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Matrix Metalloproteinases Are Expressed during Ductal and Alveolar Mammary Morphogenesis, and Misregulation of Stromelysin-1 in Transgenic Mice Induces Unscheduled Alveolar Development

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The matrix-degrading metalloproteinases stromelysin-1, stromelysin-3, and gelatinase A are expressed during ductal branching morphogenesis of the murine mammary gland. Stromelysin-1 expression in particular correlates with ductal elongation, and in situ hybridization and three-dimensional reconstruction studies revealed that stromelysin-1 mRNA was concentrated in stromal fibroblasts along the length of advancing ducts. Transgenic mice expressing an activated form of stromelysin-1 under the control of the MMTV promoter/enhancer exhibited inappropriate alveolar development in virgin females. Ultrastructural analysis demonstrated that the basement membrane underlying epithelial and myoepithelial cells was amorphous and discontinuous compared with the highly ordered basal lamina in control mammary glands. Transgenic mammary glands had at least a twofold increase in the number of cells/unit area and a 1.4-fold increase in the percent of cycling cells by 13 wk of age compared with nontransgenic littermates. In addition, transgenic glands expressed β-casein mRNA, but not protein, and resembled the proliferative and differentiated state of an animal between 8 and 10 days pregnant. An analysis of metalloproteinase expression in the glands of normal pregnant females demonstrated that the same matrix metalloproteinase family members, including stromelysin-1, were expressed in connective tissue cells surrounding epithelial clusters during the time of lobuloalveolar development. These results suggest that metalloproteinases may assist in remodeling ECM during normal ductal and alveolar branching morphogenesis, and that disruption of the basement membrane by an activated metalloproteinase can affect basic cellular processes of proliferation and differentiation.

INTRODUCTION

The development of mammary ductal structures involves a complex interplay between epithelium and mesenchyme that is dependent upon circulating hormones for stimulation and synchronization with reproductive events, but which is also influenced by local factors to provide signals that influence glandular growth, differentiation, and morphogenesis. Substantial remodeling of the extracellular matrix (ECM) accompanies structural changes in the mammary gland during ductal development, lactation, and involution, and recent work has identified a potential role for a family of ECM-degrading enzymes, the matrix metalloproteinases (MMPs), in these reorganizational events (Talhouk *et al.*, 1991, 1992; Sympson *et al.*, 1994).

The MMPs are secreted, zinc-containing enzymes that degrade extracellular matrix components at a neutral pH (Alexander and Werb, 1991; Matrisian, 1992; Birkedal-Hansen *et al.*, 1993 for review). The prototypic member of the family, interstitial collagenase, is the only enzyme known to degrade fibrillar collagens, thus suggesting that these proteases are important regulatory molecules in matrix turnover and remodeling. Other family members have much

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broader substrate specificities. The stromelysins (stromelysin-1, stromelysin-2, and matrilysin) degrade basement membrane collagens, proteoglycans, and matrix glycoproteins. Gelatinase A and B rapidly cleave denatured collagens, as well as basement membrane collagens and elastin. The substrate for stromelysin-3 is not well defined, although a truncated form of the enzyme has activity against substrates similar to that observed for other MMP family members (Murphy *et al.*, 1993).

The tight regulation of most of the MMP family members, which can occur at the level of gene transcription, enzyme activation, and the balance between MMPs and their natural inhibitors the TIMPs (Matrisian, 1990, 1992 for review), suggests that these enzymes play critical roles in normal biological processes. MMPs have been shown to be expressed at the time of menstruation (Marbaix *et al.*, 1992; Rodgers *et al.*, 1994), mammary gland and uterine involution (Blair *et al.*, 1986; Woessner and Taplin, 1988; Lefebvre *et al.*, 1992; Strange *et al.*, 1992; Talhouk *et al.*, 1992), and wound healing (Stricklin *et al.*, 1993; Saarialho-Kere *et al.*, 1994). In addition, high levels of MMP expression are associated with inflammatory diseases such as arthritis and periodontis, and in malignant neoplasms associated with tumor invasion and metastasis (Brinckerhoff, 1991; Birkedal-Hansen *et al.*, 1993; Stetler-Stevenson *et al.*, 1993 for review). Synthetic inhibitors of MMPs have been shown to be effective in altering tumor growth (Davies *et al.*, 1993), angiogenesis (Galardy *et al.*, 1994), TNF-α processing (Gearing *et al.*, 1994; McGeehan *et al.*, 1994) and organ morphogenesis (Nakanishi *et al.*, 1986).

To better understand the role of MMPs in normal biological processes, we used the murine mammary gland as a model system. Several MMPs, including stromelysin-1, were expressed during ductal and alveolar morphogenesis. Transgenic mice overexpressing an active form of stromelysin-1 were utilized to provide insights into possible roles for MMPs in mammary gland morphogenesis, proliferation, and differentiation.

MATERIALS AND METHODS

cDNA Probes

To identify the expression of various MMP family members, the following cDNA probes specific for each murine MMP were utilized: a 532-bp *Kpn*I/*Xho*I (+719 to +1251) fragment of mouse stromelysin-1 (Ostrowski *et al.*, 1988); a 730-bp fragment of mouse stromelysin-2 representing nucleotides that encode the Zn^{+2} binding site to the C-terminal region of the protein (Gack *et al.*, 1994); a 1045-bp *Pvu*II/*Pst*I (+992 to +2037) fragment of mouse stromelysin-3 (Lefebvre *et al.*, 1992); a 700-bp *Apa*I/*Hin*dIII fragment of mouse matrilysin amplified by 3'RACE polymerase chain reaction (PCR) (Wilson *et al.*, 1995); an 815-bp *Kpn*I/*Bam*HI (+1646 to +2462) fragment of mouse collagenase amplified by PCR of mouse genomic DNA using the primers 5'-CGCGAATTCACCACCACCACACACA-3' and 3'-CGCGGTACCTTGTTATCCTTCCTG-5' (Henriet *et al.*, 1992); a 965-bp *Pvu*II/*Pst*I (+822 to +1786) fragment of mouse gelatinase A amplified by reverse transcriptase (RT)-PCR from RNA isolated from NIH3T3 cells using the primers 5'-GGTGGCAATGCTGATGGACA-3' and 3'-TTGGTTCTCCAGCTTCAGGT-5' (Reponen *et al.*, 1992); a 835-bp *Eco*RI/*Kpn*I (+2267 to + 3102) fragment of mouse gelatinase B that was PCR amplified from mouse genomic DNA using the primers 5'-CGCCCATCCTGATTCTTGCGTGCTA-3' and 3'-CGCGGTACCACATGGTGGAGCACAA-5' (Tanaka *et al.*, 1993); and a 350-bp Sau3A/Sau3A (+189 to +520) fragment of mouse TIMP-1 (Edwards *et al.*, 1986). For identification of stromelysin-1 transgene expression, a 492-bp *Bgl*II/*Hin*cII (+1127 to +1619) fragment corresponding to sequences in the 3' region of the rat stromelysin-1 cDNA (Matrisian *et al.*, 1985) was utilized. Casein mRNA was detected with a 540-bp *Hin*dIII/*Bam*HI fragment of a mouse β-casein cDNA (Richards *et al.*, 1981) obtained from Dr. Mina Bissell. The cDNA inserts were ^{32}P-radiolabeled by random priming to a specific activity of 10^9 cpm/μg and utilized for Northern hybridization as described (McDonnell *et al.*, 1991). For in situ hybridization analyses, these cDNAs were subcloned into pGEM7zf(+) (Promega, Madison, WI) and utilized as templates for the generation of ^{35}S-labeled riboprobes.

Generation of Transgenic Mice

The constructs pMMTV-P/V-STR1 and pMMTV-V/G-STR1 were created by first replacing the SV40 promoter in the expression vector pKCR3 (Matrisian *et al.*, 1986) with the complete mouse mammary tumor virus long terminal repeat (MMTV LTR). Full length rat stromelysin-1 cDNAs with or without mutations resulting in a proline to valine substitution at amino acid 93 or a valine to glycine substitution at amino acid 92 (Park *et al.*, 1991) were inserted into the *Eco*RI site of the rabbit β-globin exon 3. *Xho*I fragments were purified by CsCl centrifugation and microinjected into (C57BL × DBA)F1 fertilized eggs as described (Hogan *et al.*, 1986). Transgenic founder animals were identified by Southern blot analysis of *Eco*RI-digested tail DNA hybridized with the full length stromelysin-1 cDNA (Matrisian *et al.*, 1985) under high stringency conditions. The number of copies of transgene DNA that integrated into the genome was determined by comparing the relative intensity of the hybridization signal to control DNAs containing 1 and 10 genome equivalents of the same DNA that was injected. Transgenic lines were generated by mating founder animals to (C57/BL6 × DBA)F1 males and females.

Northern Hybridization

Two micrograms of poly(A)+ RNA, isolated over an oligo dT cellulose column from total RNA samples, were separated on a 1.2% agarose-formaldehyde gel and transferred to nitrocellulose membrane as described (McDonnell *et al.*, 1991). Blots were probed with cDNAs specific for each MMP family member under conditions of high stringency (50% formamide, 5× SSC for hybridization; 0.1 × SSC wash at 50°C). Filters were stripped by boiling in water before hybridizing with different probes. Hybridization with the endogenously expressed cyclophilin gene (1B15) (Danielson *et al.*, 1988) was used to determine if the RNA was intact and if roughly equal amounts were loaded in each lane.

Whole Mount Staining of Mammary Glands

Inguinal and thoracic mammary glands were removed, fixed in 4% paraformaldehyde overnight, defatted in 100% acetone, and stained with iron hematoxylin (0.1% w/v hematoxylin, 0.1 M $FeCl_3$, 0.17N HCl in 95% EtOH) for 3 h (Medina, 1973). Whole mount glands were destained in 0.025N HCl in 50% EtOH, and stored in methyl salicylate indefinitely.

In Situ Hybridization

Paraffin-embedded formaldehyde-fixed sections of mammary tissue (5 μm) were placed onto Superfrost Plus slides (Fisher Scientific,

Pittsburgh, PA) and analyzed for MMP expression by in situ hybridization by a modification of the method described by Smith *et al.* (1990). The slides were prehybridized for 2–4 h and ^{35}S-labeled riboprobes specific to the 3′ regions of the various MMPs (1.2×10^6 cpm/slide) were added and hybridized overnight at 55°C as previously described (McDonnell *et al.*, 1991). The slides were dipped in photographic emulsion, developed, and counterstained with hematoxylin after a 2- to 3-wk exposure at 4°C. No hybridization above background was observed with the sense probes.

Three-dimensional Reconstruction of Mammary Ductal Structure

Five-micron serial sections of virgin wild-type mammary tissue were processed for in situ hybridization with an antisense probe specific for mouse stromelysin-1. Cross-sectional profiles of a mammary duct and its corresponding stromelysin-1 hybridization pattern were generated using a Bioquant computer image-based analysis system (R&M Biometrics, Nashville, TN). Profiles were filled solid utilizing Adobe Photoshop (Adobe Systems, Apple Computer) and the three-dimensional surface was rendered using Voxel View (Vital Images, Fairfield, IA) on a Silicon Graphics workstation.

Antibody Generation and Immunohistochemistry

Rat stromelysin-1 protein was isolated from the conditioned medium of Chinese hamster ovary cells transfected with the cDNA encoding full length rat stromelysin-1 as previously described (Matrisian *et al.*, 1986). Antibodies were generated by the repeat injection of purified protein into rabbits, and the polyclonal antiserum obtained was affinity-purified against the first 173 amino acids of recombinant mouse stromelysin-1 protein as previously described (Wright *et al.*, 1994).

Paraformaldehyde-fixed, paraffin-embedded sections were dewaxed, hydrated through graded ethanols, treated with 15% acetic acid in 1× TBS (to block endogenous phosphatase activity), exposed to blocking solution (10 mM Tris-Cl, pH 7.4, 100 mM $MgCl_2$, 0.05% Tween 20, 1% bovine serum albumin, and 5% fetal calf serum) for 1 h, and incubated at room temperature overnight in blocking solution with the affinity-purified anti-stromelysin antibody (1:200 dilution). The sections were washed with 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20, incubated with alkaline-phosphatase–conjugated secondary antibody for 3 h at room temperature, and visualized using nitro blue tetrazolium and 5-bromo-4-chloro-O-3-indolyl phosphatase colorimetric substrates (Promega). A 0.1% sirius red solution was utilized as a counterstain to visualize matrix components (Lopez-De Leon and Rojkind, 1985).

Tissues were similarly processed with an antibody to mouse casein (1:5000 dilution; kindly provided by Dr. Charles Daniel, University of California at Santa Cruz) (Robinson *et al.*, 1993), and an antibody to mouse vimentin (1:5000 dilution; ICN ImmunoBiologicals).

Ultrastructural Evaluation of Mammary Tissue

Mammary tissues from one control and two transgenic mice were dissected into small pieces (<2-mm cubes) and fixed in 3% glutaraldehyde buffered to pH 7.4 with 0.1 M sodium cacodylate. After several rinses in buffer solution, samples were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated, and embedded in Embed 812 (Electron Microscopy Sciences, Ft. Washington, PA). Sections 1–1.5μm in thickness were stained with alkaline toluidine blue for light microscopic examination and blocks were trimmed to include one or more of the smallest components of the duct system. Thin sections were stained with uranyl acetate and lead citrate before examining in a Hitachi H-800 electron microscope. Two or more samples from each animal were thoroughly examined and representative areas were photographed.

Morphometric Assessment of Mammary Glands

Inguinal and thoracic mammary glands of mice at various stages in juvenile development were removed, fixed in 4% paraformaldehyde, and routine 5-μm sections were stained with hematoxylin and eosin. These histological sections, obtained from similar regions of the inguinal mammary glands of three wild-type and transgenic animals at each age time point, were utilized for morphometric analysis. The number of epithelial cells present in each mammary tissue section was scored in a blind study by perimeter estimation using a Bioquant computer image-based analysis system (R&M Biometrics). Perimeter values for epithelial elements were quantitated utilizing Bioquant and divided by the basal width per cell to determine the number of epithelial cells/field. The average epithelial cell width (either wild type or transgenic) was determined to be 6 μm. Small and medium-sized epithelial elements were analyzed in 10 fields (1000 μm^2/field) situated adjacent to the characteristic lymph node in each gland. Values for very large, longitudinally sectioned ducts were excluded. The average number of cells/1000 μm^2 field was expressed as the mean ± standard deviation, and statistically compared using Student's *t*-test for paired samples.

BrdU Cell Proliferation Analysis

Before analysis of cell proliferation rates, three animals per group were synchronized with respect to their estrus cycle by the intraperitoneal (I.P.) injection of pregnant mare serum (5 international units [I.U.]) followed 40 h later by human chorionic gonadotropin (5 I.U.) to override ovarian hormones and induce superovulation in female mice (Hogan *et al.*, 1986). 5-bromo-2-deoxyuridine (BrdU) was injected I.P. at a dosage of 75 mg/kg into wild-type and V/G STR1 line 5 transgenic female mice of various ages in development. After exactly 2 h, the mice were killed, and the right third thoracic and fourth inguinal glands were removed for BrdU labeling analysis. Tissues were fixed in 4% paraformaldehyde, processed, and 3-μm sections were denatured in 2N HCl for 20 min at 37°C, neutralized in boric acid/borate buffer, and then exposed to trypsin (20 ng/ml) at 37°C for 3 min. BrdU was detected using a 1:400 dilution of a rat monoclonal antibody specific for BrdU (Accurate Chemicals, Westbury, NY) and avidin-biotin complex immunohistochemistry (Vector Labs, Burlingame, CA).

RESULTS

Expression and Localization of MMPs in the Developing Mouse Mammary Gland

The expression of the mRNA for members of the matrix metalloproteinase family was examined during the branching development of the juvenile virgin mouse mammary gland by Northern blot analysis. Of the metalloproteinases tested, the mRNA for stromelysin-1 and gelatinase A were the most abundant (Figure 1). These transcripts were highly expressed between 5 and 10 wk of development, when the ducts are actively growing and branching. The mRNA levels for these MMPs decreased at 13 wk of age and were expressed at low levels in the virgin adult. Very low levels of stromelysin-3 mRNA were also detected during early times (5–7 wk) of branching morphogenesis in the murine mammary gland (observable with a longer exposure of the gel depicted in Figure 1). At the level of sensitivity achieved with this technique, there was no detectable mRNA for stromelysin-2 (Figure 1),

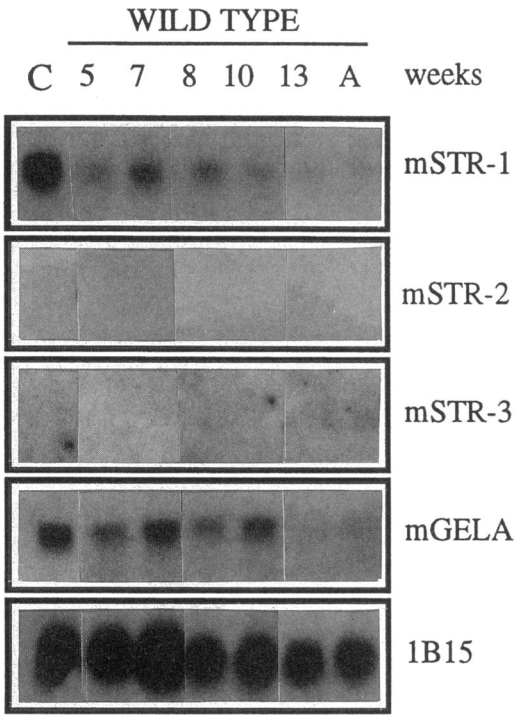

Figure 1. Expression of metalloproteinase mRNA in the virgin mouse mammary gland. Poly A(+) mRNA (2 μg) was isolated from mammary glands of virgin wild-type female mice at the indicated ages of virgin development and analyzed for the presence of the following MMP family members: murine stromelysin-1 (mSTR-1), stromelysin-1 (mSTR-2), stromelysin-3 (mSTR-3), and gelatinase A (mGELA). A, adult; C, control. For control, poly A(+) RNA (2 μg) was isolated from tumors made in nude mice by subcutaneous injection of a transformed rat fibroblast cell line (B77) known to produce rat stromelysin-1 mRNA (Matrisian *et al.*, 1985). Hybridization with a probe for cyclophilin (1B15) was used as a loading control.

matrilysin, collagenase, gelatinase B, or TIMP-1 in juvenile or adult virgin mammary glands.

Using in situ hybridization with probes that distinguish between the different MMP family members, the mRNA for stromelysin-1, gelatinase A, and to a lesser extent stromelysin-3, were localized to cells in the mammary stroma (Figure 2). Stromelysin-1–expressing cells appeared to be clustered around many of the ductal elements sectioned both longitudinally and in cross-section, (Figure 2, A, B, D, and E) as well as located in stromal cells in more isolated positions within the mammary fat pad (Figure 2E). The expression of gelatinase A was similar but appeared to be more widespread, i.e., extended a greater distance from the epithelial structures (Figure 2, G and H). Stromelysin-3 expression, in contrast, was localized to stromal cells immediately adjacent to occasional ductal elements (Figure 2, F and G). TIMP-1 mRNA was detectable at low levels by in situ hybridization in stromal cells immediately adjacent to an occasional ductal unit (Figure 2I); the low quantity of TIMP-1 mRNA expressed was apparently below the level of detection by Northern blot analysis.

To confirm expression of MMPs in developing mammary tissue, metalloproteinase activity in gland extracts was assessed by zymography utilizing gelatin gels. Eight-week mammary tissue displayed activity at a molecular weight of ~68 kDa, and this band disappeared with the addition of the metal chelator EGTA in the incubation medium (our unpublished results). Gelatinase-A activity was therefore confirmed in developing mammary tissue, although similar analyses with α-casein as the substrate failed to reveal additional metalloproteinase activity. Because zymography with α-casein is less sensitive than gelatin zymography, we analyzed for the expression of stromelysin-1 protein by immunohistochemistry using an affinity-purified antibody to stromelysin-1. Positive immunostaining was observed in stromal fibroblast-like cells surrounding epithelial elements and in isolated stromal cells, consistent with the pattern observed by in situ hybridization (Figure 3A). These results indicate that both 72-kDa gelatinase A and stromelysin-1 protein were produced in developing mammary glands, although they do not provide information regarding the activation state of the enzymes. Areas of substrate degradation were present in α-casein gels in both the presence and absence of EGTA (our unpublished results), suggesting that other proteases may also be present and play a role in juvenile mammary gland development.

Cells expressing high levels of stromelysin-1 were identified as fibroblasts in adjacent sections processed for in situ hybridization and immunohistochemistry with an antibody to mouse vimentin (our unpublished results). These findings are in contrast to the myoepithelial cell expression of stromelysin-1 protein in involuting rat mammary tissue reported by others (Dickson and Warburton, 1992; Li *et al.*, 1994). The reason for this difference is not clear, although differences in reagents and conditions may contribute to the variability in the findings. Although we cannot rule out the possibility that levels of stromelysin-1 mRNA and protein below the level of detection of our assays are present in myoepithelial cells, the co-localization of mRNA and protein by these techniques suggest that stromelysin-1 is produced by fibroblastic cells in the mouse mammary gland.

The spatial pattern of stromelysin-1 expression along an epithelial duct was analyzed utilizing 3D-imaging techniques. Sequential 5 μm sections of mammary tissue, analyzed for in situ hybridization with a stromelysin-1-specific riboprobe and counterstained with hematoxylin, were utilized for the reconstruction. At 6 wk of age, stromelysin-1 mRNA was observed only in isolated stromal cells, and was not associated with ductal elements, including multicellu-

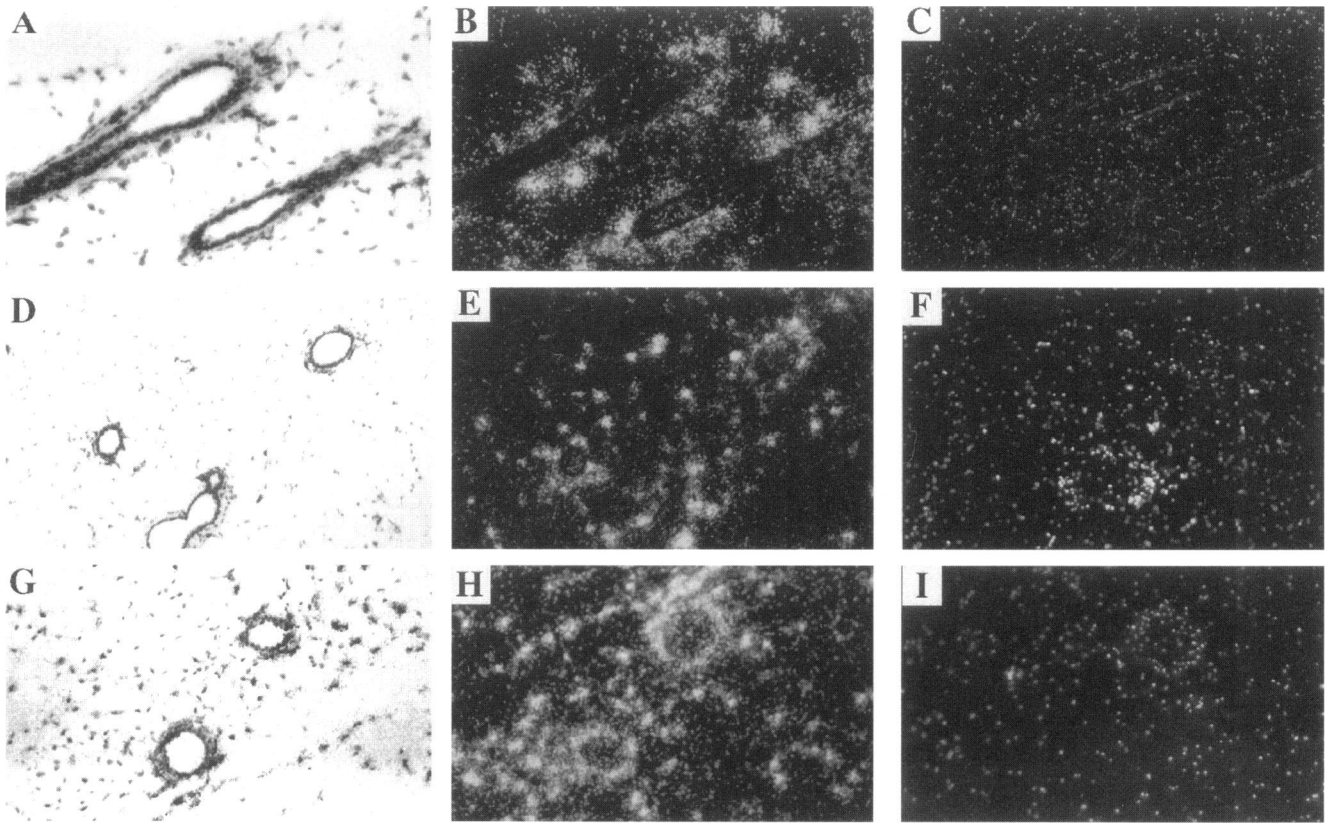

Figure 2. Localization of metalloproteinase mRNA in the virgin mouse mammary gland by in situ hybridization. Sections of 10-wk old virgin mammary gland tissue were analyzed by in situ hybridization using either the antisense (A, B, and D–I) or sense (C) probes for mouse stromelysin-1 (A–E), stromelysin-3 (F), gelatinase A (G and H), and TIMP-1 (I). Photographs were taken using a ×20 objective with brightfield (A, D, and G) or darkfield (B, C, E, F, H, and I) illumination. The images shown in A, B, and C, in D and E, and in F–I are from identical or adjacent sections. The sense control for the stromelysin-1 probe (C) is representative of sense control hybridization for all probes used in this study.

lar elements indicative of terminal end buds. At 8 and 13 wk of age, abundant expression of stromelysin-1 in stromal cells surrounding ductal elements was observed. The structures depicted in Figure 4 represent two views of a medium-sized duct from a 8-wk old virgin female mouse that divides into two branches that move away or toward the viewer. Stromelysin-1–expressing cells are nonuniformly present in the stroma along the entire length of these ducts. Areas that have more intense staining for stromelysin-1 mRNA, in general, have more stroma and stromal cells, although not all stromal cells stain positively for stromelysin-1 mRNA. In this same mammary gland, a medium sized duct that ended in a single layer of epithelial cells and a thin rim of stroma in which there was no stromelysin-1 mRNA detected was observed. This duct was presumed to represent a more mature duct, in that the multicellular, highly proliferative terminal end bud region had apparently regressed to a single, quiescent cell population. These results are consistent with the observation that total stromelysin-1 mRNA expression as measured by Northern blot analysis decreases as the animals reach adulthood when ductal branching subsides (Figure 1).

MMTV-Stromelysin-1 Transgenic Mice

The previous studies demonstrate that stromelysin-1 and gelatinase A mRNA and protein is highly expressed in the stroma surrounding developing mammary ductal structures. We found the expression of stromelysin-1 to be of particular interest, because this MMP is not widely expressed in fetal or adult normal tissues, in contrast to the expression pattern of gelatinase A (Reponen *et al.*, 1992; Wright and Matrisian, unpublished data). To further investigate potential roles for stromelysin-1 in mammary morphogenesis, we have utilized a transgenic mouse model system that allows for overexpression of this MMP in the mammary gland during periods of high ductal branching activity (5- to 10-wk of age). Transgenic mice expressing rat stromelysin-1 under control of the MMTV LTR were generated by injecting one-cell embryos with DNA fragments from either pMMTV-P/V-

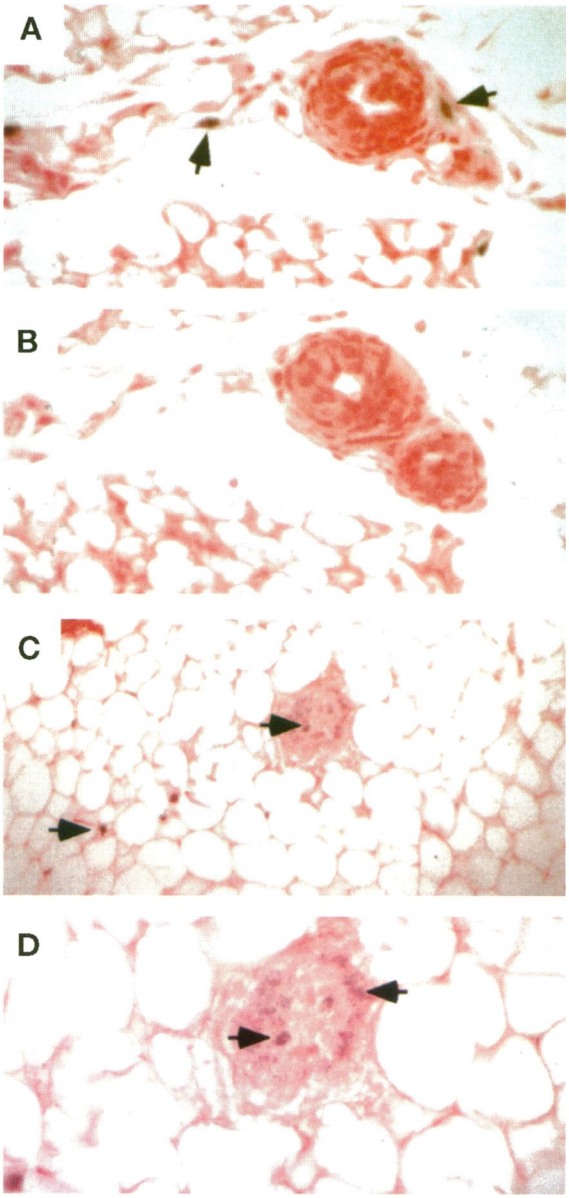

Figure 3. Stromelysin-1 protein in cells of wild-type and transgenic juvenile mammary glands. Immunohistochemical analysis of wild-type (A and B) and transgenic (C and D) mammary tissue at 12 wk of age utilizing an affinity-purified antibody recognizing rodent stromelysin and sirius red counterstain that preferentially stains collagenous matrix proteins. Representative cells staining positive with the stromelysin antibody are indicated with arrowheads. (B) No primary antibody control. Photographs were taken with a ×20 (A–C) or ×40 (D) objective.

STR1 or pMMTV-V/G-STR1. The vector utilized for both constructs contains exons two and three of the rabbit β-globin gene and thus provides a splice site upstream of the rat stromelysin-1 cDNA (Figure 5A). The stromelysin-1 sequences contain a mutation in the nucleotide sequence encoding the PRCGVPDV region, which is highly conserved among MMPs and important for controlling enzyme latency (Figure 5B; Birkedal-Hansen *et al.*, 1993 for review). The proline[93] to valine or the valine[92] to glycine alteration results in the production of the active form of the stromelysin-1 protein (Sanchez-Lopez *et al.*, 1988; Park *et al.*, 1991), thus circumventing any dependence upon enzyme activation by exogenous factors. The expression of stromelysin-1 protein from these constructs was tested by transfection into the human breast cancer cell line Hs578t. The production of both the ~58-kDa and ~48-kDa forms of the enzyme, indicative of the autoproteolysis of the proenzyme to the mature form, was detected by immunoprecipitation.

A total of four founder mice injected with pMMTV-P/V-STR1 DNA and two founders injected with pMMTV-V/G-STR1 DNA were generated (Figure 5C). Four transgenic mouse lines were established; P/V-STR1 lines 23 and 25 contained approximately 33 and 20 copies of the pMMTV-P/V-STR1 fragment, respectively, and V/G-STR1 lines 5 and 7 contained approximately 25 and 7 copies of the pMMTV-V/G-STR1 DNA fragment, respectively. Females from all of these lines have been able to suckle their young successfully. Results presented are from P/V line 23 mice unless stated otherwise, and are representative of the phenotype observed in the two P/V and the two V/G lines.

Tissue Specificity of Transgene Expression

Transgene expression was assayed by hybridizing poly(A)+ RNA extracted from various tissues from male and female mice with a probe specific for the 3′ region of rat stromelysin-1. This probe does not hybridize under high stringency conditions to mouse stromelysin-1 or to rat stromelysin-2 sequences, which show approximately 90% identity to rat stromelysin-1 (Breathnach *et al.*, 1987; Ostrowski *et al.*, 1988). Transgene expression was detected in the mammary glands, salivary glands, and brain of 10-wk old transgenic female mice in addition to brain, salivary gland, and testes in 10-wk old males (Figure 6; our unpublished observations). In virgin female mammary glands, the expression of the transgene was detected by Northern blot analysis at 7 wk of age, peaked between 8–13 wk, and declined in the adult (representative experiment shown in Figure 6). No signal was detected when the rat stromelysin-1 probe was hybridized to mRNA isolated from mammary glands of wild-type females. mRNA from other tissues in the male and female mice (i.e., kidney, liver, stomach, and lung) did not express either the rat stromelysin-1 transgene or endogenous stromelysin.

In addition to expression of the stromelysin-1 transgene, mRNAs for mouse stromelysin-1, stromelysin-3, and gelatinase A, but not matrilysin, collagenase, ge-

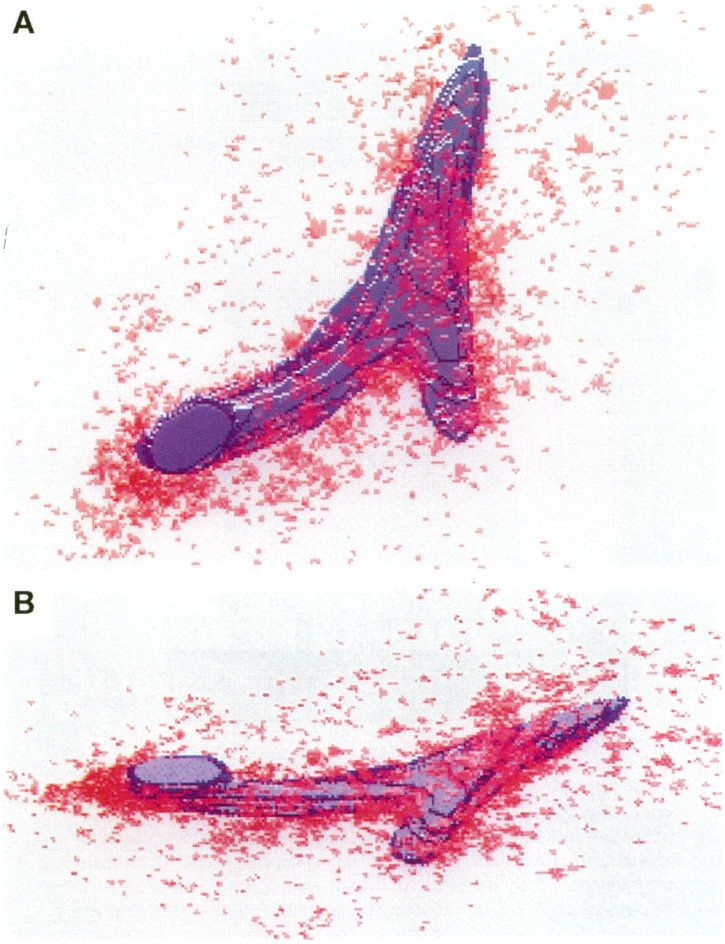

Figure 4. Three-dimensional reconstruction of a branching mammary duct. Serial 5-μm sections representing 100 μm of an inguinal mammary gland from an 8 wk virgin B6D2F1 mouse were processed for in situ hybridization with an antisense probe to mouse stromelysin-1 and a three-dimensional surface rendering was accomplished using Voxel View. The ductal epithelium is depicted in blue and the in situ hybridization signal is depicted in red. (A) The most vertical duct is a medium-to-large-sized duct extending from the general direction of the nipple, which branches into two smaller ducts that extend away from the viewer (the shorter branch) or horizontally and toward the viewer (the longer branch). Note that stromelysin-1 mRNA is observed at the cleft point. (B) The same duct viewed at an oblique angle to demonstrate that cells expressing stromelysin-1 mRNA surround the ducts.

latinase B, or TIMP-1, were identified in transgenic juvenile virgin mouse mammary tissue (Figure 6). Interestingly, the levels of mRNA for these MMP family members appeared to be higher in the transgenic animals than in wild-type control littermates (compare similar time points in Figures 1 and 6). This suggests that the production of the stromelysin-1 transgene by the mammary epithelium induces the expression of these endogenous MMPs.

In sections of mammary tissue from virgin transgenic females, mRNA for the rat stromelysin-1 transgene was localized in the epithelial cells in small and medium-sized glandular elements and ductal structures by in situ hybridization (Figure 7). No hybridization was observed in the larger ducts. Low levels of hybridization were observed in stromal cells surrounding epithelial units; it is likely that this hybridization represents cross-reactivity with the endogenous mouse stromelysin-1 under the conditions used for the in situ hybridization, although the same probe shows no evidence of cross-hybridization by Northern blot analysis (Figure 6). When the transgenic tissue was analyzed with the probe specific for mouse stromelysin-1, the endogenous expression was confined to cells in the stromal component of the mammary matrix, as previously demonstrated. The expression of stromelysin-1 protein was analyzed by immunohistochemistry using an antibody that recognizes both mouse and rat stromelysin. Stromelysin-1 immunoreactivity was detected in epithelial cells as well as stromal cells of 8- to 9-wk old virgin female transgenic mice (Figure 3, C and D), compared with the stromal expression of stromelysin protein in wild-type littermates (Figure 3A). Western blot analysis using the same antibody indicated that there was at least a threefold increase in the level of stromelysin protein in mammary gland extracts from transgenic as compared with nontransgenic littermates (our unpublished results).

Effects of Overexpression of Activated Stromelysin-1 on Mammary Gland Development

Whole-mount preparations of the mammary glands from wild-type (Figure 8, A, C, and E) and transgenic (Figure 8, B, D, F, and G) mice from 8 to 13 wk after

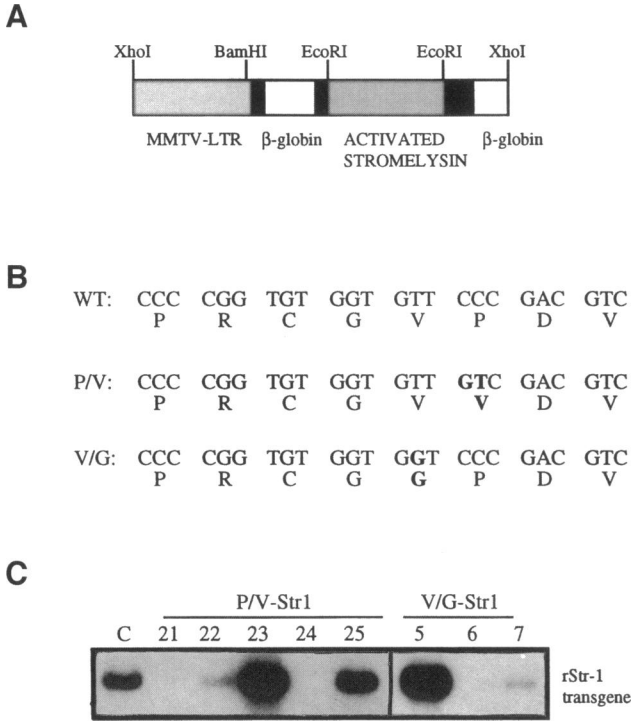

Figure 5. Transgenic mice expressing stromelysin-1 under the control of the MMTV LTR. (A) Structure of the MMTV-stromelysin-1 construct. The light gray region corresponds to 1.5 kb of the MMTV LTR (drawing not to scale). The black (filled) region corresponds to the second and third exons of the rabbit β-globin gene. The white regions correspond to the second intron and 3′ flanking region of the β-globin gene. A 1.2-kb rat stromelysin cDNA (dark gray region) was inserted into the third exon of the β-globin gene. Pertinent restriction enzyme sites are indicated. (B) Wild-type (WT) and mutant rat stromelysin-1 cDNA sequences. cDNA sequence information showing the nucleotide changes (in bold) that convert the proline at position 93 to valine (P/V = the pMMTV-P/V-STR1 construct) and valine at position 92 to glycine (V/G = the pMMTV-V/G-STR1 construct). Amino acids are indicated using the single letter code, and mutations are indicated in bold. (C) Southern hybridization of 10 μg genomic DNA from founder animals (#22, 23, 25, 5, and 7) and wild-type littermates (#21, 24, and 6) utilizing a 1.2-kb full length ^{32}P-labeled rat stromelysin-1 cDNA probe. C = 10 μg of genomic DNA from a normal mouse with 130 pg (10 genome equivalents) of pMMTV-P/V-STR1 DNA.

birth revealed a striking alteration in the developmental pattern of the terminal end regions of the glands in transgenic virgin females of all lines analyzed. Both the thoracic and inguinal glands of multiple F2 generation, heterozygous animals were analyzed at each time point. Ductal elongation and development of the initial branching structure appeared to be the same in wild-type and transgenic animals (Figure 8, compare E and F), with the mammary epithelium growing to fill the entire mammary fat pad (Figure 8G). However, the terminal regions of transgenic glands exhibited multiplicity of budding beginning at 8 wk of age (Figure 8, compare A and B) and the formation of alveolar-like structures by 12 and 13 wk of age (Figure 8, D and F, respectively), indicating an alteration in normal end-branching activity similar to that seen in early pregnant animals (Figure 8H, wild-type gland, 10 days pregnant).

The transgenic mammary gland appeared to have an increased number of epithelial cells compared with an age-matched nontransgenic animal (Figure 8, compare E and F). The number of epithelial cells per unit area in a defined region of wild-type and transgenic glands was quantitated morphometrically (Table 1A). The average number of cells/unit area decreased with age in the control animals as the growing and branching tips of the ducts extended past the characteristic lymph node located near the nipple in the inguinal gland. In contrast, the number of epithelial cells in the transgenic animals did not decrease but rather increased to 13 wk of age, reflecting the alveolar-like proliferation occurring at this time throughout the

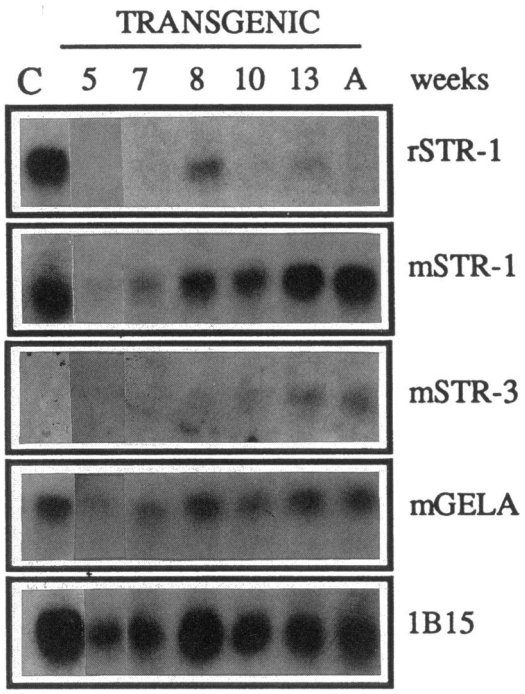

Figure 6. Northern blot analysis of transgenic mouse mammary gland RNA. Northern hybridization of 2 μg of poly A(+) RNA from P/V-STR1 line 23 transgenic female mammary glands utilizing ^{32}P-labeled cDNA probes specific for rat stromelysin-1 (rSTR-1), mouse stromelysin-1 (mSTR-1), mouse stromelysin-3 (mSTR-3), mouse gelatinase A (mGELA), and cyclophilin (1B15, loading control). The Northern blot depicted in Figure 1 represents samples processed on the same filter and with identical exposure times so that MMP levels can be directly compared. C = control; 2 μg of poly A(+) RNA isolated from tumors made in nude mice by subcutaneous injection of a transformed rat fibroblast cell line (B77) known to produce rat stromelysin-1 mRNA (Matrisian *et al.*, 1985) and that is an identical reproduction of the signal shown for this control in Figure 1 to facilitate comparison of relative MMP levels.

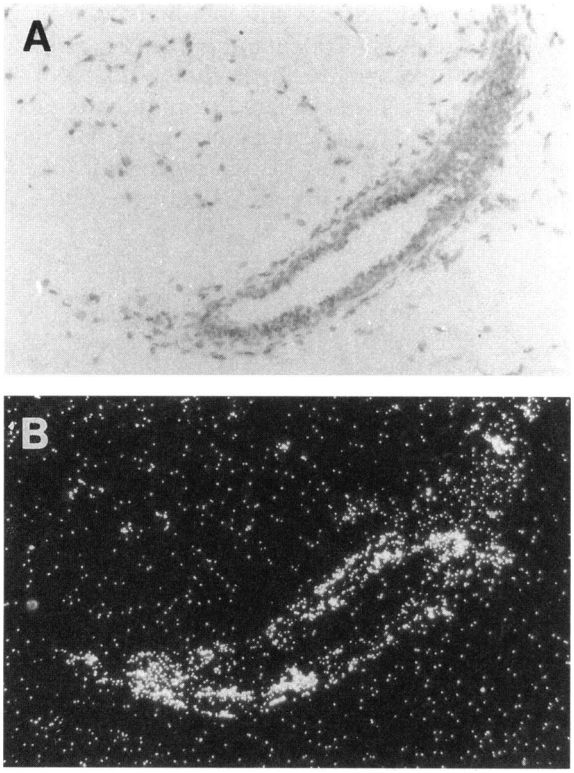

Figure 7. Localization of endogenous mouse and transgenic rat stromelysin-1 in transgenic mouse mammary tissue by in situ hybridization. Adjacent sections of 8 wk P/V-STR1 line 23 transgenic mammary tissue were hybridized with the antisense probe for rat stromelysin-1. The photographs shown were taken using either brightfield (A) or darkground (B) illumination with a ×20 objective.

gland. At 13 wk of age, transgenic animals had at least twice the number of epithelial cells/unit area compared with wild-type controls ($p < 0.001$).

To determine if the increased number of epithelial cells in the transgenic animals was the result of increased alveolar proliferation, the percentage of cycling cells was determined by bromodeoxyuridine incorporation into the mammary glands of hormonally synchronized mice (Table 1B). The percent of labeled nuclei in control animals at 7–9 wk of age was 17–18%, decreasing with time to 12% at 13 wk of age, and is consistent with previously published values (Sapino *et al.*, 1990). The transgenic animals had a slightly higher proliferation index at early times, and 1.4 times the number of labeled nuclei at 13 wk ($p < 0.01$). It is interesting that the increase in proliferative index correlates better with the increase in the levels of endogenous metalloproteinases than the levels of transgene expression, the latter which peaks earlier but apparently initiates a gradual increase in stromal stromelysin-1, in particular, that parallels the increase in proliferative index.

Histological evaluation confirms the presence of multiple budding structures in transgenic animals consisting of a single epithelial cell layer, which is characteristic of alveolar buds in mammary epithelium during early pregnancy (Figure 9, A–C). These structures are much simpler than those observed during ductal elongation, in which the growing terminal end bud consists of multiple layers of epithelium and a dense stromal cap (Williams and Daniel, 1983).

To determine if the mammary epithelial cells were functionally as well as morphologically differentiated, the expression of the milk protein casein was analyzed. Northern blot analysis of transgenic and wild-type mammary tissue revealed the expression of β-casein mRNA in 13 wk transgenic mammary glands and not in age-matched controls (Figure 9D). However, no detectable casein protein was produced in the alveolar-like structures produced by the virgin stromelysin-1 transgenic females between 8 and 13 wk as analyzed by immunohistochemistry using an antibody to mixed casein proteins (our unpublished results). In the normal pregnant mammary gland, β-casein mRNA expression was detectable as early as 8 days of pregnancy (Figure 9E), and low levels of casein protein production were detected in the epithelium of a 10-day pregnant female (our unpublished results). These results suggest that the histological phenotype observed in our 13-wk transgenic epithelium corresponds to the differentiation state of mammary epithelium present in a normal gland of an animal that is between 8 and 10 days pregnant.

The most striking feature of the mammary tissue at the ultrastructural level was the disorganized appearance of the transgenic mammary basal lamina (Figure 10). Matrix components immediately adjacent to transgenic epithelial and myoepithelial cells appeared amorphous and at points discontinuous when compared with the highly ordered basal lamina structure present in wild-type control tissue (Figure 10, compare C and D). This disruption of the mammary basement membrane was observed in all the epithelial structures examined in transgenic mice, although the glandular elements were not uniformly affected as the membrane surrounding approximately 30% of each element appeared more normal and continuous. No such discontinuities were observed in any of the normal glandular structures examined. Subtle alterations in laminin, IV collagen, and perlecan immunostaining were also observed in the MMTV-STR1 glands during juvenile development (our unpublished results). To our knowledge, this observation represents the first direct ultrastructural evidence of basement membrane disruption by a matrix metalloproteinase in vivo.

Expression of Metalloproteinases in Early Alveolar Morphogenesis

The stimulation of inappropriate alveolar development in STR1 transgenic mice combined with the localization of endogenous stromelysin-1 around growing and branching ducts during juvenile mammary gland development suggests that stromelysin-1 might be involved in both ductal and lobuloalveolar branching morphogenesis. To examine the expression of MMP family members during early lobuloalveolar development, mRNA was isolated from female mice between 2 and 12 days of pregnancy and analyzed for MMP mRNA expression by Northern blotting. This technique revealed the presence of stromelysin-1 dur-

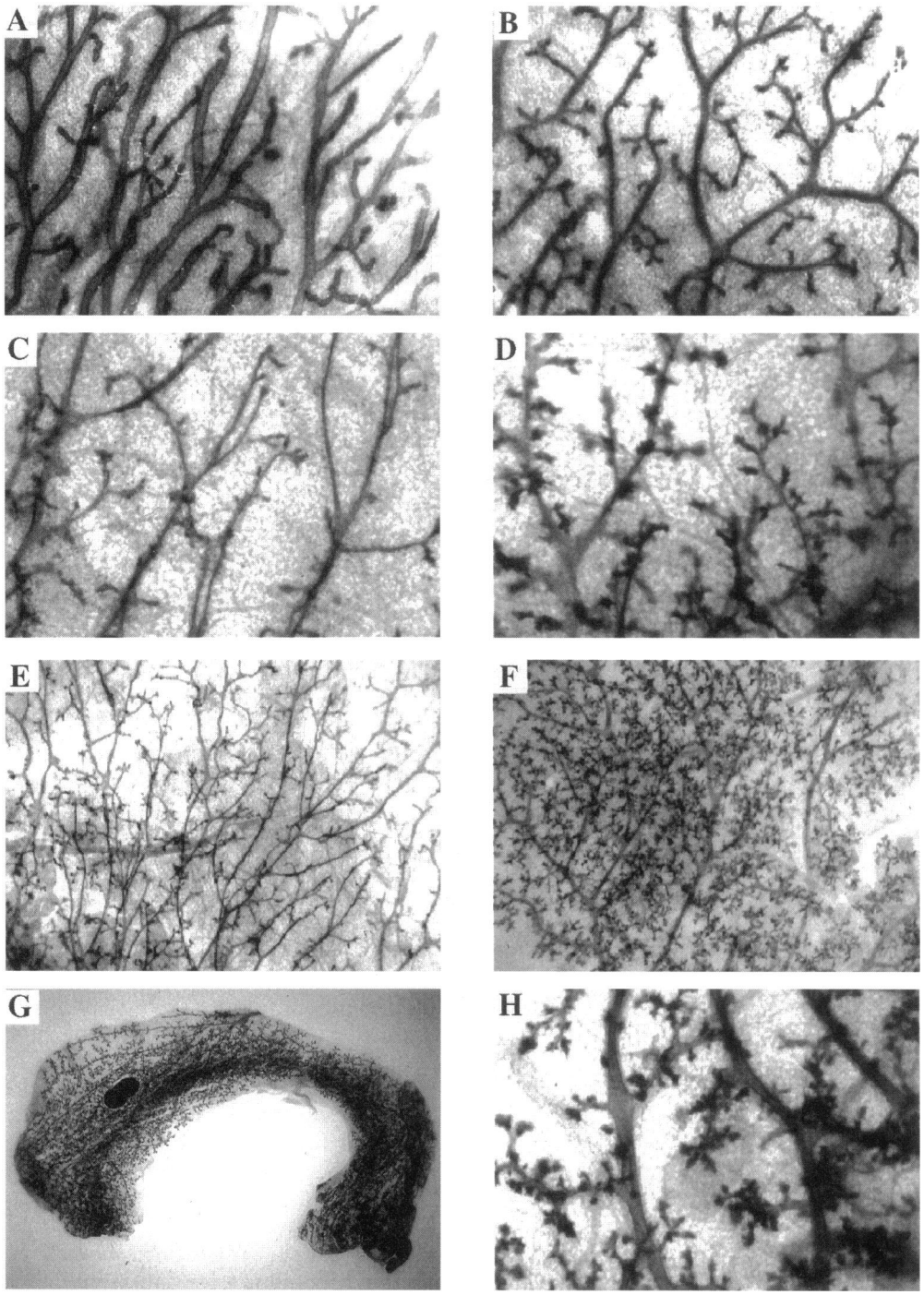

Figure 8. Morphological appearance of transgenic and wild-type juvenile mammary glands. Iron hematoxylin-stained whole mounts of thoracic mammary glands from wild-type (A, C, and E) and P/V-STR1 line 23 transgenic (B, D, and F) virgin female littermates at 8 (A and B), 12 (C and D), and 13 (E and F) wk of age. Panel G depicts a low power magnification of an inguinal gland isolated from a 13-wk transgenic female, and panel H depicts a mammary whole mount of a thoracic gland from a 10-day pregnant wild-type female. Photographs were taken with ×25 (A–D), ×7.5 (E and F), ×3 (G), and ×30 (H) objectives.

Table 1. Increased epithelial cell proliferation in virgin mammary glands of stromelysin-1 transgenic mice

A	Age (weeks)	Average number of cells/unit area[a] Transgenic	Nontransgenic	Ratio (T/NT)
	6	26.6 ± 0.5	23.3 ± 2.2	1.04x
	8	23.4 ± 5.1	21.3 ± 4.0	1.09x
	10	24.4 ± 7.5	16.0 ± 1.1	1.50x
	13	38.1 ± 2.4	18.2 ± 1.4	2.09x[b]
B	Age (weeks)	Average percentage BrdU incorporation Transgenic	Nontransgenic	Ratio (T/NT)
	7	18.53 ± 1.99	17.8 ± 2.24	1.04x
	9	20.50 ± 2.69	17.53 ± 1.06	1.17x
	13	16.9 ± 2.34	12.4 ± 1.36	1.36x[c]

For each time point, three nontransgenic (NT) or transgenic (T) mice were used. In the morphometric analysis (A), ten 1000 μm^2 areas from corresponding regions of each inguinal gland from transgenic mice and their nontransgenic littermates were scored from histological sections. In determination of the rates of cell proliferation (B), nuclei of 500 epithelial cells from corresponding regions of each inguinal gland from transgenic mice and their nontransgenic littermates were scored from immunohistochemical sections as positive or negative for BrdU incorporation. Values in A and B are expressed as mean ± standard deviation.

[a] 1 unit area = 1000 μm^2.

[b,c] Statistically significant differences were observed for comparisons between nontransgenic and transgenic glands at 13 wk of age ($p < 0.001$ and $p < 0.01$, respectively, Students *t*-test).

ing early alveolar morphogenesis, with mRNA levels peaking at 6 days of pregnancy (Figure 11A). Expression of gelatinase A was also observed with the highest levels at 6 days of pregnancy, and lower levels of stromelysin-3 mRNA were detected beginning at 6 days of pregnancy. This suggests that the same MMPs that are present during juvenile gland development are present during early alveolar morphogenesis (compare Figures 1 and 11). Localization of stromelysin-1 in the stromal component surrounding the developing alveoli at 8 days of pregnancy was observed by in situ hybridization (Figure 11B). Gelatinase A showed a similar pattern of expression (our unpublished results). The presence of stromelysin-1 and gelatinase-A mRNA precedes that of β-casein, which is induced after 8 days of pregnancy (Figure 9C). This suggests a potential involvement of these MMPs in the remodeling of the mammary matrix, which may facilitate epithelial outgrowth indicative of the differentiated pregnant phenotype.

Consequences of Transgene Expression in Male Mice

Male transgenic mice from all four lines were examined at the stages in juvenile development described above (5–13 wk). Despite expression of the transgene in the brain, salivary glands, and testes, no obvious gross or histologic abnormalities of these organs were found. Infertility of several P/V-STR1 Line 25 males has been noted, but no histological abnormalities have been observed to date.

DISCUSSION

MMPs in Ductal and Lobuloalveolar Branching Morphogenesis

The development of branched organs is dependent upon instructive interactions between the expanding epithelium and the surrounding mesenchymal cells and extracellular matrix (Wessells, 1977). The classical model describing mechanisms of branching was first proposed by Wessells, who suggested that the stromal matrix of a developing salivary gland promotes the growth and expansion of epithelial cells, degrades glycosaminoglycans at the sites of ductal elongation, and lays down collagen fibers in the cleft between lobules (Wessells, 1977). Matrix metalloproteinases, as enzymes that degrade extracellular matrix components, have long been postulated to play a role in branching morphogenesis. Support for this hypothesis comes from studies that demonstrate that salivary gland morphogenesis is altered in the presence of exogenous collagenase or collagenase inhibitors in vitro (Nakanishi *et al.*, 1986), and studies in which the presence of TIMP-1 in murine lung bud cultures reversed the branching effect of lung rudiments treated with growth factors known to stimulate metalloproteinase activities (Ganser *et al.*, 1991). To obtain more direct information on the role of metalloproteinases in mammary gland morphogenesis, we initiated studies to examine the expression of various MMP family members in the mammary gland at the time of ductal development.

Temporal expression of several members of the MMP family (stromelysin-1, stromelysin-3, and 72-kDa gelatinase A) correlated with ductal branching morphogenesis in female mice between 5 and 10 wk of age. These MMPs were localized in stromal fibroblast-like cells surrounding small and medium epithelial ducts and occasional isolated cells in the mammary stroma (Figure 2). The expression pattern of stromelysin-1 was particularly interesting, in that the mRNA was highly expressed during juvenile mammary gland development and decreased to undetectable levels in adult animals, whereas the mRNA for stromelysin-3 was expressed at much lower levels during mammary gland morphogenesis and the mRNA for gelatinase A is widely expressed in fetal and adult tissues (Reponen *et al.*, 1992) (Figure 1). In situ hybridization of serial sections of a 6-wk old virgin female mammary gland revealed, however,

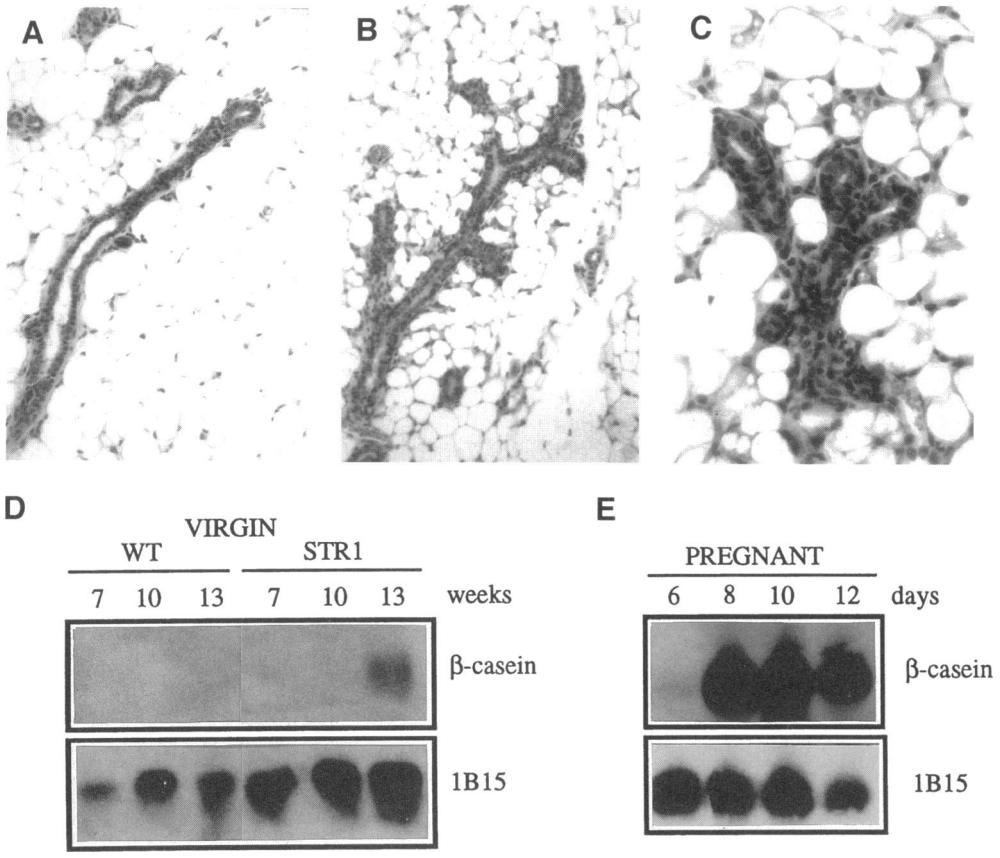

Figure 9. Differentiated characteristics of the mammary gland of stromelysin-1 transgenic mice. Representative sections of 10-wk wild-type (A) and P/V STR1 line 23 transgenic (B and C) virgin female mammary glands stained with hematoxylin and eosin. Photographs were taken with a ×20 (A and B) or ×40 (C) objective. Northern blot analysis of 2 μg of poly A(+) mRNA from virgin wild-type and P/V STR1 line 23 transgenic mammary tissue (D) and pregnant wild-type mammary tissue (E) utilizing a ^{32}P-labeled probe specific for β-casein. Cyclophilin (1B15) was utilized as a loading control.

that stromelysin-1 mRNA was not highly expressed in the stroma immediately adjacent to club-shaped terminal end buds. Although stromelysin-1 is known to degrade several matrix components in vitro, including proteoglycans, the lack of stromelysin-1 mRNA expression in the cap surrounding a terminal end bud suggests that this enzyme does not participate in the glycosaminoglycan and matrix degradation that has been proposed to determine the branched pattern of an organ (Wessells, 1977). Instead, stromelysin-1 mRNA was nonuniformly expressed along the length of what appeared to be newly branching ducts (Figure 4), and was absent in ducts that had apparently completed elongation and no longer exhibited the multilayered terminal end bud structure. The clustered expression of stromelysin-1 mRNA around the duct appeared in general to correlate with an increase in the relative amounts of stroma and stromal cells in these areas, but it is not clear if there is an absolute increase in stromelysin-1 expression per cell in any of these regions. Stromelysin-1 protein correlated with mRNA expression, although it is not known if the enzyme produced is actively degrading ECM because both enzyme activation and the level of MMP inhibitors present can effect the final activity of metalloproteinases. However, based on these observations, we suggest that stromelysin-1 may participate in the remodeling and restructuring of the matrix that occurs along a newly formed duct, and that its expression decreases once the normal ductal architecture is achieved.

Interestingly, the overproduction of stromelysin-1 in the mammary epithelium of juvenile virgin mice expressing activated stromelysin-1 under the control of the MMTV LTR results in structural changes resembling alveolar development normally observed in an 8- to 10-day pregnant animal (Figure 8). A similar observation was made by Sympson *et al.* (1994) in which activated stromelysin-1 was expressed under the control of the whey acidic protein (WAP) promoter. The WAP promoter directs transgene expression to the mammary gland during mid-pregnancy and lactation in contrast to the earlier expression of the MMTV promoter. Due to the difference in expression patterns of the two transgenes, we focused on the effects of stromelysin-1 on early development, while Sympson *et al.* (1994) focused on pregnancy and lactation. However, low levels of stromelysin-1 were apparently produced in the WAP-STR1 transgenic mice during juvenile development because precocious alveolar formation was observed in virgin, 10-wk females (Sympson *et al.*, 1994). These results could be interpreted as evidence for a role of metalloproteinases in

the morphological changes that occur during lobuloalveolar development in early pregnancy. The same subset of MMPs expressed during juvenile ductal morphogenesis were expressed during lobuloalveolar branching (Figure 11). It is possible that localized degradation of the extracellular matrix or basement membrane stimulates epithelial cell morphogenesis into lobular units, similar to that proposed for ductal branching morphogenesis by terminal end buds (see above). Indeed, we have observed substantial basement membrane disruption by ultrastructural analysis in virgin MMTV-STR1 mammary glands (Figure 10), and Sympson *et al.* (1994) have reported discontinuities in laminin and IV collagen in lactating WAP-STR1 transgenic mice. This degradation is initiated by the expression of the transgene, but may be potentiated by the enhanced production of endogenous MMPs (Figure 6). It is not clear if a similar mis-expression of stromelysin-1 would also effect ductal branching morphogenesis. No obvious difference in initial ductal patterning in young MMTV- or WAP-STR1 transgenic mice was observed compared with age-matched wild-

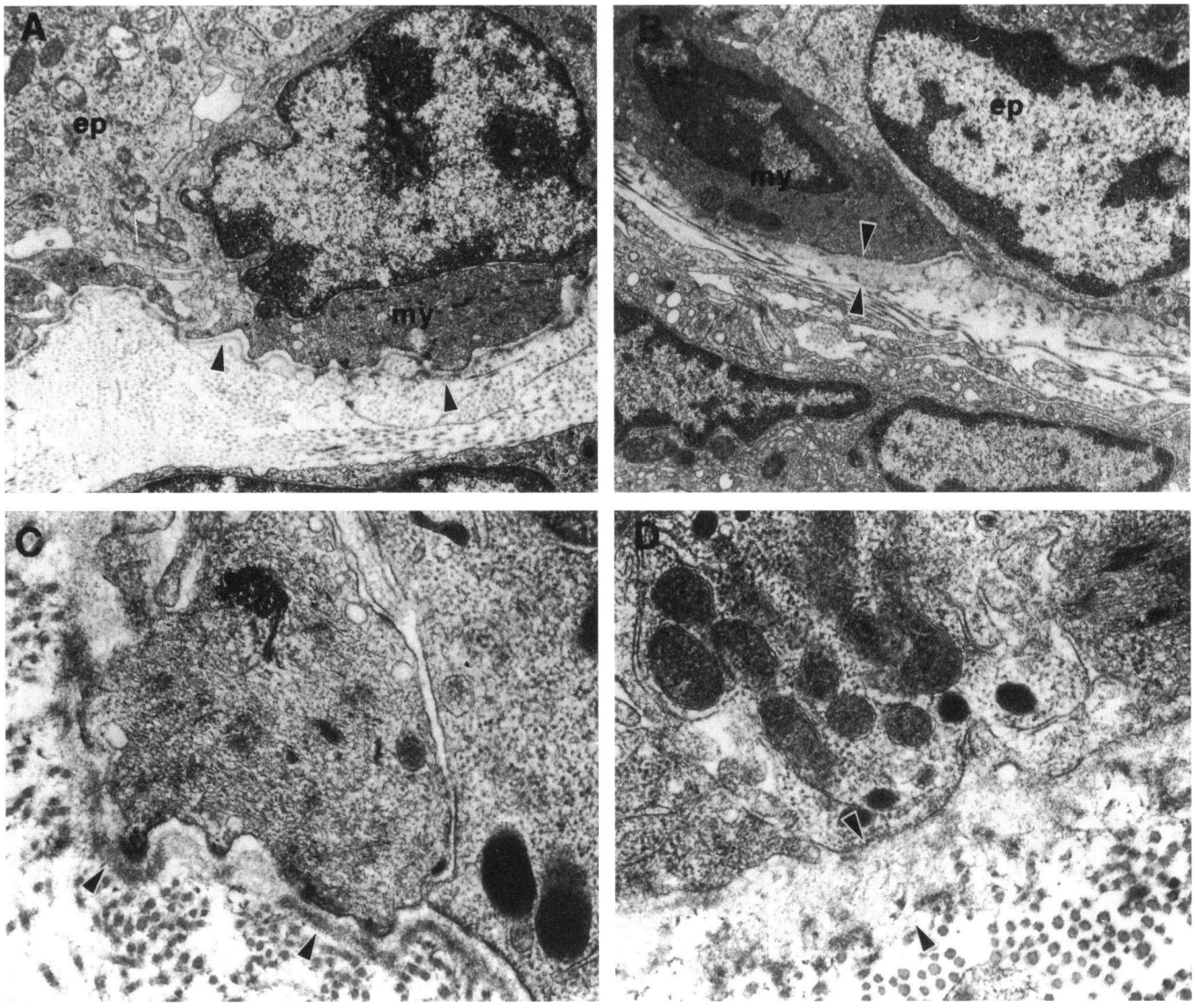

Figure 10. Ultrastructure of wild-type and stromelysin-1–transgenic mammary tissue. Transmission electron micrographs of mammary tissue from 11-wk old wild-type (A and C) and P/V STR1 line 23 transgenic (B and D) mice. Epithelial (ep) and myoepithelial (my) cells are separated from the adjacent mammary stroma by a clearly distinguished basal lamina (at arrowheads) in the wild-type gland (A, 11,500×). Portions of the extracellular matrix and of periductal stromal cells are observed in the bottom of the low power magnifications (A and B). At higher power, the basal lamina resolves as two components: the lamina densa (at arrowheads) and lamina lucida (clear area adjacent to the cell border) (C, 34,500×). In the transgenic tissue, the epithelial basement membrane appears less well organized (area between two arrowheads, panel B, 11,500×), and at high power reveals an amorphous region that lacks a distinct lamina lucida and densa (D, 34,500×).

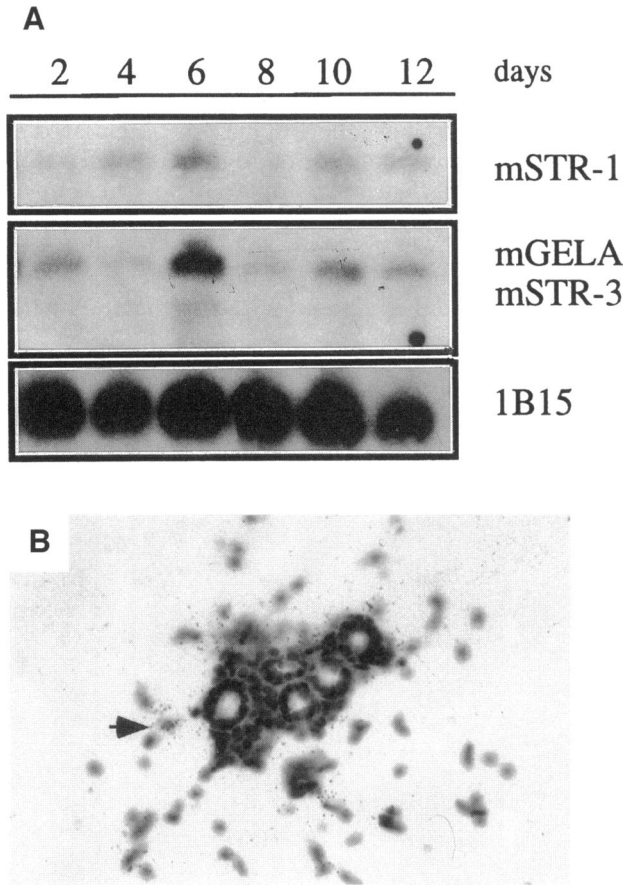

Figure 11. Expression of metalloproteinases during lobuloalveolar development in the pregnant mouse mammary gland. (A) Poly A(+) mRNA (2 μg) was isolated from mammary glands of wild-type female mice at the indicated days of pregnancy and analyzed for mRNA corresponding to murine stromelysin-1 (mSTR-1), gelatinase A (mGELA), stromelysin-3 (mSTR-3), and rodent cyclophilin (1B15). (B) Sections of wild-type mammary gland tissue at 8 days of pregnancy were analyzed by in situ hybridization using the antisense probe for mouse stromelysin-1. Photographs were taken with the ×20 objective using brightfield illumination. A representative stromal cell with silver grains indicating stromelysin-1 mRNA is indicated with an arrowhead.

type controls (Figure 8; Sympson *et al.*, 1994); however, transgene expression is first detected by Northern blot analysis at 7 wk of age in the MMTV-STR1 animals (Figure 6), a few weeks after the onset of ovarian function. It is not clear, therefore, if an elevation in MMP levels during earlier stages of ductal elongation and branching would affect the ultimate ductal pattern formation in murine mammary glands.

Although it is tempting to speculate that stromelysin-1–mediated matrix degradation initiates lobuloalveolar morphological changes based on these transgenic mouse models, it is not clear that they recapitulate a normal event. The expression of endogenous stromelysin-1 in virgin mice does not induce alveolar formation in the absence of pregnancy as is observed with stromelysin overexpression in the transgenic animals. The effect in the transgenic mice may be due to elevated levels of stromelysin-1 produced, or may be related to the expression of the enzyme in epithelial cells as opposed to the localization of endogenous stromelysin-1 in stromal cells. This difference in cell-type expression may affect the specific substrates digested, therefore altering the consequences of stromelysin-mediated matrix degradation. Determining the role of stromelysin-1 and other MMPs in ductal or lobuloalveolar development of the mammary gland will require a gene ablation approach to answer these questions.

MMPs in Cellular Differentiation and Proliferation

Although lobuloalveolar formation and overt differentiation run somewhat parallel courses, they are in fact partially uncoupled during most of pregnancy (Topper and Freeman, 1980). As pregnancy proceeds, rates of cellular proliferation decrease as the cells reach their fully differentiated state. Changes associated with this progression have been studied both in vitro and in vivo, and three alveolar cell types (A, B, and C cells) have been identified and classified according to their ultrastructural morphology and degree of functional differentiation (Topper and Freeman, 1980). Overproduction of stromelysin-1 protein by epithelial cells in transgenic mouse mammary glands increased cell number and cell proliferation (Table 1) and affected the differentiated state of the cells as determined by the appearance of β-casein mRNA (Figure 9; Sympson *et al.*, 1994), although the production of β-casein protein was not observed. The cells forming alveolar-like structures in the transgenic animals most likely correspond to A- or B-like cells between approximately 8 and 10 days of pregnancy, capable of rapid growth, although not yet containing the intricate cellular machinery required for full secretory activity.

What is not clear from these studies is whether the consequence of stromelysin-1 misexpression is to induce lobuloalveolar morphogenesis and the structural changes in turn alter cellular proliferation and differentiation states, or the degradation of the basement membrane alters the proliferative and differentiated state of the epithelial cells and this in turn alters the morphology of the gland. There are several reasons to suspect that ECM degradation may directly effect epithelial cell differentiation. Local environmental signals, provided via the ECM as well as by communication with adjacent cells, appear to be intricately involved in regulating mammary phenotype. Studies regarding functional differentiation of mammary epithelial cells in vitro have identified an instructive role for ECM components on the expression of differentiation-specific genes (Barcellos-Hoff *et al.*, 1989; Li *et al.*,

1987; Streuli *et al.*, 1995). During mammary gland involution, an increase in the expression of MMPs correlates with discontinuities in basal laminal structure and a loss of differentiation markers (Dickson and Warburton, 1992; Lefebvre *et al.*, 1992; Strange *et al.*, 1992; Talhouk *et al.*, 1992; Li *et al.*, 1994). Local infusion of TIMP, a specific MMP inhibitor, results in retention of a lactational phenotype and a delay in involution, suggesting that matrix degradation by MMPs can control the functional state of murine mammary glands (Talhouk *et al.*, 1992). This is in contrast to what we and others (Sympson *et al.*, 1994) observed in stromelysin-1 transgenic mice, in which the differentiated phenotype was induced when basement membrane was inappropriately degraded early in mammary gland development. The effect of ECM components on the differentiated phenotype of mammary epithelium is therefore apparently also influenced by additional external factors, for example circulating hormones, that determine how the cell responds to changes in the integrity of basement membrane structures.

Cell:matrix interactions are known to alter how a cell responds to external stimuli. The attachment of cells such as capillary endothelial cells to matrix molecules can regulate cell growth in response to growth factors as a result of modulations in cell shape (Ingber, 1990). In the mammary gland, epithelial cell shape is altered by attachment to ECM components (Streuli *et al.*, 1991). It is therefore possible that the disruption of the basement membrane in our in vivo system leads to alterations in cell:matrix interactions and changes in cell shape which, in turn, results in the enhanced proliferation and differentiation observed in the stromelysin-1 transgenic mice. Alternatively, stromelysin-1 action may be more indirect, stimulating proliferation of mammary epithelial cells by the release and/or activation of growth factors or growth-factor–like molecules present in the mammary matrix that mimic a normal mammogenic signal. Regardless of whether the initiating signal is direct or indirect, the mammary gland may then follow a pre-determined program of proliferation and differentiation that results in the formation of alveolar structures as opposed to, for example, uniform layers of epithelial cells. In this scenario, the disruption of the mammary gland basement membrane by the mis-expression of stromelysin-1 overrides the necessity for external hormonal stimuli to induce the lactogenic phenotype, and the mammary gland develops to a stage parallel to that observed in an 8- to 10-day pregnant animal before arresting. Presumably the addition of normal hormonal signals during pregnancy results in the completion of lactogenic differentiation, as these animals are capable of suckling normal-sized litters.

It is interesting that the induction of a 1.4-fold increase in the number of proliferating cells in the stromelysin-1–expressing transgenic mice results in an estimated twofold increase in the number of cells in the gland at 13 wk of development. With a cycling time of approximately 21 h (Daniel and Silberstein, 1987), it would appear that this difference in proliferative index should result in a much greater number of cells in the transgenic mammary glands. The difference in cell number may be an underestimation, as primary ducts could not be excluded from the cell count if they were analyzed in cross-section, and these ducts do not display alveolar expansion. However, we have also recently determined that there is a fourfold increase in programmed cell death in the mammary glands of the P/V STR1 line 23 transgenic mice as compared with nontransgenic littermates at 13 wk of age (Witty *et al.*, 1995), and a similar observation has been made in the WAP-STR1 mice (Boudreau *et al.*, 1995). These results indicate that the turnover of mammary epithelial cells is accelerated by the disruption of the basement membrane, suggesting that ECM may play a role in controlling cell survival as well as the proliferation and differentiation state of these cells.

Combining the results of overexpressing an activated MMP with the evidence for expression of endogenous stromelysin in the developing mammary gland, we hypothesize that MMPs function during normal mammary morphogenesis by participating in the remodeling of the basement membrane and extracellular matrix during ductal and lobuloalveolar morphogenesis. Following ductal morphogenesis, the decrease in the levels of MMPs is associated with the epithelial cells maintaining a quiescent state that is controlled, at least in part, by their association with underlying basement membrane. Disruption of epithelial cell:matrix interactions in the STR1 transgenic mice results in the unscheduled induction of epithelial cell proliferation and differentiation that would normally occur only under the influence of lactogenic hormones. Lobuloalveolar morphogenesis may be influenced by these disruptions in the basement membrane, but may also be an inherent consequence of the initiation of a predetermined pathway of events in the mammary gland. Further development of completely functional lactation requires additional external signals. These studies support a critical role for basement membrane and extracellular matrix in determining cellular responses to external stimuli, and further investigations may provide important insights into the molecular mechanisms that regulate such complex biological processes as cell proliferation, apoptosis, and differentiation.

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