, respectively, with the M2 mutant stromelysin-1 protein displaying faster kinetics of activation than the M1 (Sanchez-Lopez et ai., 1988). Eight SL-M2 founder mice (F0) were generated. Two of the seven surviving founder mice that showed the highest copy number, M2-5 and M2-21 (Fig. 1 B), were bred further and their offspring characterized for expression of the transgene and consequences of such expression. Four SL-M1 founder mice were generated, of which a single viable founder, M1-9, survived. The M2-5, M2-21, and M1-9 heterozygotes all had similar phenotypes (as described below).

Tissues from transgenic mice were examined by reverse transcription-PCR for tissue-specific expression of the stromelysin-1 transgene. The stromelysin-1 transgene was expressed in mammary glands of pregnant female mice (Fig. 1 C) and at low levels in brain, but not in liver, kidney, skin, or Harderian gland (data not shown). The expression of a WAP transgene in brain has been described previously (Gunzburg et ai., 1991). The transgene was not expressed in mammary glands of adult male transgenic mice. The transgenic mice expressed both the rat stromelysin-1 transgene mRNA and the endogenous mouse stromelysin-1 mRNA (Fig. 1 C). The endogenous gene was expressed at all stages of mammary gland development, although expression was by far the highest in involution (Talhouk et ai., 1992). Surprisingly, the transgene was expressed at low levels in adult (70-d-old) female virgin mammary gland. As expected from the activity of the WAP promoter, expression of the transgene increased in late pregnancy and lactation and decreased during involution, when normalized to the RNA present in the whole gland. Because total RNA and mRNA in mammary gland increase 100-fold over the course of pregnancy and another 10-fold during early lactation (Nakhasi and Quasba, 1979), we consider the total expression of the transgene to have increased at least 100-1,000-fold during pregnancy

and lactation.

Although little endogenous stromelysin-1 protein was detected by immunocytochemistry during lactation in mammary tissue from normal mice (Fig. 2 A), there was abundant stromelysin-1 protein in transgenic mammary tissue, localized to the proximity of the basement membrane surrounding the aiveoli (Fig. 2 B). By in situ hybridization, the expression of endogenous stromelysin was confined to walls of blood vessels (data not shown). These data support the conclusion that at least some of the transgenic protein is secreted toward the basement membrane. It also appeared that the activated transgene product accumulated to a greater extent than the latent endogenous gene product. Although not all areas of the gland stained uniformly for the stromelysin-1 protein (Fig. 2 B), this pattern of expression is not surprising, because endogenous expression of WAP is not uniform in the gland (Blackie, L., and M. J. Bissell, unpublished observation).

The Stromelysin-1 Transgene Behaves Like a Morphosen, Increasing Branching Morphogenesis and Stimulating Differentiation during Mammary Gland Development

During normal development, WAP mRNA is detected in pregnancy, and reaches a high level by day 16 (Pittius et al., 1988). It has been assumed that the WAP gene is transcriptionally inactive before this stage of development (Hennighausen et al., 1991); however, we detected the transgenic rat stromelysin-1 mRNA even in nonpregnant mice, albeit at a low level. Our data for stromelysin-1 RNA expression led us to examine the virgin female gland by using whole-mount preparations.

Branching morphogenesis in the mammary gland is under hormonal control of the ovary. At puberty (about 3 wk of age in mice), the mammary epithelial cells proliferate, resulting in the lengthening and branching of the ductal tree until the whole mammary fat pad is filled (Daniel and Silberstein, 1987). Glands taken from normal 70-d-old virgin females displayed the typical ductal branching pattern of postpubertal mammary glands (Fig. 3 A). However, the mammary glands of the M2-5 and M1-9 transgenic virgin females (Fig. 3, B and E, respectively) showed precocious maturation, with more branches from primary and secondary ducts filling in the spaces in the dactal network. This is similar to the morphology of a normal 9-12-day pregnant gland (Fig. 3 F). In the transgenic mice the frequency of branching, as measured by interbranch distance of the primary ducts, doubled, and the number of alveolar-like end buds increased dramatically (Table I). Examination of transgenic glands from the start of branching morphogenesis at day 35 until its completion at day 70 revealed that the transgenic virgin phenotype became apparent between day 40 and day 60, first appearing in the most mature areas of branching, away from the still actively growing areas within terminal end buds, although the exact timing differed from animal to animal (data not shown). This phenotype was not observed in the mammary glands of male transgenic mice, confirming that WAP expression depends on the presence of female hormones (Pittius et al., 1988). In histological cross-sections, the increased branching seen in the transgenic whole-mount preparations was evident as increased numbers of ducts and alveolar end buds (compare Fig. 3, C with D). The excess branching persisted into pregnancy and was readily apparent in whole-mount preparations even in midpregnancy (data not shown).

Our results suggest that the morphology of the developing gland is exquisitely sensitive to periceilular proteolysis. The increased cellularity present in the transgenic virgin gland was similar to that found in normal glands during early to midpregnancy (Fig. 3 F), suggesting that stromelysin-1, directly or indirectly, stimulates epithelial cell growth and differentiation. We investigated the possibility that the more differentiated morphology in the transgenic virgin gland had functional consequence by examining the expression of mRNA for milk proteins. RNA blot analysis showed that the glands from transgenic virgin mice, but not those from normal virgin mice, were highly induced for β-casein expression. β-casein was detected as early as day 40, in parallel with the appearance of the precocious morphological phenotype, arid reached a maximum by day 70 (Fig. 4 A). Although the animals are grouped by age, weight is also a factor in the rate of pubertal development. Thus, some morphological and functional phenotype could be observed as early as day 40 (lane 2 of transgenic), and was present by day 60 in most animals. When total RNA from glands of normal pregnant mice was analyzed, we found that the transgenic 70-d virgin glands expressed β-casein at a level similar to that present in glands from a normal 9-d-pregnant mouse (Fig. 4 B). However, neither the transgenic nor normal virgin mice expressed endogenous WAP even at day 70 of development. The inability of the transgenic virgin mice to express WAP could be a reflection of the normal developmental process because WAP is expressed at low levels prior to day 16 of pregnancy (Pittius et al., 1988) or it could reflect the presence of inhibitors of WAP synthesis (Chen and Bissell, 1989), which exist in the virgin gland (Lin, C. Q., R. J. Coffey, and M. J. Bissell. 1993. *Mol. Biol. Cell.* Suppl. 4:22a). These data suggest that the expression of β-casein mRNA correlates with the extent of branching morphogenesis.

Expression of the Stromelysin-1 Transgene Affects Expression of the Milk Protein Genes during Pregnancy

By day 14 of pregnancy, mRNA for the full complement of milk proteins, including WAP, begins to accumulate in mammary epithelial cells at very high levels, and the cells acquire a more mature phenotype, becoming lactationally competent under appropriate stimuli in culture. To determine whether the stromelysin-1 transgene affected this stage of development, we isolated total RNA from whole mammary tissue extracts of 15-d-pregnant mice. RNA blot analysis showed that mRNA for both β-casein and WAP was substantially reduced in transgenic mammary glands (Fig. 5 A), suggesting that the transgene affected the functional differentiation of the gland. Although there were equivalent total cross-sectional areas of alveoli in normal and transgenic glands (Fig. 5 C), the alveoli of the transgenic glands were slightly smaller, less differentiated in appearance, and lacking in fat droplet inclusions.

To test whether the decrease in expression of β-casein and WAP mRNA in transgenic mice was due to a loss of epithelial cells, or whether epithelial cells produced less milk protein mRNA, we isolated mammary epithelial cells from 15-d-pregnant normal and transgenic mice by enzyme digestion and separated them from other cell types such as fibroblasts and adipocytes. The concentration of both β-casein and WAP rnRNA was substantially lower in epithelial cells expressing the transgene, whereas keratin 18 mRNA, a marker for epithelial cells, was similar in normal and transgenic mice (Fig. 5 B). This reduction in milk protein mRNA in mammary epithelial cells from transgenic mice paralleled that of the whole mammary tissue from 15-d-pregnant mice. Thus, the activity of the stromclysin-1 transgene specifically affects both the

morphology of the mammary gland and the function of the mammary epithelial cells during pregnancy.

Expression of the Stromelysin-1 Transgene Affects Basement Membrane Structure and Morphology and Ptmction of the Mammary Gland during Lactation

Endogenous WAP RNA is expressed at the highest levels during lactation. To determine whether the inappropriate expression of the stromelysin-1 transgene affected the mammary gland during lactation, we examined lactating mammary glands from normal and transgenic mice. There were distinct differences in alveolar morphology and organization between the two. In the normal gland, alveoli were well rounded and surrounded large lumina (Fig. 6, A and C). In contrast, most of the alveoli in glands from transgenic mice were convoluted, smaller, and in some cases appeared to have no central lumina (Fig. 6, B and D).

Stromelysin-1 is able to degrade many of the proteins in ECM including laminin, fibronectin, type IV collagen, and proteoglycans (Chin et al., 1985). To determine whether the expression of the transgene produced any effects on basement membrane proteins, we next investigated the quantity and distribution of basement membrane proteins in mammary glands from lactating normal and transgenic mice. There was a striking reduction in the amount of ECM protein extractable from glands of lactating transgenic mice (Fig. 7). There were few or no high molecular weight bands corresponding to laminin and type IV collagen extractable from transgenic mouse glands, as compared with normal mouse glands (Fig. 7 A). By immunoblot analysis, mammary gland extracts from normal lactating mice contained abundant basement membrane laminin A and B chains and type IV collagen, whereas these proteins were decreased to 10% or less in the transgenic mice (Fig. 7, B and C). By immunocytochemistry, laminin and type IV collagen (Fig. 8, B and D), and fibronectin (data not shown) were distributed in a patchy, discontinuous manner around the small convoluted alveoli in many areas of the stromelysin-1 transgenic mouse glands, whereas continuous basement membrane protein staining was seen under the epithelial cells of the normal lactating gland (Fig. 8, A and C).

We next examined the functional consequences of the reduced basement membrane proteins in the lactating transgenic mammary gland by using β-casein as a marker of tissue-specific function. By immunocytochemistry, β-casein was primarily located in the alveolar lumina, with some cellular staining (Fig. 8, E and F). In areas of the transgenic glands in which alveoli had little imrounoreactive laminin and type IV collagen, the much reduced amounts of immunoreactive β-casein were localized to the shrunken lumina of the alveoli (Fig. 8 F). Although the intensity of β-casein immunostaining in the lumina of the transgenic gland was comparable to that in normal lactating tissue (Fig. 8 E), the cellular staining was less, and the area was considerably smaller and resembled an early involuting gland (Talhouk et al., 1992), rather than a lactating gland.

Discussion

We used transgenic techniques to directly determine the role of ECM in regulating tissue-specific function in mammary gland in vivo. As one approach to gleaning information from

the complex, interactive system of ECM components, we chose to target basement membrane generally, but in a tissue specific context. Earlier studies have shown that functional perturbation of MMP activity delays mammary gland involution: Implantation of slow-release pellets containing the MMP inhibitor TIMP-1 into involuting gland generate a halo of functional alveoli (Talhouk et al., 1992). The time course of stromelysin-I mRNA expression during involution correlates with the loss of tissue-specific function (Talhouk et al., 1992; Strange et al., 1992), and immunostaining shows that stromelysin-1 is highly expressed in myoepithelial cells (Dickson and Warburton, 1992). By in situ hybridization we also find stromelysin-1 expressed in stromal cells (Thomasset, N., C. J. Sympson, Z. Werb, and M. J. Bissell, unpublished results). Stromelysin-1 is therefore a good candidate for a functional regulator of the ECM-mammary cell interactions. We decided to overexpress this MMP under the control of a mammary-specific promoter, WAP, to study the effect that an enzyme of broad specificity has on the development and function of the mammary gland.

Nothing is known about physiological activation of prostromelysin, although the enzyme can be activated by a cascade of serine proteinases (such as plasminogen activator) and subsequently catalyze an autolytic activation in vitro (Birkedal-Hansen et al., 1993; Mignatti and Rifkin, 1993). Therefore, we chose to use the autoactivating mutant enzymes to bypass the requirement for a functional activator. Transgenic rat stromelysin-1 mRNA is expressed at a much lower level than endogenous stromelysin-1 RNA but is clearly morphogenic, suggesting that enzyme activation may be critically controlled in developing gland. Not only does stromelysin-1 have a cleavage specificity that targets many components of the ECM, but this enzyme also serves to proteolytically activate other members of the MMP family (Birkexial-Hansen et al., 1993) and is therefore likely to be key as an initiator of a cascade of MMP activity. Several other MMPs are expressed in mammary gland, notably gelatinase A (72 kD gelatinase) (Talhouk et al., 1991, 1992; Dickson and Warburton, 1992) and stromelysin-3 (Lefebvre et al., 1992).

The targeted expression of rat stromelysin-1 to mammary epithelial cells led to three distinct phenotypes that depended on the stage of development of the mammary gland: (a) excess branching and precocious development in the transgenic virgin glands; (b) reduced tissue-specific gene expression in midpregnancy; and (c) a discontinuous basement membrane and reduction in alveolar size and function during lactation. Our data provide direct evidence for the hypothesis that basement membrane remodeling is central to the regulation of mammary epithelial cell growth and function during various stages (prepuberty, puberty, gestation, lactation, and involution) of postnatal mammary development and suggest that stromelysin-1 has growth-promoting activity for virgin mammary epithelial cells in vivo.

Hyperplasia of the Transgenic Virgin Gland

The postnatal development of mammary epithelium has three distinct phases: In the virgin gland, postpubertal steroids instruct ductal expansion by stimulating the growth of cells in the bulbous terminal end buds. During the first two weeks of pregnancy, smaller side branches develop from the major ducts, their tips differentiating to form alveolar-like structures. During the final week of pregnancy, the alveolar sacs distend and synthesize lipid droplets and milk proteins (Knight and Peaker, 1982; Daniel and Silberstein, 1987; Forsyth, 1991). The virgin mouse mammary

gland is not quiescent but is subject to cyclic stimulation and regression during the estrus cycle. Elegant studies have shown that the composition of the basement membrane of human gland changes at different stages of the menstrual cycle (Ferguson et al., 1992). Some proteins (for example, collagens I, Ill, VI, and VII) appear to be stable, and others (laminin, fibronectin, tenascin, collagens IV and V) are withdrawn and resynthesized under hormonal instruction.

In the virgin female, the stromelysin-1 transgene behaved as a morphogen, inducing the ducts to branch, proliferate, and differentiate. Activation of the WAP-driven transgene is likely to derive from a short burst of activation of milk protein expression during estrus. Although we do not understand the mechanism that underlies this morphogenic property of stromelysin-1, there are various possibilities: (a) loss of a growth inhibitor (active suppression of growth by the basement membrane, or proteolytic destruction of a negative cytokine); (b) generation of a growth stimulator, perhaps by fragmentation of ECM (for example, to reveal cryptic sites on critical molecules such as laminin) or mobilization of an ECM-tethered growth factor. In support of these ideas, Zhou et al. (1993) described a hyperplastic condition of human smooth muscle cells in vivo that was linked to a deficiency of basement membrane collagen or5 (IV) and 0~6 (IV) chains. Furthermore, many classes of growth factor have been shown to bind ECM components and are likely to be immobilized there. These include fibroblast growth factor, Writ, TGF-a, and insulin-like growth factor family members (Flaumenhaft and Rifkin, 1991; Bernfield et al., 1992; Jones et al., 1993). Expression of all of these families is represented in developing mammary gland, together with receptors and effective signaling responses (Gavin and McMahon, 1992; Sllberstein et al., 1992; Fielder et al., 1992; Shackleford et al., 1993). Experiments with transgenic animals have implicated a number of these growth factors in the developmental process (Dickson et al., 1991).

The virgin transgenic glands progress only to the equivalent of midpregnancy and do not proliferate or develop beyond that stage (i.e., they do not branch further and do not express WAP). It has previously been assumed that WAP is not expressed in normal virgin gland (Pittius et al., 1988; Hennighausen, 1991). However, our results clearly indicate that the WAP promoter is active in the virgin gland albeit at a much lower level. Although β-casein is expressed in transgenic virgin mice, WAP is not detected. This indicates that despite the fact that the WAP promoter is active (hence, the expression of the transgene and branching), the endogenous WAP mRNA does not accumulate. The mechanism of WAP mRNA stability is not understood and may relate to the putative soluble inhibitors of WAP detected in tissue culture (Chela and Bissell, 1989).

Ductal hyperplasia is also observed in mice overexpressing human growth hormone, activated growth factors and their activated receptors, and protooncogenes (for review see Adams and Cory, 1991). The same pattern is seen in transgenic animals bearing an MMTV-Wnt-1 construct and in epithelial cells transfected with Wnt-1 and grown in reconstituted glands (Tsukamoto et al., 1988; Edwards et al., 1992). Wnt-1 overexpression and hyperplasia is succeeded by development of numerous mammary carcinomas. Dysfunctional morphogenesis appears to cause a significant increase in the rate of neoplastic lesions. We find that the WAPstromelysin-1 transgenic lines are also predisposed to the evolution of mammary gland tumors (unpublished observations). We suggest that stromelysin-1 may contribute to the transformation process by causing abnormal patterns of cell growth.

Reduced Tissue-Specific Gene Expression in Midpregnancy

ECM is critical to the synthesis of milk proteins by mammary epithelial cells in culture (Streuli et al., 1991 and references therein). Detailed studies have attributed the transcriptional sensitivity of β-casein milk protein to a signal derived from the interaction of laminin with its cognate receptor (Streuli et al., 1991; Streuli, C. H., C. Schmidhauser, N. Bailey, P. Yurchenco, A. Skubitz, and M. J. Bissell, manuscript submitted for publication). Laminin has also been identified as a substrate for stromelysin-1 in vitro, as has collagen IV and entactin (Birkedal-Hansen et al., 1993). The reduced amount of total laminin and collagen IV we observed by protein analysis correlated with diminished expression of milk proteins in the transgenic pregnant glands. This result would be predicted from the studies of mammary epithelial cells in culture and serves to validate these studies as a model of physiological function.

Because virgin glands show precocious development to a stage that resembles midpregnancy, and pregnant glands show diminished function, the transgene would appear to have two opposing effects on development. The switch in expression patterns of ECM and growth factors specified by the hormonal regimen characteristic of pregnancy is likely to change the growth-response characteristics of the cells, and the relative importance of any given stimulus may be completely different in the progesterone-independent and –dependent states. The differences in growth control are also implied by the very different effects that exogenous TGF-/3 has on virgin and pregnant gland. In virgin gland, TGF-β inhibits epithelial growth, stimulates mesenchymal growth, and increases ECM synthesis (Daniel et al., 1989). In pregnant gland, TGF-B has no effect on epithelial growth and does not affect matrix gene expression (Silberstein et al., 1992).

Altered Alveolar Morphology and Function in Lactation

The cross-sectional morphology of the transgenic pregnant gland was not radically different from that of the normal pregnant gland, but the lactating gland showed a clear alteration. Alveoli were smaller and appeared frequently in tangential section, suggesting that they were small spheres and unexpanded, flattened aiveoli. WAP expression increased during lactation, with a parallel increase in expression of the transgene. The role of the ECM in the final dilation, expansion, and functional differentiation of alveoli has not been explored. The functional integrity of the basement membrane may be critical at this time. The continuity of basement membrane, visualized by immunostaining, was maintained in the transgenic pregnant gland, presumably as a result of continuous synthesis and deposition of ECM components (unpublished observations). However, the integrity of the lamina lapsed in the lactating gland, apparent as fraying by in situ immunocytochemistry. At this stage, few ECM components are transcribed, and we surmise that the diminished staining revealed a loss of ECM generated by transgenedependent proteolysis. In preliminary studies, we have critically tested the role of active stromelysin-1 in generating the transgenic phenotype: we were able to reverse some of the stromelysin-1 transgenic phenotypes by crossing these mice with TIMP-l-overexpressing transgenic animals (unpublished observations).

There have been very few examples of the genetic manipulation of proteinases and inhibitors, despite their implication in both developmental and pathological processes (Alexander and

Werb, 1991; Mignatti and Rifkin, 1993). Proteinases and inhibitors also alter morphogenesis in vitro: Embryonic salivary glands and lungs cultured with either TIMP-1 or collagenase react by forming more or fewer clefts, respectively (Fukuda et al., 1988; Matrisian, 1990). Targeting of human collagenase to mouse lung by using the haptoglobin promoter generated an emphysema-like phenotype (D'Armiento et ai., 1992). Overexpression of urokinase on an albumin promoter led to a coagulation disorder but no ECM phenotype (Heckel et al., 1990). When urokinase and plasminogen activator were overexpressed on the WAP promoter, these two proteinases were secreted into milk with no reported consequences on mammary phenotype (Pittius et al., 1988; Hennighausen et al., 1991). Thus, direct tests of the role of ECM and proteinases have been very limited. Our experiments allowed us to examine in molecular detail how stromelysin-1 works both as a morphogen to generate diverse phenotypes at various stages of mammary gland development and as a regulator of gene expression by disrupting the integrity of the basement membrane.

Acknowledgments

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Figures and Tables

FIGURE 1

Figure 1. (*A*) Schematic diagram of the WAP genomic DNA clone and the CA10:SL:M1 or M2 construct (containing the mutant rat stromelysin-1 1.8-kb cDNA insert {M1 or M2} flanked by the 2.4-kb WAP promoter and the 2.8-kb WAP 3' untranslated region). Restriction sites were described by Campbell et al. (1984) and confirmed for this study: *B*, BamHI; *R*, EcoRI; *H*, HindIII; *K*, KpnI; *P*, PstI; *S*, SalI; *Xb*, XbaI; *Xh*, XhoI. (*B*) Southern blot analysis of DNA from transgenic mice. 10 μg of tail DNA from transgenic founder (F0) mice was digested with EcoRI, transferred to a nylon membrane, and hybridized to a random primed ³²P-labeled rat stromelysin-1 cDNA. The 1.8-kb band is indicative of the stromelysin-1 DNA fragment. Asterisks indicate the two lines, M2-5 and M2-21, that were characterized and used in these studies. (*C*) Expression of the stromelysin-1 transgene in transgenic mice. RNA (4 μg) from 70-d virgin (*V*), 15-d-pregnant (*P*), 8-d-lactating (*L*), and 9-d-lactating/2-d-involuting (*I*) transgenic mice was treated with DNase and reverse-transcribed, and 120 ng was amplified by PCR with nonspecific rat/mouse stromelysin-1 primers. Equal amounts of the resulting amplified cDNA were digested with BamHI, resolved on a 4% agarose gel, and visualized by ethidium bromide staining. Although the primers amplified both endogenous mouse and transgenic rat stromelysin-1 sequences, the rat sequence contained a BamHI restriction enzyme site, which upon cleavage generated a 169-bp fragment and a 38-bp fragment. The 169-bp fragment was used to indicate the presence of the rat stromelysin-1 transgene. The endogenous mouse stromelysin-1 was not cleaved. Total RNA from rat (*R*) and mouse (*M*) involuting mammary glands was used as a positive and a negative control, respectively. For additional controls, the reaction was performed without RNA (−) or with total rat RNA (+) but without reverse transcription. Two different samples were analyzed for 15-d-pregnant (*P*), 8-d-lactating (*L*), 9-d-lactating/2-d-involuting (*I*), and the negative control (−). Molecular weight (×10⁻³) markers are indicated on the left.
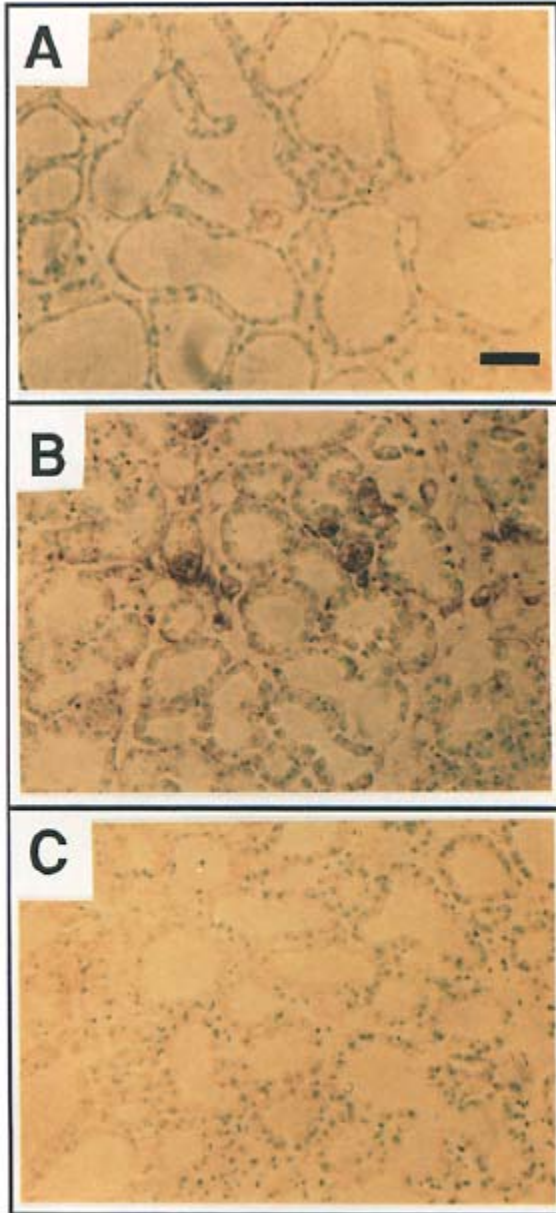
FIGURE 2

Figure 2. Immunohistochemical localization of stromelysin-1 in mammary tissue from 8-d-lactating mammary glands. (*A*) Normal mammary tissue incubated with human anti-stromelysin-1 monoclonal antibody. (*B*) Transgenic (*M2-5*) mammary tissue incubated with anti-stromelysin-1 monoclonal antibody. (*C*) Transgenic (*M2-5*) mammary tissue incubated without the primary stromelysin-1 antibody. Stromelysin-1 protein present in transgenic lactating mammary glands but not in normal glands is evident as abundant deposits mainly around small convoluted alveoli in the proximity of the basement membrane. In the normal gland a small amount of staining is seen around a small blood vessel near the center of the field. Bar, 50 μm.
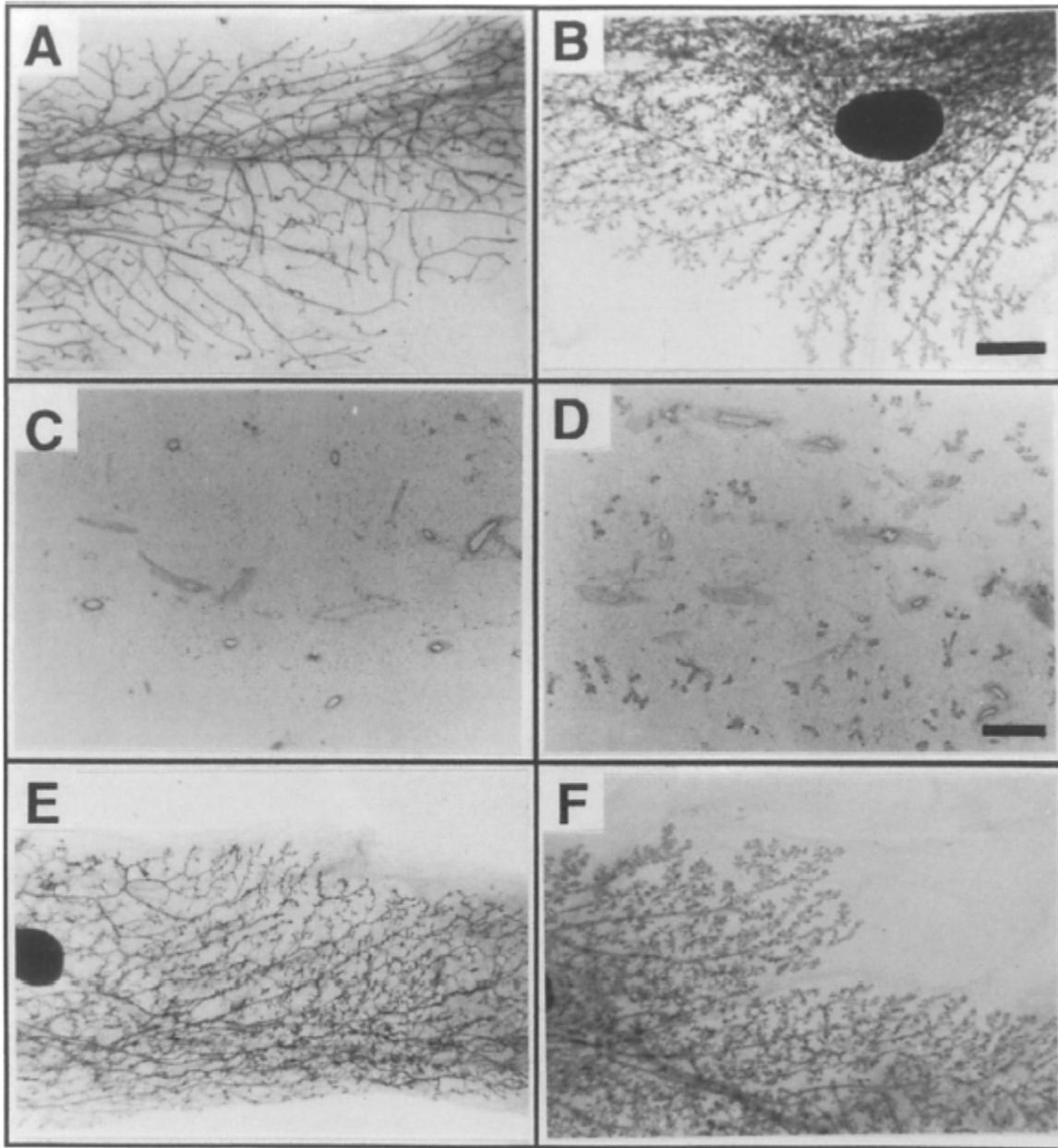
FIGURE 3

Figure 3. Structure of normal and transgenic mouse mammary glands during development. Whole-mount (*A, B, E,* and *F*) and hematoxylin-eosin-stained sections (*C* and *D*) of normal and transgenic mouse mammary glands are shown. (*A*) Normal 70-d virgin, (*B*) transgenic 70-d virgin (*M2-5*), (*E*) transgenic 72-d virgin (*M1-9*), and (*F*) normal 12-d-pregnant mammary glands. The dense oval structures seen in *B* and *E* are lymph nodes. The transgenic virgin mammary glands are highly branched and resemble the morphology of the normal 12-d-pregnant glands. This increased branching morphology is evident by comparing the hematoxylin and eosin-stained sections of normal virgin gland (*C*) with those of M2-5 transgenic gland (*D*). Bar, 250 μm.

FIGURE 4

Figure 4. Expression of β-casein in normal and transgenic virgin mouse mammary glands during development. RNA (1 μg) from (A) mammary tissue extracts of normal and transgenic virgin mice at various stages during development or from (B) mammary tissue extracts of normal pregnant mice was blotted and hybridized with cDNA probes for β-casein and WAP. The ethidium bromide-stained 28S band of total rRNA is shown as a control.
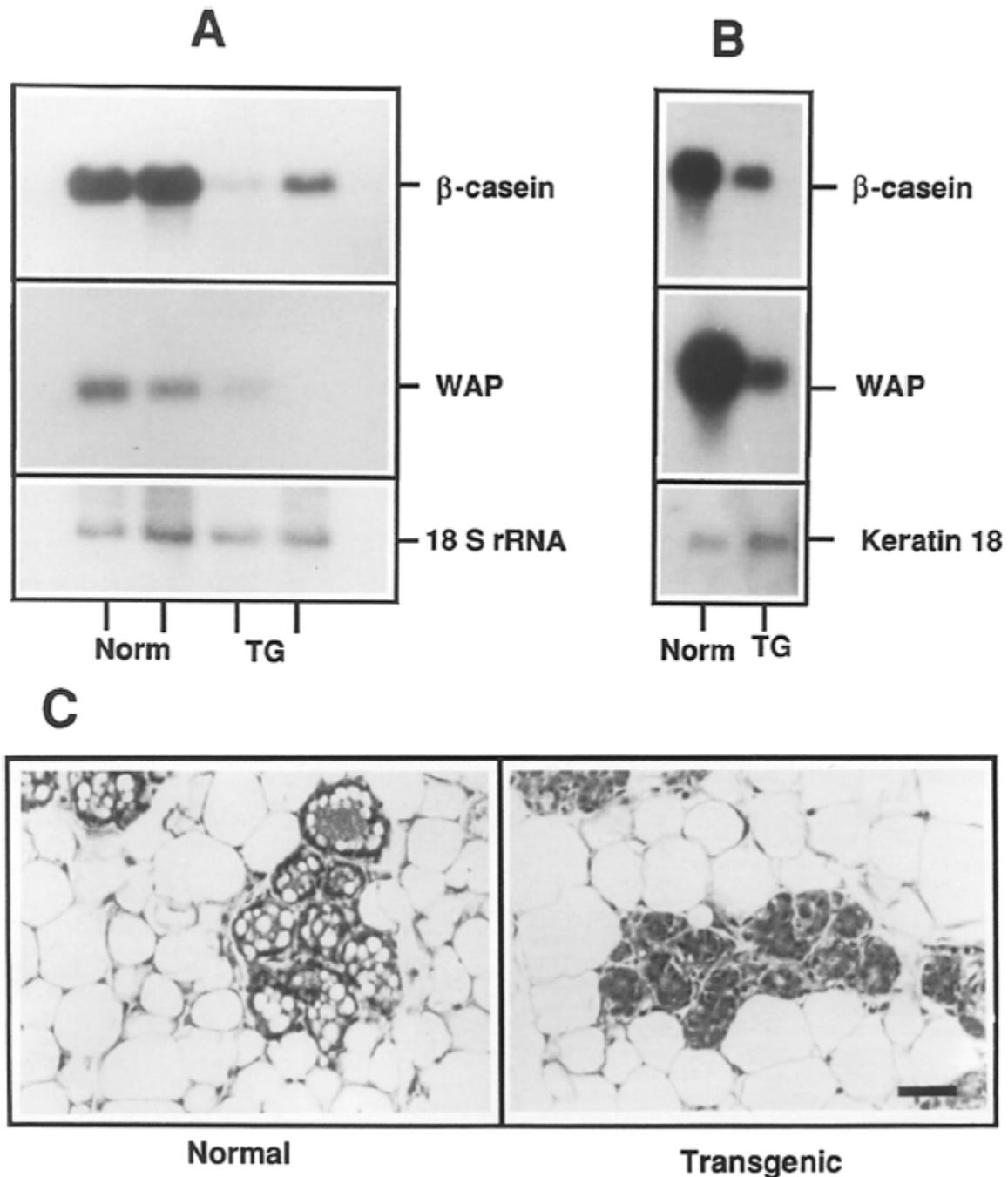
FIGURE 5

Figure 5. Morphology and expression of β-casein and WAP mRNA in mammary glands of 15-d-pregnant normal mice (*Norm*, two lanes from different mice) and M2-5 (*TG, left lane*) and M2-21 (*TG, right lane*) transgenic mice. (*A*) RNA blot (2 μg) from mammary tissue extracts of 15-d-pregnant mice. (*B*) RNA blot (2 μg) from mammary epithelial cells isolated from 10 pooled mammary glands of 15-d normal and of M2-5 and M2-21 transgenic (*TG*) pregnant mice. (*C*) Hematoxylin-eosin-stained sections of normal and transgenic mammary glands from 15-d-pregnant mice. Bar, 50 μm.
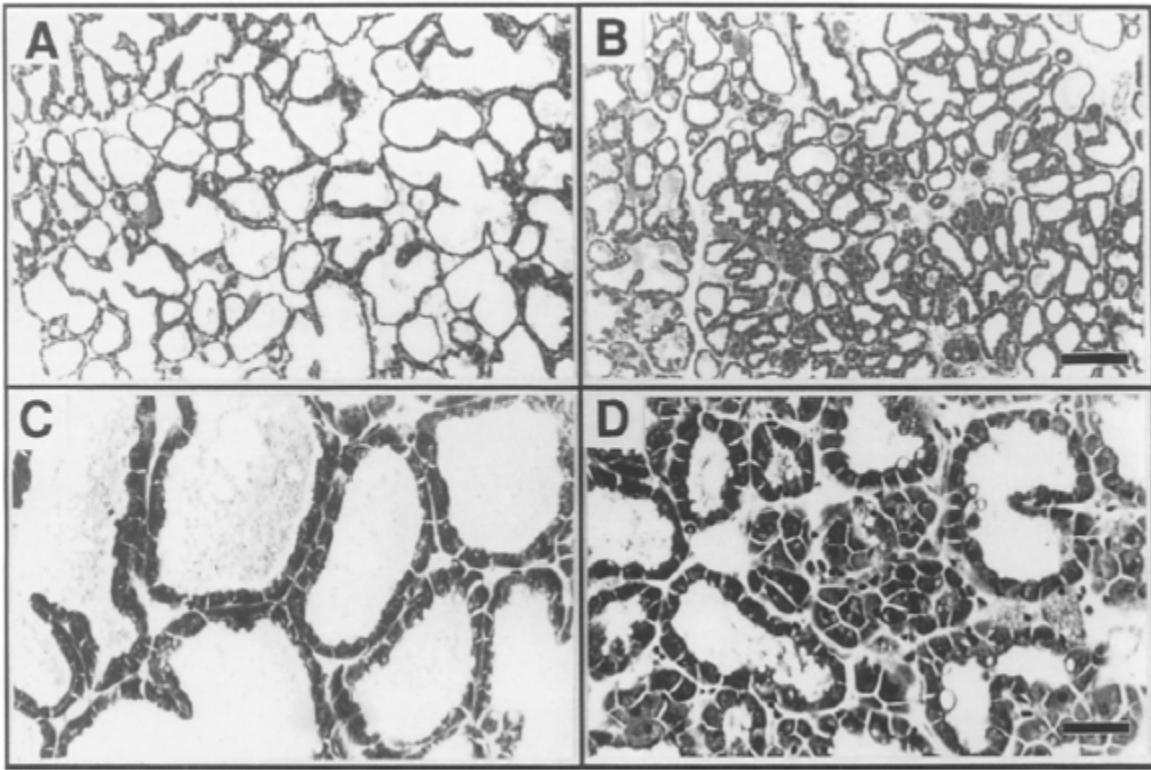
FIGURE 6

Figure 6. Histological appearance of 8-d-lactating mammary tissue from normal and transgenic (M2-21) mice. Paraffin sections were stained with hematoxylin and eosin. Compare the well-rounded alveoli with abundant lumina in normal mice (*A* and *C*) with the numerous convoluted, small alveoli in transgenic mice (*B* and *D*). Bars: (*A* and *B*) 100 μm; (*C* and *D*) 25 μm.

FIGURE 7

A Coomassie

92
66
45
31
21

Normal TG

B Laminin

A
B
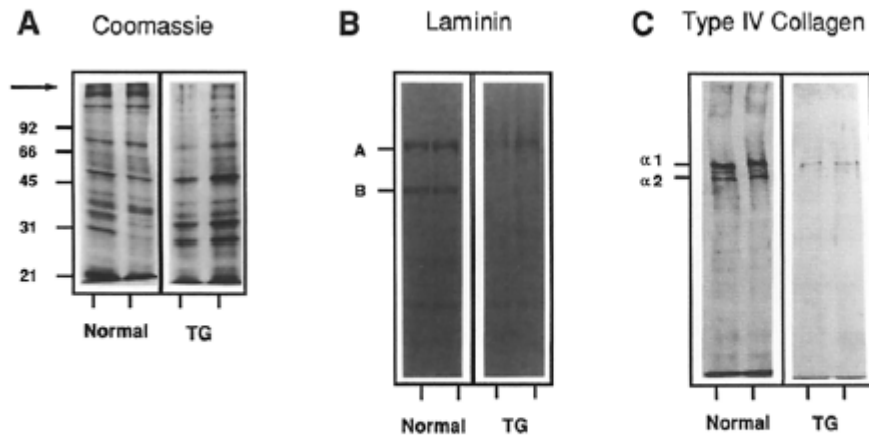
Normal TG

C Type IV Collagen

α1
α2

Normal TG

Figure 7. Basement membrane proteins in mammary glands of transgenic and normal mice. (*A*) Extracts of mammary tissue from normal and M2-5 transgenic (*TG*) lactating mice were resolved on 12% SDS-polyacrylamide gels. The arrow points to the high molecular weight proteins, most of which are components of the basement membrane and ECM components. Molecular weight ($\times 10^{-3}$) markers are also shown. (*B* and *C*) Immunoblot analysis of mammary tissue extracts shown in *A*. Proteins were blotted onto Immobilon-P membranes and incubated with rabbit polyclonal antibodies to mouse laminin (*A* and *B* chains) (*B*) or type IV collagen (α1 and α2) (*C*), followed by incubation with alkaline phosphatase-conjugated anti–rabbit IgG. Tissue extract samples were obtained from two different 8-d-lactating transgenic mice; normal tissue extract samples were obtained from 1-d-lactating mice. The protein pattern seen in normal mice was indistinguishable at 1 and 8 days of lactation.
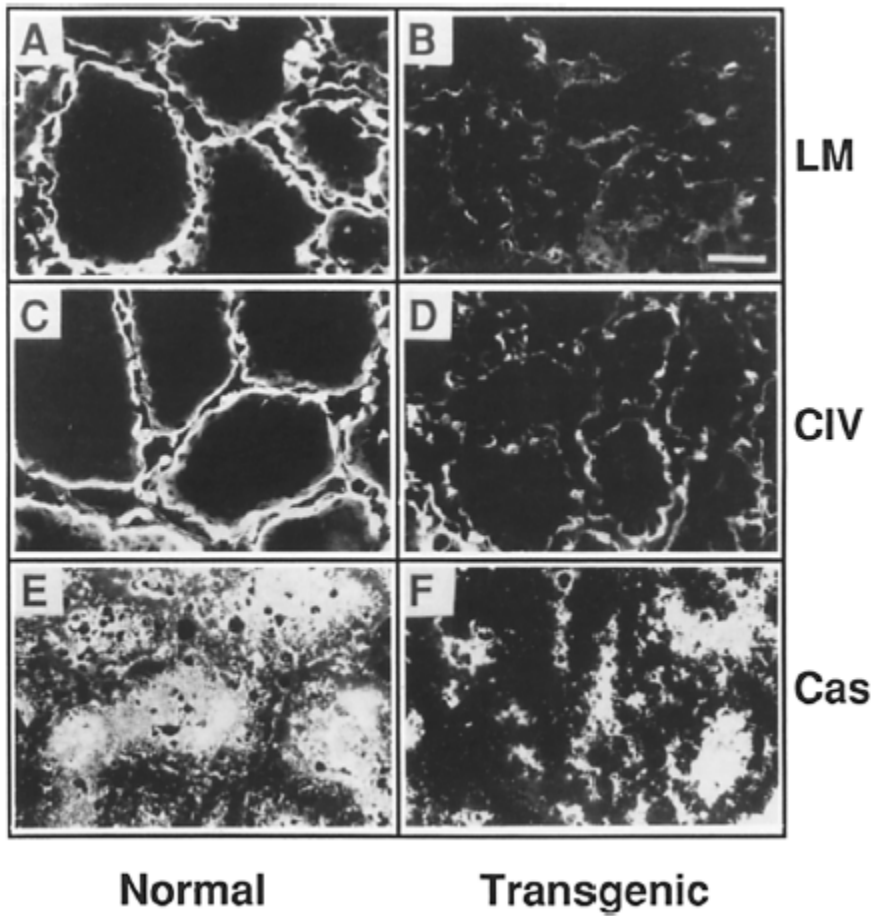
FIGURE 8

Figure 8. Immunolocalization of laminin, type IV collagen, and β-casein in lactating mammary glands of transgenic and normal mice. Photomicrographs of (*A* and *B*) laminin (*LM*), (*C* and *D*) type IV collagen (*CIV*), and (*E* and *F*) β-casein (*Cas*) were localized by immunofluorescence in 8-d-lactating mammary glands in normal mice (*A*, *C*, and *E*) or M2-21 transgenic mice (*B*, *D*, and *F*). Note the patchy distribution of LM and CIV in the basement membrane of many areas of the transgenic mice, compared with the continuous distribution of both of these components in normal tissue. β-casein was localized in luminal areas in both normal (*E*) and transgenic (*F*) glands, but much more β-casein was present in the normal gland. Bar, 50 μm.

TABLE 1

Table I. Branching Morphogenesis in Virgin Mammary Gland of Stromelysin-1 Transgenic Mice

Mice	Age (days)	Interbranch distance* (μm)	Number of alveolar-like buds per 10^4-μm^2 area of gland
Normal	60	56.1 ± 3.7	7.2 ± 2.6
Transgenic[†]	60	33.4 ± 2.3§	27.8 ± 6.9§
Normal	70	77.3 ± 4.0	7.8 ± 4.1
Transgenic[†]	70	32.2 ± 2.3§	42.9 ± 16.8§

For each determination, four mice were used, and eleven 10^4-μm^2 areas of each inguinal gland were scored from whole-mount preparations. Values are expressed as mean ± SD.
* Distance between secondary branches on primary ducts.
† Results from SL-M2-5 and M2-21 transgenic lines were pooled.
§ For all comparisons between normal and transgenic mice, the differences were significant ($p \leq 0.0005$; Student's t test).