Dominant-Negative Interference of the Transforming Growth Factor β Type II Receptor in Mammary Gland Epithelium Results in Alveolar Hyperplasia and Differentiation in Virgin Mice

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Abstract

Transforming growth factor (TGF)-β1 and TGF-β3 are normally expressed at high levels in the mammary gland during quiescence and at all stages of development, except lactation. Exogenously added TGF-β1, -β2, and -β3 have been shown to regulate growth and differentiation of mammary epithelial cells in vitro and in vivo. TGF-βs signal through a heteromeric complex of type I and type II serine/threonine kinases. The type II receptor is necessary for ligand binding and growth suppression by TGF-βs. Deletions of the cytoplasmic domains of several kinase receptors known to function in multimeric complexes have been shown to act as dominant-negative mutations. To evaluate the role of endogenous TGF-βs in the growth and differentiation of the mammary gland in vivo, we have targeted expression of a truncated, kinase-defective TGF-β type II receptor to mammary epithelial cells in transgenic mice using the mouse mammary tumor virus promoter/enhancer. Transgene expression was localized to the epithelial cells of terminal ducts and alveolar buds. At approximately 20 weeks of age, virgin female transgenic mice demonstrated varying degrees of mammary epithelial hyperplasia. Mammary glands from transgenic, virgin animals exhibited alveolar development and expression of the milk protein, β-casein. The data suggest that impaired responsiveness in the epithelium to endogenous TGF-βs results in inappropriate alveolar development and differentiation in the mammary gland. We conclude that endogenous TGF-β signal to the epithelium to maintain quiescence in the mammary glands of virgin animals.

Introduction

The development of the mouse mammary gland is primarily a postnatal event (1). At about 3–4 weeks of age, epithelial end buds drive growth and dichotomous branching, resulting in a highly branched ductal system that extends to the edge of the fat pad by the end of puberty. During pregnancy, the mammary gland undergoes alveolar development, in which there is rapid growth, morphogenesis, and terminal differentiation of mammary epithelial cells. Involution at the end of lactation involves the loss of alveolar structures and return to the original ductal pattern. Endocrine hormones and local growth factors regulate these processes (2). The mammary gland provides a good model for the study of basic developmental processes, including branching morphogenesis and terminal differentiation. Repeated rounds of morphogenesis and differentiation occur in the adult animal, so that the roles of hormones, growth factors, and epithelial-mesenchymal interactions can be readily studied. Also, the organ is easily accessible, and targeted overexpression or mutation of specific genes in the mammary gland is usually not immediately lethal to the animal.

TGF-β represents a large family of multifunctional peptides that may act as inductive signals in the embryo and are involved in the regulation of growth, ECM accumulation, and differentiation (3, 4). TGF-βs are direct mitogens for certain mesenchymal cells; however, the growth of most epithelial cell types is inhibited by TGF-βs (5). The expressions of TGF-β1, -β2, and -β3 have been localized to the mouse mammary gland at different functional stages (6). During puberty, each isoform is expressed within the epithelium of quiescent mammary ducts and actively growing end buds. TGF-β3 is expressed in the myoepithelium and in precursor cells in the end bud. TGF-β1 is expressed in ductal cells of the quiescent gland. TGF-β2 and -β3 levels increase during pregnancy and are localized to the epithelium of ducts and alveoli. Expression of all three TGF-β genes is dramatically reduced during lactation. Exogenous TGF-βs have been shown to inhibit growth and differentiation of mammary ap-

Received 10/27/97; revised 12/18/97; accepted 1/20/98.

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1. This work was supported by NIH Grants CA42572 and CA48799 and the Frances Williams Preston Laboratories of the T. J. Martell Foundation (to H. L. M.). R. S. is also supported by NIH/National Institute for Arthritis, Musculoskeletal, and Skin Diseases Grant 5P30 AR4 1943 and American Cancer Society Grant IN-250386. H. J. was funded by Training Grant 17-B4-LL024 from the Department of Defense. Transgenic founder mice were generated by the Transgenic Mouse/ES Cell Shared Resource, supported in part by National Cancer Institute Cancer Center Support Grant CA68485.

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3. The abbreviations used are: TGF, transforming growth factor; ECM, extracellular matrix; MMTV, mouse mammary tumor virus; WAP, whey acidic protein; LTR, long terminal repeat.
Ilethelial cells in cell culture (7), in organ culture (8), and in vivo (9–11). Administration of exogenous TGF-β1, -β2, or -β3 into actively branching mouse mammary glands using slow-release pellets resulted in reversible growth inhibition and involution of mammary gland end buds (6, 9). Transgenic mice that express a constitutively active TGF-β1 under the control of the MMTV promoter/enhancer demonstrated fewer lateral branches and a reduction in total ductal tree volume (11). It has been proposed that TGF-βs normally act to maintain ductal spacing during active branching at puberty and to regulate lactogenesis. TGF-β1 inhibits expression of β-casein, a differentiation marker for mammary epithelium, in HC11 mouse mammary epithelial cells (7). Synthesis and secretion of caseins in mammary explant cultures was also suppressed by TGF-β1 (8). Targeted expression of active TGF-β1 to the pregnant mammary gland using a WAP promoter resulted in inhibition of alveolar development in two of four mouse lines, whereas ductal development, including branching morphogenesis, was not impaired (10). Alveolar development was not affected in the MMTV-TGF-β1 transgenic mice and administration of TGF-β1 to mammary glands at day 15 of pregnancy using slow-release pellets did not inhibit further lobulo-alveolar development (6, 11). The different phenotypes observed with overexpression of TGF-β1 under control of the MMTV and WAP promoters is likely due to differences in temporal and spatial expression of the transgene.

TGF-βs signal through heteromeric complexes of type I and type II serine/threonine kinase receptors (reviewed in Refs. 12–14). These receptors were first identified by competitive binding assays and chemical cross-linking studies (reviewed in Refs. 12 and 13). Later, studies using mutant cells that were resistant to TGF-β demonstrated that the type I and type II receptors were functional in signal transduction (15, 16). Receptors for the TGF-β superfamily have been cloned (reviewed in Ref. 14), and studies indicate that the type II receptor is necessary for ligand binding and growth suppression by TGF-β. When overexpressed, a truncated, kinase-deficient type II receptor can act as a dominant-negative mutant. These truncated TGF-β type II receptors have been shown to block responsiveness of cells in culture to all three TGF-β isoforms (17–21). Inhibition of DNA synthesis by TGF-β1 and -β2 was blocked in Mv1Lu mink lung epithelial cells (17, 18); TGF-β1 mediated gene expression was blocked in cardiac myocytes in culture (19); and in vitro capillary morphogenesis was inhibited in endothelial cells expressing a truncated TGF-β type II receptor (20, 21). The truncated receptor has also been shown to inhibit TGF-β signaling in vivo. Expression of the dominant-negative receptor in transgenic mice blocks TGF-β-induced growth inhibition of keratinocytes (22), results in pancreatic acinar cell proliferation (23), and results in alterations in chondrocyte differentiation (24). To study the role of signaling by TGF-β through the type II receptor in mammary gland development in vivo, we have constructed transgenic mouse lines that express a truncated TGF-β type II receptor (DNIIR; Ref. 17), which is targeted to mammary gland epithelium using the MMTV promoter/enhancer. The data suggest that signaling by endogenous TGF-βs through the type II receptor suppresses development and differentiation of alveolar epithelium in the virgin mammary gland.

Results

Construction of MMTV-DNIIR Transgenic Mice. An expression plasmid (MMTV-DNIIR) containing the cDNA sequence encoding a human truncated TGF-β type II receptor (17) and the complete MMTV LTR promoter/enhancer was generated (Fig. 1). The truncated receptor contains the complete extracellular and transmembrane domains and is capable of binding ligand and interacting with type I receptors (17). The kinase domain of the mutant receptor has been deleted, and overexpression of this receptor in mink lung epithelial cells in culture has been shown to block inhibition of DNA synthesis by TGF-β1 and -β2 in a dominant-negative manner (17). The MMTV LTR has been shown to direct gene expression primarily to mammary gland epithelium (25–28).

Transgenic mice were generated by injecting the Xhol fragment of the MMTV-DNIIR expression plasmid into single-cell mouse embryos. Seven transgenic mouse lines were established, and three lines with the highest numbers of integrated plasmids, designated MMTV-DNIIR-5, -7, and -8, were used for experiments. All three lines demonstrated a similar phenotype, but the experiments described here will focus on line MMTV-DNIIR-8.

Expression of DNIIR mRNA. To determine whether the truncated receptor was expressed in the mammary glands of transgenic mice, RNA was extracted from the mammary glands of homozygous (Fig. 2, Lanes D–H) and hemizygous transgenic (Fig. 2, Lanes A–C) and wild-type (Fig. 2, Lanes I–M) virgin mice in the diestrus stage (Fig. 2) or the estrus stage (data not shown) of the estrous cycle. RNA was analyzed by Northern blot hybridization to a human TGF-β type
II receptor extracellular domain cDNA probe (DNIIR). At 13–15 weeks, DNIIR mRNA expression was very low (data not shown); however, higher levels of transgene expression were detected in mammary glands from older homozygous and hemizygous mice between 18 and 23 weeks of age (Fig. 2). Expression was detected in mammary glands from hemizygous and homozygous mice in the diestrus (Fig. 2, Lanes A–H) and estrus stage (data not shown). Hybridization to the endogenous mouse type II receptor mRNA was not detected with this probe (data not shown), and no hybridization was detected in wild-type mice (Fig. 2, Lanes I–M).

To determine the localization of transgene expression in the mammary gland, sections of mammary glands from 20-week-old, virgin wild-type (Fig. 3D) and MMTV-DNIIR-8 transgenic mice (Fig. 3, A and B) were hybridized to an antisense 35S-labeled riboprobe, corresponding to the extracellular domain of the human TGF-β type II receptor. Expression of the transgene was localized to epithelial cells of terminal ducts and small alveolar-like structures in the mammary gland (Fig. 3, A and B, large arrows; see also Fig. 5B). Expression was not detected in stromal cells or the epithelium of the larger ducts (Fig. 3A, small arrow). No hybridization was detected between MMTV-DNIIR-8 sections and an 35S-labeled sense probe (Fig. 3C). Hybridization to the antisense probe was not detected in wild-type mammary glands (Fig. 3D), suggesting that this probe is specific for the human transgene.

**Morphology of Mammary Glands from Virgin MMTV-DNIIR Transgenic Mice.** The morphology of the mammary gland ductal structure was compared in hematoxylin-stained whole-mount preparations of thoracic mammary glands from homozygous (Fig. 4, E and F) and hemizygous (Fig. 4, C and D) MMTV-DNIIR-8 transgenic and wild-type (Fig. 4, A and B) virgin mice at 20 weeks of age and in the diestrus (Fig. 4) or estrus stage (data not shown). Mammary glands from transgenic mice demonstrated development of alveolar-like buds (Fig. 4, D and F, arrows), which began to fill in the spaces between the ductal network. This alveolar hyperplasia was observed for mice in diestrus (8 of 9 MT-DNIIR-8 versus 2 of 7 wild-type mice) and estrus (9 of 11 MT-DNIIR-8 versus 3 of 6 wild-type mice). It was later shown that the one transgenic mouse in diestrus that did not exhibit hyperplasia did not express the truncated receptor (Fig. 2, Lane B); however, transgene expression was detected in the two mice in estrus that did not exhibit hyperplasia (data not shown). Homozygous mice exhibited a more striking level of hyperplasia than hemizygous mice, with 4 of 6 homozygous mice, demonstrating a higher level of hyperplasia (Fig. 4E compared to Fig. 4C), compared to 3 of 14 hemizygous mice exhibiting this level of hyperplasia. The morphology observed in the virgin female transgenic mice was similar to that of mammary glands from pregnant wild-type mice at early to midgestation (9.5 days postcoitum; Fig. 4, G and H). Alveolar hyperplasia was less apparent in the mammary glands of 13-week-old transgenic mice and correlated with a very low level of transgene expression (data not shown).

To further characterize the observed hyperplasia, H&E-stained paraffin sections were examined and showed increased numbers of terminal ducts and alveolar buds in mammary glands from MMTV-DNIIR-8 (Fig. 5B) and MMTV-DNIIR-7 (Fig. 5C) transgenic mice relative to mammary glands from wild-type mice (Fig. 5A), which demonstrated only simple ducts. Secretory material was apparent in ducts of mammary glands from both transgenic and wild-type virgin female mice, but alveolar structures, some containing apparent secretions, were observed only in mammary glands from transgenic animals (Fig. 5, B and C). These histological observations, along with the appearance of the whole-mount mammary gland preparations from transgenic female mice, suggested that dominant-negative interference of signaling to the epithelium by endogenous TGF-β2 was permitting epithelial differentiation, characteristic of pregnancy in the virgin animals. Thus, expression of β-casein, a protein found in the differentiating mammary gland, was examined.
Expression of the Truncated Receptor Promotes Differentiation. The abundance of caseins in milk make these proteins useful markers of differentiated mammary cell function. β-Casein is normally induced in the mammary gland early in pregnancy (29). To determine whether morphological development and DNIIR mRNA expression correlated with functional epithelial differentiation, expression of β-casein mRNA and protein was examined in mammary glands from wild-type and MMTV-DNIIR-8 homozygous and hemizygous transgenic mice in diestrus. Northern blot analysis showed that five of seven mammary glands from virgin transgenic mice expressed β-casein mRNA (Fig. 2, Lanes A–H). Fewer (two of five) mammary glands from virgin age-matched wild type mice in diestrus expressed β-casein (Fig. 2, Lanes I–M). β-Casein protein was detected by immunohistochemistry as a secreted protein within alveolar buds (Fig. 6A, large arrows) and large ducts (data not shown) of MMTV-DNIIR-8 transgenic mammary glands. The intensity and pattern of staining was similar to that seen in 12.5-day-pregnant wild-type mice (Fig. 6D). Interestingly, β-casein protein was not detected in the sections from wild-type mammary glands that did (Fig. 6B) or did not (Fig. 6C) express the β-casein mRNA. The data suggest that impaired responsiveness to endogenous TGF-βs results in inappropriate differentiation of mammary epithelium giving, an early- to midpregnant phenotype in virgin animals.

Stromelysin Expression in Wild-type and MMTV-DNIIR-28 Mammary Glands. Expression of the dominant-negative TGF-β type II receptor in the mammary epithelium resulted in alveolar hyperplasia. To examine potential mechanisms for this interaction, the expression of the matrix metalloproteinase, stromelysin-1, was examined using Northern analysis. Stromelysin-1 is expressed in the mammary stroma (30, 31), and expression is inhibited by TGF-β1 (32, 33). In addition, overexpression of stromelysin-1 in the mammary gland (30, 31) resulted in increased differentiation and alveolar hyperplasia, similar to those observed in MMTV-DNIIR-8 mammary glands. We hypothesized that altered expression of stromelysin could result in the alveolar hyperplasia seen in the MMTV-DNIIR-8 mammary glands. A 32P-labeled cDNA probe to stromelysin-1 was used to hybridize a Northern blot containing RNA from mammary glands of homozygous transgenic (Fig. 2, Lanes A–F) and wild-type (Fig. 2, Lanes G–K) mice. There was no consistent alteration in the expression of stromelysin-1 mRNA in transgenic mice relative to wild-type mice, as determined by Northern analysis. These results indicate that the alveolar hyperplasia observed in the MMTV-DNIIR mammary glands is most likely not the result of altered expression of stromelysin-1 mRNA.

Discussion

This report demonstrates that impaired responsiveness to endogenous TGF-βs in mammary epithelium results in varying degrees of inappropriate alveolar hyperplasia and differentiation in virgin female mice. For these studies, we used a truncated TGF-β type II receptor regulated by the MMTV promoter/enhancer to functionally impair receptor function in
mammary epithelium in vivo. The kinase-deficient mutant used is able to compete with wild-type receptors for ligand and type I receptors, thereby acting as a dominant-negative mutant (17). Dominant-negative mutants of the fibroblast growth factor receptor have been used in transgenic mice to demonstrate a role for endogenous fibroblast growth factors in lung and skin development (34, 35). The truncated TGF-β type II receptor has been used in transgenic to examine the role of endogenous TGF-βs in skin development (22), pancreatic homeostasis (23), and maintenance of the skeletal system (24). Previous studies have involved exogenous administration of TGF-βs to mammary glands to indirectly deduce how TGF-βs may normally act. The response of cells subjected to high doses of TGF-βs at inappropriate times
demonstrates the sufficiency of TGF-βs for a response but does not address the question of necessity. The use of a truncated receptor in the mammary gland in vivo permits an examination of the normal role of endogenous growth factors and can demonstrate the necessity of TGF-β in for specific aspects of mammary gland development.

Previously, truncated TGF-β type II receptors have been shown to block responsiveness of cells in culture to TGF-βs (17–21, 36). The advantages of using the TGF-β type II receptor dominant-negative mutant in transgenic mice include: (a) the function of the receptor is inhibited in specific tissues at specific times, depending on the DNA regulatory element chosen, which reduces the problem of embryonic or neonatal lethality associated with targeted disruption of TGF-β or TGF-β receptor genes and allows for the study of the normal role of TGF-βs in adult mice; and (b) the response to all

\[ \text{Role of Endogenous TGF-βs in Mammary Epithelium} \]

![Fig. 5. Histology of wild-type and MMTV-DNIIR transgenic virgin mammary glands. H&E-stained mammary gland sections from wild-type (A), MMTV-DNIIR-7 (B), and MMTV-DNIIR-8 (C) virgin mice at 20 weeks are shown. Arrows, alveolar buds. DNIIR mRNA expression in B corresponds to Fig. 3B.}

![Fig. 6. Localization of β-casein in wild-type and MMTV-DNIIR-8 transgenic virgin mammary glands by immunohistochemistry. Sections from wild-type (B and C) and MMTV-DNIIR-8 transgenic (A) virgin mammary glands at 20 weeks were incubated with an anti-β-casein (A–C) polyclonal antibody. MMTV-DNIIR-8 virgin mammary glands demonstrated β-casein protein in ducts and the lumen of new alveoli (A, arrows). Staining was similar to that seen in wild-type 12.5-day-pregnant glands (D, arrow). B, β-Casein protein was not detected in alveolar buds in sections from a wild-type mouse that expressed β-casein mRNA. β-casein was also not detected in sections from wild-type mammary glands in which β-casein mRNA was not detected. Staining was not detected in sections incubated with the secondary antibody alone (data not shown).}
TGF-β isoforms is blocked (17), which avoids any problems with functional redundancy that are often associated with genetically null mice (37, 38). One disadvantage of the dominant-negative strategy is that high levels of the transgene relative to the endogenous type II receptor are required for the dominant-negative effect. Fortunately, the endogenous type II receptor appears to be expressed at very low levels in the quiescent mammary gland (data not shown), although this may account for some of the variations in the level of hyperplasia and differentiation observed. Another disadvantage is that the truncated TGF-β type II receptor could potentially block signaling by other TGF-β superfamily members by binding to their associated type I receptor (39). However, mammary glands from MMTV-DNIIR mice do not display the same phenotype as activin β-null mice (40), and Bottinger et al. (23) have shown that a similar dominant-negative receptor does not block signaling by activins in hepatocytes in primary culture.

Addition of TGF-β to actively branching mammary glands using slow-release pellets resulted in inhibition of branching morphogenesis and involvment of end buds, which normally drive the growth process (6, 9, 41). The histology of TGF-β inhibited end buds appeared normal, resembling quiescent ducts. Overexpression of TGF-β in the mammary glands of transgenic mice using the MMTV LTR resulted in decreased lateral branching and decreased growth but not involvment of the end buds (11). These data, along with the observed pattern of expression of the TGF-β isoforms in the mammary gland, suggest that the TGF-βs may act as the normal regulator of ductal spacing, maintaining patterning and suppressing adventitious lateral branching in the developing, actively branching mammary gland (6). Disorganized branching was not detected in mammary glands from MMTV-DNIIR transgenic mice relative to wild-type mice. This observation suggests TGF-β does not act on the epithelium to regulate this process. However, transgenic mice that express the dominant-negative receptor in the mammary gland stroma demonstrate increased and disorganized lateral branching, suggesting a role for TGF-β action in the stroma in maintenance of ductal spacing.4 At 20 weeks, MMTV-DNIIR mammary glands from virgin transgenic mice demonstrate development of alveolar buds. This pattern is similar to what is seen normally at early to midgestation in mammary glands and suggests endogenous TGF-βs act on the epithelium to prevent development of alveoli and maintain quiescence in the virgin mammary gland.

It has been proposed that the TGF-βs may also act to prevent accumulation of milk proteins before lactation. TGF-βs have been shown to inhibit differentiation-specific gene expression in hormone-induced HC11 cells (7), mammary epithelial cells from pregnant mice in cell culture (42), explant cultures from pregnant mammary glands (8, 43), and transgenic mice overexpressing TGF-β1 specifically in the pregnant mammary gland under control of the WAP promoter (10). Mammary tissue from lactating animals did not respond to TGF-βs, and the expression of all three isoforms of TGF-β is reduced during lactation (8, 41, 43). In the MMTV-DNIIR mice, impaired responsiveness to TGF-β resulted in expression of β-casein, supporting a role for endogenous TGF-β in preventing inappropriate expression of the differentiated phenotype.

The mechanism by which impaired responsiveness to TGF-β results in alveolar hyperplasia and expression of β-casein is not clear, but several possibilities exist. The TGF-βs are known to regulate the accumulation of ECM, and the integrity of the ECM regulates morphogenesis and differentiation of mammary epithelial cells (44–47). Transgenic mice that overexpress the stromelysin gene, an ECM metalloproteinase, in mammary epithelium demonstrate a phenotype similar to the MMTV-DNIIR mice, including precocious alveolar budding and induced expression of the β-casein gene (30, 31, 46). TGF-βs are known to suppress the expression of matrix proteinases (33); however, we did not detect any consistent differences in stromelysin expression in transgenic and wild-type mice.

TGF-βs may act to maintain quiescence of the mammary ductal tree by inducing apoptosis in cells that stochastically differentiate in the absence of the appropriate endocrine hormones. WAP- and casein-expressing cells have been detected scattered throughout virgin mammary glands during estrus (48, 49). The fate of these cells is not clear. Overexpression of TGF-β1 in the mammary gland has been shown to result in increased apoptosis when expressed in lobular progenitor or epithelial stem cells (48). Impaired responsiveness to TGF-βs might result in increased survival of differentiated cells, causing expansion of this cell population.

TGF-βs inhibit the growth of many epithelial cell types, including mammary epithelial cells (50). TGF-βs may act to maintain quiescence in the mammary gland by inhibiting growth of epithelial cells. In this model, impaired responsiveness to TGF-βs would directly result in increased DNA synthesis in normally quiescent cells, sufficient to result in the formation of alveolar buds. TGF-α is known to stimulate epithelial cell proliferation and virgin transgenic mice, which overexpress the TGF-α gene and also demonstrate alveolar hyperplasia, similar to that observed in the MMTV-DNIIR mice (51–53).

These data indicate that a functional type II TGF-β receptor is required for maintenance of a quiescent mammary ductal structure in virgin mice. Expression of a truncated type II TGF-β receptor under the control of the MMTV promoter/enhancer resulted in a phenotype similar to what is observed in early to midpregnant wild-type mammary glands, including alveolar hyperplasia and expression of the β-casein gene. Recently, evidence has accumulated suggesting that the type II TGF-β receptor functions as a tumor suppressor (54). Inactivation of the type II receptor has been detected in some tumor types and restoration of a functional type II receptor by stable transfection suppresses tumorigenicity of receptor-negative cells (54, 55). The strategy of overexpressing a truncated TGF-β type II receptor that acts in a dominant-negative manner in a transgenic context will also allow us to

4 H. Joseph, A. Gorska, P. Sohn, H. L. Moses, and R. Sema. Overexpression of a kinase deficient TGF-β type II receptor in mouse mammary stroma results in increased epithelial branching, manuscript in preparation.
examine the roles of endogenous TGF-β in mammary carcinogenesis.

Materials and Methods

Generation of Transgenic Mice. The MMTV-DNIIR expression plasmid was prepared by inserting the 0.6-kb EcoRI-XhoI fragment of the DNIIR from pRH102 (17) into the EcoRI site of pKCR containing the complete MMTV LTR. The pRH102 fragment contained the extracellular and transmembrane domains of the human TGF-β type I receptor and a FLAG epitope tag. The 3.2-kb XhoI fragment was purified using the low melting agarose gel method (NuSieve GTG low melting temperature agarose; FMC Bioproducts) and microinjected into (C57BL/6×DBA/F2) F1 fertilized eggs (56). Transgenic mice were identified by Southern blot analysis (57) and by PCR analysis, as described previously (28). The three of seven founder mice that showed the highest number of integrated plasmids, as determined by Southern blot analysis (lines 5, 7, and 8), were bred to (C57BL/6) mice to establish transgenic lines.

DNA Isolation and Detection. DNA was isolated from 0.5-cm pieces of tail tip tissue by proteinase K digestion. DNA was further purified by phenol/chloroform/isooamyl alcohol extraction. DNA was purified with Alu enzyme, separated on 1.0% agarose gel, and transferred to a Nylon filter (Hybbon-N, Amersham, Buckinghamshire, England). A 900-bp DNIIR-specific band was detected by hybridization with a 32P-labeled probe made from the BglII-XhoI fragment of the MMTV-DNIIR expression plasmid containing 446 bp of rabbit β-globin exon 3. PCR analysis was done using the following primers for the β-globin fragment: 5′-β-globin, 5′-CTA CTG CGT AGC AGG TTG CCT; and 3′-β-globin, 5′-ACA GCT ATG AGT AGG AGT AGT CAG. PCR was carried out for 35 cycles with 54°C as the annealing temperature (28). PCR product was detected on a 1% agarose gel with 0.5 μg/ml ethidium bromide.

Extraction of RNA and Northern Blot Hybridization. Total RNA was isolated from mammary gland tissue as described previously (58). Poly(A) RNA was resolved by electrophoresis and then transferred to Nylon membrane (Hybond-N). The membranes were probed with the following 32P-labeled cDNAs: 0.6-kb EcoRI-XhoI fragment from pRH102 for DNIIR, 540-bp HindIII-BamHI fragment of pT7BC-1 for β-casein (Ref. 59; obtained from Mina Bissell, Lawrence Berkeley Laboratory, Berkeley, CA), stromelysin-1 (Ref. 31; kindly provided by Lynn Matrisian, Vanderbilt University, Nashville, TN), and 0.7-kb BamHI-PstI fragment from plasmid SP65 1815 for cyclophilin.

Morphological Assessment of the Mammary Glands. The inguinal and third thoracic mammary gland of wild-type and MMTV-DNIIR transgenic mice were resected and fixed in freshly prepared 4% paraformaldehyde for 16 h. Glands were processed for whole mounts as described (9). Whole mounts were stained with hematoxylin, cleared with methylcellulose, and photographed with an Olympus Research Stereo Microscope.

In Situ Hybridization. The DNIIR 0.6-kb EcoRI-XhoI fragment from pRH-102-4 was inserted into SP72 and SP73 vectors to make riboprobes in the sense and antisense orientation. For the antisense DNIIR riboprobe, the SP73DNIIR plasmid was linearized with EcoRI enzyme and transcribed with T7 polymerase. For sense DNIIR riboprobe, the SP72DNIIR plasmid was linearized with XbaI, and T7 polymerase was used. Single-stranded RNA was labeled with 35S-labeled UTP (100 μCi/ml; NEN; specific activity, 1400 Ci/mmol). In situ hybridization was performed for 18 h at 55°C as described previously (60).

Histology and Immunohistochemical Detection of β-Casein. For β-casein immunohistochemistry and for H&E staining, paraffin-embedded sections were used. Mammary glands were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (4 μm) were stained with H&E or used for β-casein immunohistochemistry as described previously (8). β-Casein antibody (kindly provided by Charles Daniel, University of California at Santa Cruz, Santa Cruz, CA) was diluted 1:2000. Biotinylated secondary antibodies were detected with the Vectastain ABC kit (Vector Laboratories).

Acknowledgments

We thank Charles Daniel, Lynn Matrisian, and Mina Bissell for providing antibody and cDNA reagents. We would also like to thank Rebecca Townsend for her assistance and advice.

References


Role of Endogenous TGF-βs in Mammary Epithelium


