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RETRACTION

Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis

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Retraction of: *Development* **131**, 3469-3479.

The authors contacted the journal when they became aware of a number of errors involving the re-use of lanes and panels in multiple figures of the paper. Specifically, the vinculin lanes in Fig. 6H and Fig. 1E are identical, and two of these lanes are also duplicated in Fig. 7D. In addition, the vinculin lanes 1-3 in Fig. 7C are duplicated in lanes 4-6, and in Fig. 9 the Bcl-X_L bands in lanes 2 and 3 are identical. Finally, Fig. 3B is replicated (with aspect changes) from a previous paper (Fig. 2C of *J. Biol. Chem.* **278**, 17421-17429).

It has not been possible to fully resolve these anomalies, and therefore the authors and the editors of the journal believe that the most appropriate course of action is to retract the article. The authors apologise for any inconvenience this may have caused. This complies with the policies and practices of the journal.

Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis

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Summary

Emerging data suggest that metastasis-associated protein 1 (MTA1) represses ligand-dependent transactivation functions of estrogen receptor-alpha in cultured breast cancer cells and that MTA1 is upregulated in human breast tumors. However, the role of MTA1 in tumorigenesis in a physiologically relevant animal system remains unknown. To reveal the role of MTA1 in mammary gland development, transgenic mice expressing MTA1 under the control of the mouse mammary tumor virus promo[illegible]g terminal repeat were generated. Unexpectedly, we found that mammary glands of these virgin transgenic mice exhibited extensive side branching and precocious differentiation because of increased prolif[illegible] of ductal and alveolar epithelial cells. Mammary glands of virgin transgenic mice resemble those from wild-type mice in mid-pregnancy and inappropriately express β-casein and β-catenin protein. Increased ductal growth was also observed in the glands of ovariectomized male mice, as well as of transgenic male mice. MTA1 dysregulation in mammary epithelium and cancer cells triggered down-regulation of the progesterone receptor-B isoform and up-regulation of the progesterone receptor-A isoform, resulting in an imbalance in the native ratio of progesterone receptor A and B isoforms. MTA1 transgene also increased the expression of progesterone receptor-A target genes Bcl-X (*Bcl2l1*) and cyclin D1 in mammary gland of virgin mice and, subsequently, produced a delayed involution. Remarkably, 30% of MTA1 transgenic females developed ductal hyperplastic nodules, and about 7% exhibited mammary tumors within 18 months. These studies establish, for the first time, a potential role of MTA1 in mammary gland development and tumorigenesis. The underlying mechanism involves the upregulation of progesterone receptor A and its targets, Bcl-X_L and cyclin D1.

Key words: Mammary gland development, Transgenic mice, MTA1, Progesterone receptors, Cyclin D1, Bcl-X_L, Bcl2l1

Introduction

Mammary gland growth and maturation consist of a series of highly ordered events that are regulated by complex interactions among many steroid hormones and growth factors (Medina et al., 1[illegible] Hennighausen and Robinson, 2001). For these normal glands to develop, however, there must be a balance between cell proliferation, cell differentiation and cell death throughout the development. Perturbations in this balance can lead to abnormalities in mammary gland development. During the prepubertal stage, mammary gland development becomes hormone dependent and this continues at the onset of puberty. A large body of studies using hormone depletion, gene targeting and transgene expression approaches has identified the estrogen receptor (ER) and progesterone receptor (PR) to be essential in mammary gland development (i.e. ductal elongation and branching during puberty and the appearance of alveolar units during estrus) (reviewed by Couse et al., 1999; Connely et al., 2003). This has been further shown by ERα (*Esr1* – Mouse Genome Informatics) knockout mice, which display grossly impaired ductal epithelial cell proliferation and branching (Lubahn et al., 1993; Bocchinfuso et al., 2000), and by PR (*Pgr* – Mouse Genome Informatics) knockout mice, which display significant ductal development but decreased arborization and an absence of alveolar differentiation (Lydon et al., 1995). Perturbation of PR-A and PR-B isoforms by PR-A transgene (TG) has also been shown to cause aberrant ductal morphology, hyperlateral branching, and hyperplasia in virgin mammary glands (Shyamala et al., 1998).

However, these phenotypes are not limited to PR-A transgenic mice, as the deregulation of other regulatory gene products such as cyclin D1, a regulator of cell cycle progression, also causes hyperplasia (Wang et al., 1994). In particular, cyclin D1 directly activates ERs in a ligand-independent manner (Zwijsen et al., 1998). Results from cyclin D1 knockout mice suggest an essential role of cyclin D1 in the development of mammary glands (Fantl et al., 1999). Together, these observations indicate that cyclin D1 may constitute an important downstream target of diverse upstream signals in normal mammary gland development.

Because chromatin remodeling plays an essential role in the expression of genes, factors that control chromatin remodeling

in the vicinity of ER-target promoters are likely to play an important role in the development of both normal mammary gland and breast cancer. One such ER co-modulator is metastasis-associated protein 1 (MTA1), originally identified as an overexpressed gene in rat metastasis tumors (Toh et al., 1994). In in vitro models, MTA1 has been shown to interact with ERα and inhibits estradiol-induced stimulation of ER transactivation function (Mazumdar et al., 2001). MTA1 overexpression in breast cancer cells also correlates with aggressive phenotypes (Kumar et al., 2003). It is not clear, however, what role MTA1 plays in the context of complete mammary gland development. To determine the effects of MTA1 during postnatal mammary gland development, we have generated transgenic mice expressing MTA1 under the control of the mouse mammary tumor virus long terminal repeat (MMTV). We observed that MTA1 dysregulation in mammary epithelium caused increased cell proliferation, hyper-branched ductal structure formation and precocious development, and resulted in the development of hyperplastic nodules and mammary gland tumors in virgin mice.

Materials and methods

Generation of transgenic mice and Southern blot analysis of genomic DNAs

An MMTV-human MTA1-TG construct was created by subcloning T7-tagged MTA1 cDNA using sites *Hin*dIII-*Xba*I (blunted) into *Hin*dIII-*Eco*RI (blunted) of the MMTV-SV40-BssK vector (Huang et al., 1981). The transgene was excised from plasmid DNA, and the 6.2 linear fragment-containing promoter sequences, MTA1-coding and 3′ untranslated regions and SV40 polyadenylation signals was injected to the pronuclei of a B6D2F1/J mouse embryos. The Southern blot of tail DNA digested with *Eco*RI and *Xho*I restriction enzymes was used to identity founder animals. *Eco*RI and *Xho*I are the flanking sites of 2 kb MTA1 cDNA. Several MMTV-MTA1 founder m[illegible] expected 2 kb MTA1 band were identified and kept [illegible] lines 30-33. These results were confirmed by PCR using a unique forward primer to the T7-epitope encoding region ([illegible]-CAGCAAA[illegible]GGTCGGG-ATC) and reverse primer (5′-G[illegible]TTGAGCGACT[illegible]GTCTC) corresponding to MTA1 cDNA. These primers only amplify T7-tagged MTA1 and do not recognize endogenous mouse MTA1. As expected, these primers specifically amplified a 500 bp band in MTA1-transgene positive founder lines.

RT-PCR and northern blot analysis

RT-PCR was performed using the Access Quick reverse transcription RT-PCR system (Promega, Madison, WI) according to the manufacturer's instructions. Primers for PR total were forward (5′-CTGGGGT[illegible]TCGTA[illegible]AG) and reverse (5′-ACCAATTGC-CTTGAT[illegible]ATTCC) for PRB primers were forward (5′-TCGTC-TGTAC[illegible]CTCGCC[illegible]TACCG) and reverse (5′-CGGAGGGAGTCA-ACA[illegible]GAGT). RNA was extracted from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA), denatured, analyzed on a 1% agarose gel containing 6% formaldehyde and transferred to a nylon membrane. The blots were hybridized with MTA1 cDNA probe and developed by autoradiography.

Immunoblot analysis

Total protein extracts of mammary gland were prepared and western blot analysis carried out using primary mouse antibodies against cyclin D1 (1:1000; Santa Cruz Biotecnology, Santa Cruz, CA); β-casein, progesterone receptor (PR) Ab-4 (1:100; Neomarkers, Fremont, CA); mouse PR (1:500 Novocastra Laboratories, UK); β-catenin (1:500, BD Transduction Laboratories, Lexington, KY); anti-cytokeratin18 (1:100; Progen, Heidelberg, Germany); and rabbit polyclonal BCL-X s/l (L-19) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies consisted of anti-mouse and anti-rabbit antibodies (both 1:2000) conjugated to horseradish peroxidase and visualized by an enhanced chemiluminescence system. Densitometry was performed using a computerized densitometer and proteins were quantitated from the images using Sigma gel software (Sigma, St Louis, MO)

Mammary gland whole mounts, histology and immunodetection

For whole-mount analysis, number 4 inguinal mammary glands were stained with carmine alum as previously described (Bagheri-Yarmand et al., 2003). Briefly, the glands were fixed with acetic acid/ethanol (1:3) for 2 hours and stained with 0.5% carmine/0.2% aluminum potassium sulfate for 16 hours. After briefly being rinsed with distilled water, the mammary glands were dehydrated using graded ethanol, and lipids were removed with two changes of acetone. Finally, the glands were preserved in methyl salicylate. For histological analysis, mammary gland tissue was fixed in 10% neutral buffered formaldehyde and embedded in paraffin wax according to standard methods. Sections (4 μm) were stained with Hematoxylin and Eosin. For immunostaining, deparaffinized sections were subjected to antigen retrieval. This involved boiling the sections for 10 minutes and gradually cooling them for 30 minutes in 10 mM citric acid buffer (pH [illegible]0). Sections were then incubated with rabbit polyclonal PR-IgG (1:100; DAKO, Carpinteria, CA) followed by incubation with biotin-conjugated anti-rabbit or anti-mouse secondary antibody. To specifically detect PR-A forms in IHC, we used previously characterized hPRa7 (1:50; Neomarkers, Fremont, CA). Immunostained sections were lightly counterstained in Hematoxylin according to the manufacturer's instructions, dehydrated in graded ethanol, cleared in xylene and mounted on a coverslip with peramount.

BrdU labeling and TUNEL assays

To detect bromodeoxyuridine (BrdU)-positive cells, a sterile solution of 5-bromo-2′-deoxyuridine (BrdU) (20 mg/ml; Sigma-Aldrich) in PBS (pH 7.4) was administered to mice by intraperitoneal injection (50 mg/kg). Mammary glands were harvested after 3 hours, embedded in paraffin wax and sectioned. BrdU incorporation was detected by immunohistochemistry using a mouse anti-BrdU monoclonal antibody as previously described (Tonner et al., 2002). Apoptosis was detected in paraffin wax sections by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis with terminal deoxynucleotidyl transferase (Roche Diagnostics), as previously described (Gavrieli et al., 1992). Ten random fields per section were documented by photomicroscopy, and the percentage of TUNEL-positive epithelial cell nuclei relative to the total number of epithelial cell nuclei was calculated. Mean values were determined from results from at least six different mice.

Statistical analysis and reproducibility

Results are expressed as the mean±s.e.m. Statistical analysis of the data was performed using a Student's *t*-test. The presented phenotypic changes were documented in MTA1-TG founder lines 30, 31 and 33.

Results

Generation of MTA1 transgenic mice

As a means of targeting the expression of the *MTA1* transgene to the mammary gland, we placed the *MTA1* cDNA under the control of the MMTV promoter (Fig. 1A). The MMTV promoter directs transgene expression to mammary and salivary glands in the early stages of puberty and is hormonally regulated by progesterone during estrus and pregnancy (Matsui et al., 1990). Four founders lines 30-33 showing transgene

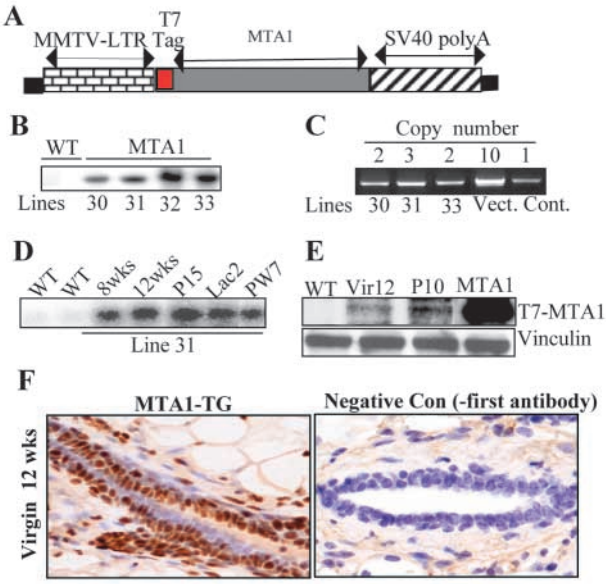

Fig. 1. Generation of MTA1-TG mice and expression of the MTA1 transgene. (A) The MMTV-MTA1 transgene. (B) Southern blot detection of the MTA1 transgene in the tail genomic DNA of transgenic (TG) and wild-type littermates of F1 from lines 30, 31, 32 and 33. (C) Representative PCR analysis of genomic DNA from potential founder mice. Lanes marked 10 and 1 show respective copy number equivalents of control MTA1 cDNA plasmid. (D) Time course of transgene expression in line 31 MTA1-TG mice a[nalyzed] by RT-PCR followed by Southern blotting. (E) Protein lysates [from] mammary gland of MTA1-TG and wild-type, virgin 12-week-o[ld] mice and pregnancy day 10, were immunoprecipitated with anti-[MTA1] antibodies and western blotted with anti-T7 antibod[y]. [MCF7-MTA1] cell lysate was used as a positive control. (F) Im[munohis]tochemical analysis of T7-MTA1 expression using anti-T7 [a]ntibodies [in] the mammary gland from 12-week-old virgin. [A] negative co[ntrol] without first antibody was shown. Note that [the] transg[ene] is located in the nucleus of the luminal epithelial [cells].

integration were identified b[y] PCR and confirmed by Southern blot analysis (Fig. 1B). Fo[under line] 3 did not transmit the transgene though the g[erm]line. Lines 30, 31, 33 expressed 2, 3 and 2 copies, respe[ctive]ly, of the transgene when compared with the intensity o[f] predetermined positive control (Fig. 1C). RT-PCR analysis [fo]llowed by Southern blotting in line 31 showed that the [MMTV]-MTA1 transgene was expressed throughout mammary [gland] and develo[pment]: virgin, pregnancy, lactation a[nd] involution (Fig. 1D). Endogenous MTA1 was also expressed in all stages of mammary gland development. The lowest level of MTA1 expression was observed during pregnancy (M[artin] et al., 2001). The T7-tagged MTA1 transgene protein was also detected by immunoprecipitation, followed by western blotting using the antibody specific to the T7 epitope (Fig. 1E). Immunohistochemical staining of the paraffin sections of mammary gland with an anti-T7 antibody revealed MTA1 in the nucleus of epithelial cells (Fig. 1F). Similar results were also obtained for lines 33 and 30.

MTA1 deregulation leads to excessive lateral branching and precocious development of virgin mammary gland

To investigate the effect of MTA1 on the development of

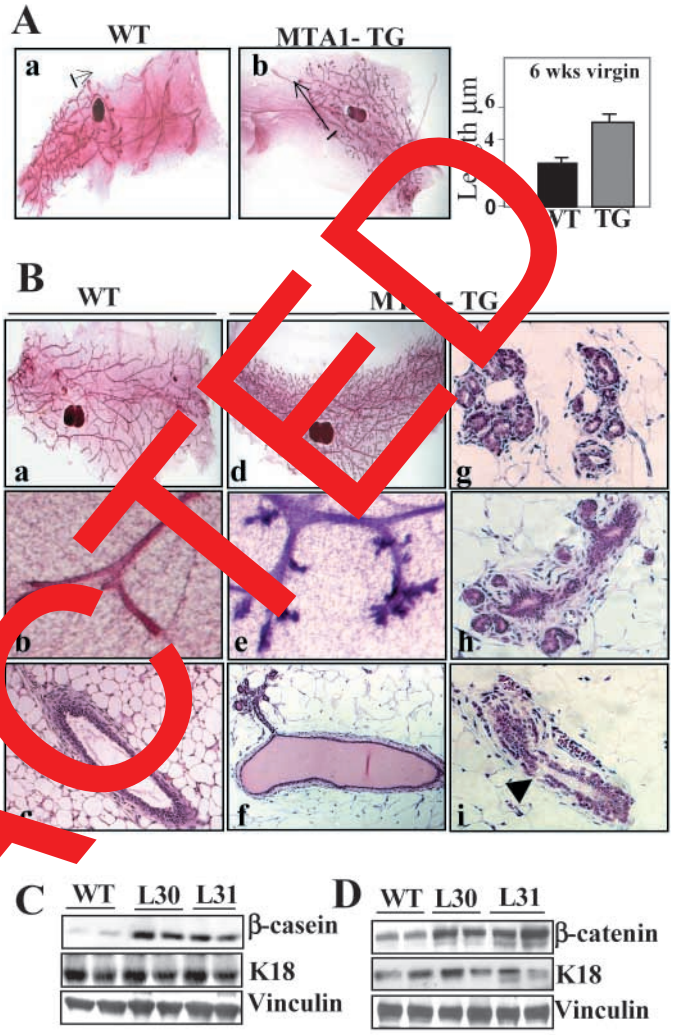

Fig. 2. Accelerated ductal extension, excessive side-branching and precocious lobuloalveolar development in wild-type virgin and MTA1-TG females. (A) Whole-mount preparations of wild type (a) and MTA1-TG mammary gland (b) at 6 weeks of age. (Right panel) Quantitative representation of distances from the center of the lymph node to the far end of terminal end-buds (five to seven mice per line). (B) Carmine Red-stained whole mounts of inguinal mammary glands from control (a,b) and MTA1-TG mice (d,e) at 12 weeks of age. Images in b and e are higher magnifications of a and d. Hematoxylin and Eosin-stained sections of mammary glands of 12-week-old wild-type (c) and MTA1-TG mice (f-i). Dilated ducts (f), increased budding (h), a lobuloalveolar-like structure (g) and an indistinct epithelial-stromal boundary (arrowhead, i) can be seen in the mammary glands of MTA1-TG mice. Western blot showing expression of β-casein (C) and β-catenin (D) in mammary glands of 12-week-old wild-type and virgin MTA1-TG mice. Cytokeratin 18 used as a control for epithelial cell content. Vinculin was used as a loading control.

mammary glands, we examined whole-mount preparations from littermates with matching estrous cycles at different developmental stages. During puberty, the MTA1-TG ducts grew faster than the ducts of age-matched wild-type mice (Fig. 2A, part b). The distance between the end of terminal end-buds (TEB) and the center of the lymph node of MTA1-TG ducts

was more than twice as long as that in wild-type mice at 6 weeks of age (Fig. 2A). MTA1-TG animals also showed extensive lateral branching when compared with the smooth surface seen in the ducts of wild-type mice (compare Fig. 2B, parts a,b with Fig. 2B, parts d,e) and often hyper-dilated ducts (Fig. 2B, part f). Furthermore, the extensive lateral branching from the mature secondary ducts resulted in a gland resembling that of a female in early pregnancy (Fig. 2B, part d). Increased budding in the mammary glands of MTA1-TG was also evident when ducts of similar lengths from control and transgenic mice were compared (compare Fig. 2B, part c and Fig. 2B, part h). Moreover, the mammary glands of virgin MTA1-TG mice contained many lobuloalveolar buds, which are normally associated with hormonal stimulation during pregnancy (Fig. 2B, part g). Some of the ducts in transgenic mice exhibited regions with indistinct epithelial-stromal boundary (Fig. 2B, part i). We consistently noticed the previously mentioned phenotypic changes in mammary glands from MTA1-TG founder lines 30, 31 and 33. To investigate whether the noticed precocious alveolar development was accompanied by functional differentiation, we examined the expression of the milk protein β-casein in the virgin mammary gland. As expected, no casein expression was detected in the gland of wild-type virgin mice (Fig. 2C). By contrast, β-casein was expressed in as early as 12 weeks in the mammary glands from virgin MTA1-TG mice (Fig. 2C). In addition, these glands also expressed increased levels of β-catenin, another indicator of precocious differentiation (Fig. 2D) (Imbert et al., 2001).

Decreased pregnancy-associated ductal and alveolar morphogenesis in MTA1-TG mice

Both whole-mount analysis and histological examination of the developmental changes occurring throughout pregnancy in mammary glands of MTA1-TG mice revealed a decreased pregnancy-associated morphogenesis as early as 7 days into pregnancy and continuing though mid-pregnancy (15 days) to day 2 of lactation (Fig. 3A; upper panels, wild-type; lower panels, transgenic). Histological studies of the gland at day 2 of lactation revealed a lower density of alveolar lobules than in corresponding glands of wild-type mice. Despite their decreased density, however, these lobules did not appear underdeveloped (Fig. 3A,B). Although the lobules do not appear underdeveloped, they do appear less distended than wild type (Fig. 3A,B).

Impaired proliferation in MTA1-TG mammary epithelium

We wanted to determine whether the phenotype observed in MTA1-TG mice during virgin state and pregnancy stages was associated with the level of proliferation in the glands. To address this, we examined the degree of proliferation in the mammary epithelium of MTA1-TG mice during virginity and pregnancy. Wild-type and transgenic female mice were pulsed labeled with the BrdU before they were sacrificed, and the proliferative indices were calculated as a percentage of the BrdU-positive cells in the ductal and alveolar regions per the total number of epithelial cells. The proliferative index of the alveolar epithelium of 6-week-old virgin MTA1-TG (13.94±2%) mice was markedly increased relative to the wild-type mice (7.3±1.71%) (Fig. 4A,B). At 12 weeks in virgin mice, the defect was even more dramatic (11.6±3.2% versus

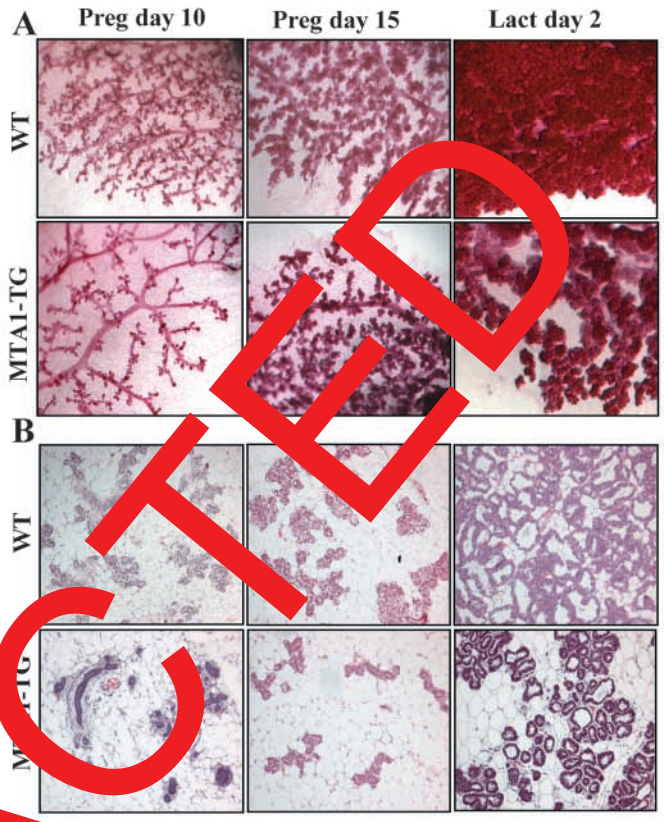

Fig. 3. Impaired pregnancy-associated morphogenesis in MTA1-TG mice. Whole-mount (A) and histological analysis by Hematoxylin and Eosin stained sections (B) of number 4 abdominal glands on days 10 and 15 of pregnancy, and day 2 of lactation in wild-type and MTA1-TG mice.

1.85±0.25) (Fig. 4A,B). By contrast, day 10 of pregnancy, the proliferation rate in MTA1-TG mice was approximately half the rate of the wild-type mice (6.4% versus 14.3%) (Fig. 4A,B).

Increased ductal growth in MTA1-TG ovariectomized mice

Ovarectomy was performed on wild-type and MTA1-TG females at 21 days of age to determine whether estradiol production at puberty is required for ductal growth and side branching in MTA1-TG glands. Ovarectomy of wild-type and MTA1 transgenic mice were performed at 21 days of age, and mice were sacrificed at 8 weeks and 12 weeks of age. As expected, the mammary gland of the ovarectomized wild-type mice consisted of an only a rudimentary ductal structure, appearing very similar to ERα knockout mice (Fig. 5A, parts a,c). By contrast, the mammary gland of MTA1-TG mice yielded a complete mammary ductal outgrowth at 12 weeks of age, similar to epithelial ductal structure seen in the wild-type mice without ovarectomy at 8 weeks of age (Fig. 5A, part d).

MTA1 induce ductal growth in male mice in the absence of hormonal stimuli

As MTA1-TG female mice show excessive side branching and lobular development in the absence of the hormonal stimulation associated with pregnancy, we asked whether this

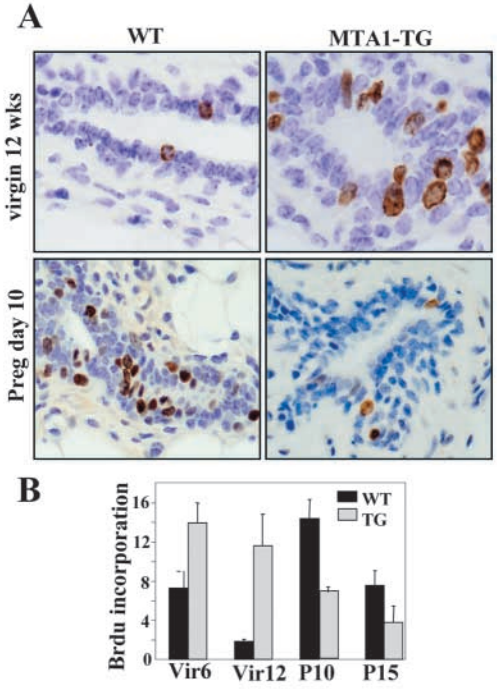

Fig. 4. Impaired cell proliferation in ductal and alveolar epithelium of MTA1-TG mice. (A) BrdU incorporation into the nuclei of mammary epithelial cells from wild type and MTA1-TG virgin at 12 weeks of age and on day 10 of pregnancy. (B) Quantitation of BrdU incorporation in the nuclei of mammary epithelial cells at 6 and 12 weeks in virgin mice, and at day 10 and day 15 of pregnancy in wild-type and MTA1-TG mice. Five thousand cells per mouse were counted, and six mice per line were examined. The proliferative index was calculated as follows: (number of BrdU labeled cells/total number of cells)×100. Student's *t*-test, $P<0.01$. Error bars indicate s.e.m.

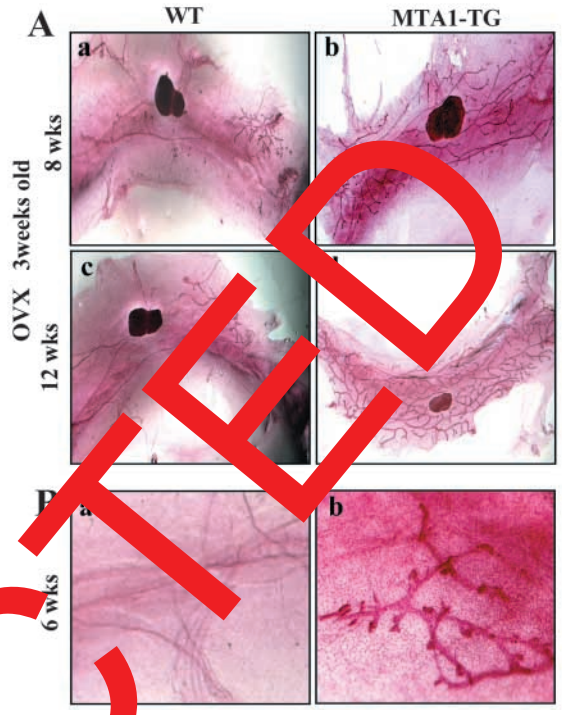

Fig. 5. Ductal growth in the mammary glands of ovariectomized MTA1-TG mice and MTA1-TG male mice. (A) Whole mounts of inguinal mammary glands stained with carmine alum from wild-type (a,b) and MTA1-TG (b,d) mice ovariectomized at 3 weeks of age and allowed after 5 or 9 weeks are shown. (B) Whole mount of 6-week-old wild-type (a) and MTA1-TG (b) male littermates.

phenotype could also be induced in males, where the complete absence of ovarian hormone function. Mammary gland develops as epidermal invaginations during embryogenesis in a process that is similar in both males and females up to day 14. At this point they are pinched off in males by androgen-induced mesenchymal constriction (Sakakura, 1987). In the wild-type mice used in this study, the epithelial rudiment in the male gland is completely destroyed in response to fetal androgens (Fig. 3, part). By contrast, in male MTA1-Tg mice this rudiment exhibited limited progression from the nipple region to the lymph nude. The ducts were covered in lobuloalveolar structures (Fig. 3B, part b).

Isoform-selective modulation of progesterone receptor expression by the MTA1-TG

It is generally accepted that appropriate cellular responsiveness to progesterone depends on the regulated expression and/or activity of the two forms of PR. Thus, inappropriate progesterone signaling caused by an imbalance in the expression and/or activities of the two forms of PR could lead to an aberration in normal mammary gland development (Soyal et al., 2002). Because, as previously discussed, we discovered that the phenotypes of MTA1-TG mice resemble those of PR-A TG mice (extensive lateral branching) (Shyamala et al., 1998), we next attempted to determine whether the extensive side branching in MTA1-TG mice glands was caused by regulation of the PR isoform by MTA1. To do so, we examined the levels of PR transcripts by RT-PCR. Because the only difference between PR-A and PR-B is in their C-terminal region, we designed primers that are specific for PR-B or for total PR (Fig. 6A). Interestingly, RT-PCR showed a marked reduction in PR-B transcripts in the MTA1-TG mammary glands, compared with wild-type glands, suggesting an increase in the level of PR-A transcripts (Fig. 6A). Immunoblot analysis to show the status of PR isoforms also revealed upregulation of the PR-A isoform in the virgin mammary glands from MTA1-TG mice (Fig. 6B). We were unable to detect either progesterone receptor isoforms in wild-type mice, possibly because of the low levels in total lysate of mammary gland (Schneider et al., 1991). In brief, the observed up regulation of PR-A and a downregulation of PR-B in the transgenic mammary gland may partially account for the lack of hormonal dependency for growth in the virgin gland and for the delayed or retarded development of alveolar-lobular structures during pregnancy.

PRs are expressed exclusively in the mammary epithelium in a pattern that is mostly segregated from proliferating cells (Seagroves et al., 2000; Sivaraman et al., 2001) and function in a paracrine manner to regulate alveolar morphogenesis in PR-negative cells (Brisken et al., 1998). To determine whether the phenotype observed in MTA1 transgenic mice was due to difference in levels of PR isoforms, we compared the expression of PRs using immunohistochemistry. Immunolocalization of PR suggested an increased expression

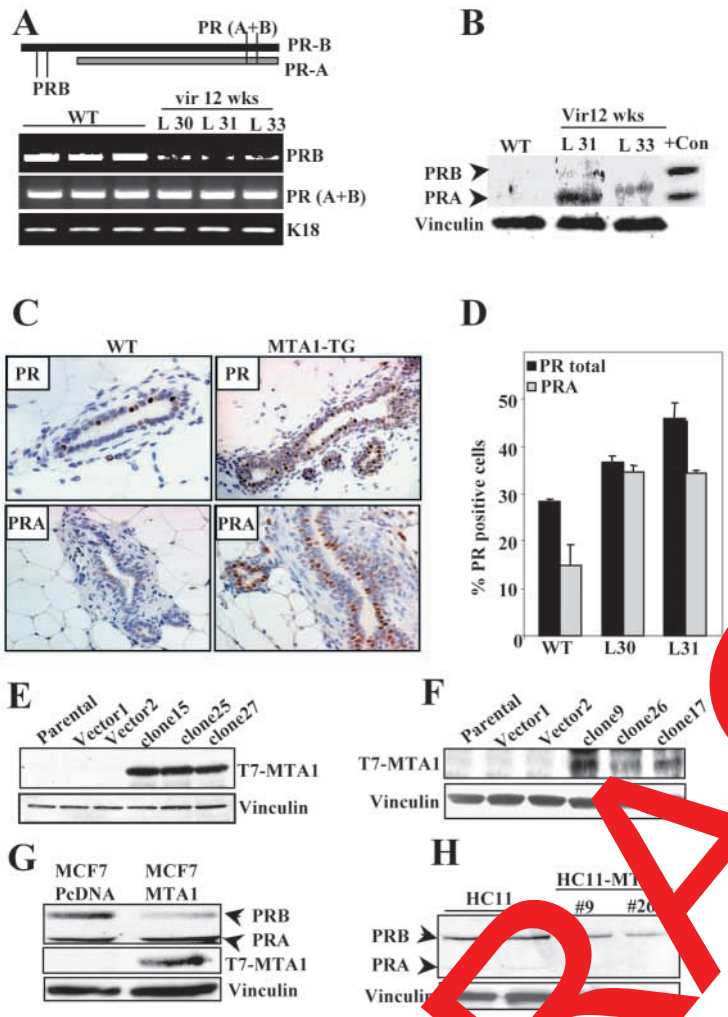

Fig. 6. Differential regulation of progesterone receptor isoform by MTA1. (A) RT-PCR analysis of PR isoforms and K18 expression in 12-week-old virgin MTA1-TG and wild-type female mice. (B) Western blot analysis of PR isoforms in 12-week-old virgin MTA1-TG (line 31 and line 33) and wild-type female mice. (C) IHC detection of PR and PRA isoform in mammary glands of wild-type and MTA1-TG mice. (D) Quantification of PR staining. Western blot analysis of T7-MTA1 expression in MCF-7/MTA1 (E) and HC11/MTA1 (F), and control clones using an anti-T7 monoclonal antibody. Western blot analysis of PR isoforms expression in MCF-7/MTA1 (G) and HC11/MTA1 (H).

of total PR in MTA1-TG mi (Fig. 6C). To cifically determine the status of these R-A PR-B forms, we then used the previously cha terized Ab hPR-a7, which selectively recognize the R-A isoform in C assays (Clarke et al., 1987). Consiste with our previous findings shown in Fig. 6A,B the mam ry glan of MTA1-TG mice showed significantly lowe vels of P B (Fig. 6C,D) and higher levels of PR-A than the l ls in d-type mice (Fig. 6C,D).

To define the effect MTA1 on e PR pathway, we next used stable 7 (Fig.) or C11 mouse epithelial cell clones ex essing 7-tagged 1 or control vector (Fig. 6F). HC11 i clonal m mmary epithelial cell line that was isolated from e mam g of mid pregnant BALB/c mice which e es strogen receptor (Ball et al., 1988; Faulds et al., 2004). examined the status of the PR isoform in MCF-7/MTA1 stable ne #25 (Fig. 6G). The MCF-7/MTA1 clones showed a reduced evel of the PR-B isoform compared with the control MCF-7/vector cells (Fig. 6G), suggesting a change in the ratio of PR isoforms. The levels of B isoform were also reduced in HC11/MTA1 cells when compared with the levels in vector-transfected HC11 cells (Fig. 6H). In brief, these findings suggested that MTA1 promotes selective downregulation of PR-B, and an alteration in the ratio of PR-A and PR-B isoforms.

Impaired c lin D and Bcl-X expression in MTA1-TG ice

Becaus clin D1 is a G1 cyclin expressed in mam ry helial cells and because the mammary glan of pregn cyclin D1 knockout females exhibited a defect similar to t in MTA1-TG females (Fantl et al., , we examined yclin D1 expression in mammary glands of wild-type and MTA1-TG. We found that cyclin D1 expres in 12-week-old virgin MTA1-TG mice was two- to thr fold greater than that in wild-type mice (Fig. 7A). B contrast, there was about a 50% reduction the cyc D1 level at day 15 of pregnancy in MTA1-TG pared with that in wild-type mice (Fig. 7A). At lactation day 2, the reduction in cyclin D1 expression was more dramatic (Fig. 7A). These results suggested that the proliferation defect in the MTA1-TG mammary epithelium of pregnant mice was due to impaired cyclin D1 expression. However, cyclin D1 was upregulated in the MCF-7/MTA1 clones, similar to MTA1-virgin mammary gland (Fig. 7B). In breast cancer cells, although some genes are regulated by progesterone through both PR isoforms, most genes are uniquely regulated through one or the other isoform and predominantly through PR-B. Expression of the gene encoding the anti-apoptosis protein Bcl-X_L is uniquely regulated by PR-A (Richer et al., 2002). We reasoned that regulation of PR isoforms in MTA1-overexpressing cell lines might affect the expression of PR downstream target genes. We observed that Bcl-X_L levels were increased in MTA1-transgenic mice during virgin and pregnancy and lactation (Fig. 7C). The HC11/MTA1 cells also showed increased levels of Bcl-X_L (Fig. 7D).

Delayed involution in MTA1-TG mice

We asked whether MTA1-TG mammary glands were able to involute correctly after cessation of lactation. At day 1 of involution, alveolar structure, which comprises a single layer of epithelial cells surrounding a lumen, is observed in mammary glands from both normal and transgenic mice (Fig. 8A, parts a,b). No distinct morphological differences are apparent between the two glands at this stage. At day 3 of involution, the alveolar structures in mammary gland from wild-type mice have started to collapse, and numerous apoptotic bodies are apparent in the ductal lumens (Fig. 8A, part c). In the mammary gland of transgenic mice, however, the alveoli have not yet begun to collapse, and remain hyperplastic (Fig. 8A, part d). This discrepancy between wild-type and transgenic glands persists even up to day 21 (Fig. 8A, parts f,h), the time at which

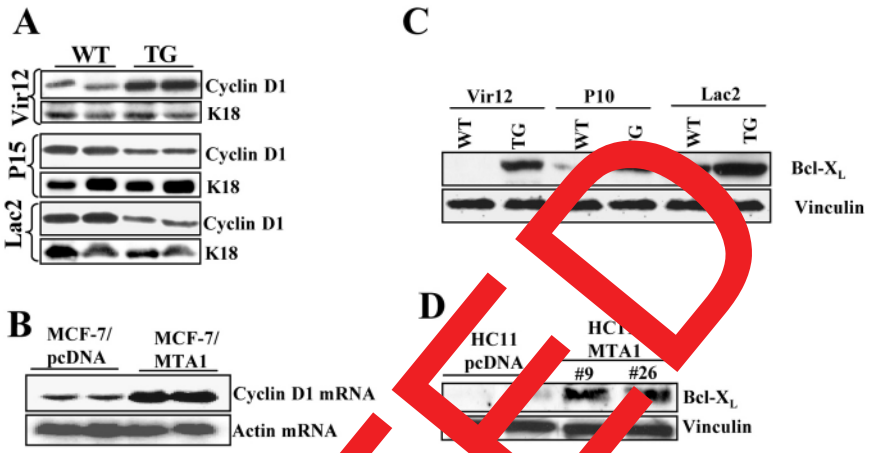

Fig. 7. Impaired cyclin D1 and Bcl-X_L expression in MTA1-TG mice. (A) Western blot analysis of cyclin D1 and cytokeratin 18 in wild-type and MTA1-transgenic from virgin 12 weeks, pregnant 15 days and lactation day 2 mammary glands. (B) Northern blot analysis of cyclin D1 mRNA level in MTA1-MCF7 overexpressing cell line. mRNA was extracted from MCF-7/PCDNA and MCF-7/MTA1 cells. Northern blots were prepared and probed with cDNAs encoding cyclin D1 and K18 as a control to permit normalization for epithelial content of the mRNA samples. (C) Western blot of total proteins from the inguinal mammary glands at various stages of development, virgin 12 weeks, pregnancy day 10, and lactation day 2 with anti-Bcl-X_L and anti-vinculin antibodies on the same blot. (D) Western blot analysis of Bcl-X_L and vinculin in HC11/pcDNA and HC11/MTA1 overexpressing cells.

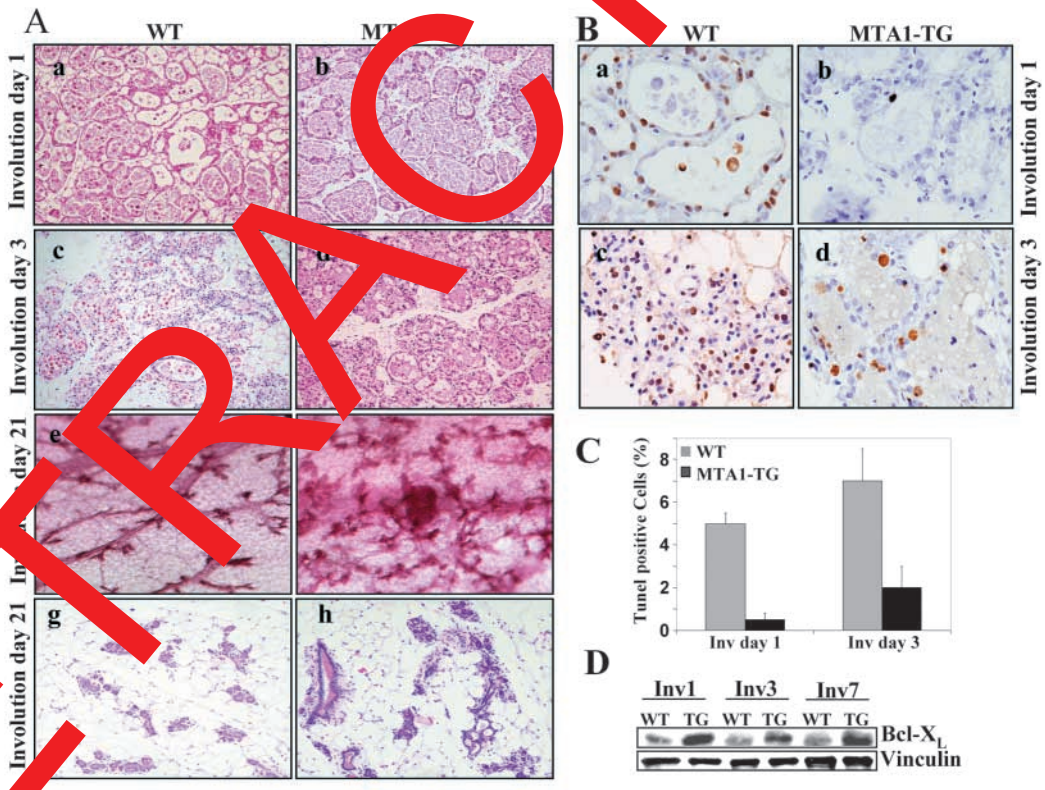

Fig. 8. Involution is severely affected in the mutant mammary glands. (A) Hematoxylin and Eosin staining of mammary glands from wild-type mice (a,c,g) and mammary glands from MTA1-TG mice (b,d,h). Day 1 of involution (a,b), day 3 of involution (c,d), day 21 of involution (g,h). Carmine Red-stained whole mounts of mammary glands at day 21 of involution (e,f). Hyperplastic nodules are present in MTA1-TG mice. (B) TUNEL analysis of wild-type (a,c) and MTA1-TG (b,d) involuting mammary gland 1 day (a,b) and 3 days (c,d) of involution. (C) Quantification of tunnel-positive cells. Ten 200× magnification fields of view were randomly counted. The apoptotic index was calculated as follows: (number of TUNEL positive cells/total number of cells)×100. Statistical analysis was performed using Student's *t*-test. Error bars represent s.e.m. (D) Western blot analysis of Bcl-X_L expression in wild-type and MTA1 transgenic mice at days 1, 3 and 7 of involution (Inv). Vinculin was used as a loading control.

involution and mammary gland remodeling is considered complete in wild-type mice (Fig. 8A, parts e,g). However, as virgin MTA1-TG mice already have a mid-pregnancy phenotype, their involution does not result in full loss of alveolar structure of differentiation (Fig. 8A, parts f,h). To determine whether the delayed involution observed in MTA1 transgenic mice was accompanied by a decrease of apoptosis, TUNEL analysis was performed on sections of mammary glands from both wild-type and transgenic mice. Significantly less apoptosis was apparent by day 1 of involution in MTA1-TG mice (0.5±0.2) compared with the wild-type mice (5±1; n=5) (Fig. 8B,C). The reduced apoptosis seen in MTA1-TG mice prompted us to investigate the levels of Bcl-X_L, which suppress apoptosis in several systems (Adams and Cory, 1998), has been shown to be upregulated at the start of involution (Heermeier et al., 1996) and may prevent epithelial apoptosis during the initial phase of involution (allowing this phase to be reversed if necessary). Western blot analysis of Bcl-X_L showed an increase in Bcl-X_L in glands from MTA1-TG at days 1, 3 and 7 of involution compared with the wild-type mice (Fig. 8D).

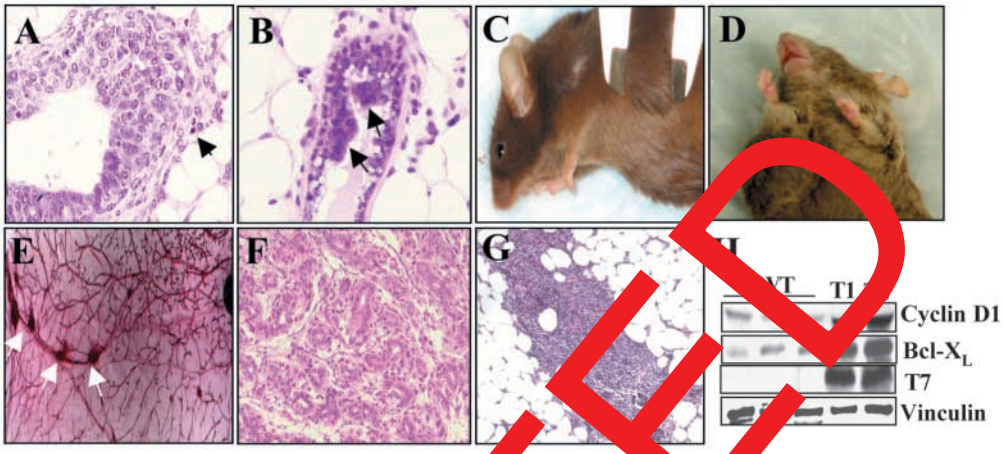

Fig. 9. MTA1 transgenic mice develop mammary gland hyperplasia, hyperplastic nodules and mammary tumors. (A) Hematoxylin and Eosin staining showing ductal hyperplasia in mammary glands from 6 weeks virgin MTA1-transgenic mice. Pleomophic nuclei and mitotic figures are present (arrows). (B) MTA1-TG mammary glands after one pregnancy showed intra-luminal focal hyperplasia. Note the ductal epithelial cells contain multi-layers and protrude into the lumen (arrows). (C) Founder mice line 30 with single large mammary tumor on the thoracic mammary gland. (D) Founder mice line 31 with a single large tumor on the thoracic mammary gland. (E) Whole-mount staining of number 4 right inguinal mammary glands from 18-month-old virgin MTA1 transgenic mice with several hyperplastic nodules (arrows). (F) Hematoxylin and Eosin-stained section of mammary adenocarcinoma in a 15-month-old multiparous MTA1-TG female. (G) Hematoxylin and Eosin-stained section of malignant lymphomas in a 24-month-old multiparous MTA1 transgenic mammary gland. (H) Western blot analysis of cyclin D1 and Bcl-X_L in mammary tumors from MTA1 transgenic mice (T1, T2) and wild-type mouse mammary tissue (WT). The blot was reprobed with T7 antibody to show transgene expression. Vinculin was used as a loading control.

MTA1 transgenic females develop hyperplastic nodules and mammary tumors

In the virgin MTA1-TG mammary glands, we observed the ductal hyperplasia (Fig. 9A), intra-luminal focal ductal hyperplasia (Fig. 9B) with areas of atypical proliferation and mitotic figures (Fig. 9A). Intriguingly, whole-mount analysis of 8- to 15-month-old MTA1 transgenic females revealed the presence of focal hyperplastic nodules (Fig. 9E). These nodules appeared in 30% of the transgenic females (21 out of 70) from three independent lines. Both nulliparous and multiparous transgenic females developed these lesions, indicating that pregnancy was not required for neoplastic transformation. In many cases, there were multifocal lesions per gland, suggesting independent stochastic transformation of the mammary epithelium (Fig. 9E). The incidence of hyperplastic nodules may be underestimated because only one mammary gland per animal (the ten glands in a female mouse) was subject to whole-mount analysis. No such lesions were observed in over 127 wild-type littermate females (data not shown). A fraction of (5 out of 70; 7%) of the transgenic females developed visible masses 1-2 cm in diameter in their mammary glands after 10-18 months (Fig. 9C,D). Pathology analysis revealed that two out of five mice masses represented full-blown mammary adenocarcinomas and three tumors represented malignant lymphomas in the mammary glands. None of more than 100 wild-type littermate females in our colony developed mammary tumors during this time. We next assessed MTA1 transgene expression in tumors of transgenic mice by western blot analysis using anti-T7 tag antibody. The tumor of line 30 (T1, adenocarcinoma) and line 31 (T2, lymphoma) displayed high levels of MTA1 transgene expression (Fig. 9H). A dramatic increase in expression of cyclin D1 and Bcl-X_L was also found in tumor samples compared with the wild-type samples (Fig. 9H).

Discussion

In this study, we describe the phenotypes of transgenic mice with deregulated expression of MTA1 during mammary gland development. We report that the overexpression of MTA1 in the mammary gland resulted in increased ductal extension, enhanced ductal branching and proliferation, an accelerated lobuloalveolar-like precocious differentiation, decreased pregnancy associated morphogenesis, delayed involution and tumorigenesis, suggesting that MTA1 is an important factor controlling mammary epithelial cells during normal mammary gland development and mammary gland cycling. Our findings of enhanced ductal extension and ductal side branching, together with increased BrdU incorporation, suggested that mammary epithelial cell proliferation was deregulated in the mammary glands of virgin MTA1-TG mice. The increased cell proliferation in the virgin stage is accompanied by elevated cyclin D1 expression in the absence of pregnancy. There was also increased ductal growth in the glands of mammary gland in the ovariectimized MTA1-TG mice, suggesting that an estrogen-independent mechanism was responsible for the increased ductal growth noted in the MTA1-TG mammary glands. In this context, it is possible that the increased cyclin D1 regulates ER-dependent pathways important in precocious differentiation, as cyclin D1 has been shown to upregulate the ER-dependent pathway in a ligand-independent manner (Zwijsen et al., 1998).

Analysis of mammary glands of virgin MTA1-TG mice revealed precocious lobuloalveolar development and increased levels of the milk protein β-casein. The MTA1 transgene may induce precocious development by extending the lifespan of differentiated mammary epithelial cells with each estrous cycle, hence causing differentiated mammary epithelial cells and milk-secreting lobuloalveoli to accumulate. Our finding that the β-catenin level was increased in virgin MTA1-TG mice is consistent with results from a previous study of MMTV-N89 β-catenin and MMTV-cyclin D1 showing precocious mammary gland development (Wang et al., 1994; Imbert et al., 2001). In addition, the early morphogenic phenotype of MTA1-TG mammary gland also resemble to mammary phenotype found in MMTV-Wnt1 transgenic mice which induce ductal

hyperbranching, adenocarcinomas in females and male ductal extension (Tsukamoto et al., 1988).

We further found that MTA1 expression in the mammary epithelium resulted in the downregulation of the PR-B isoform and upregulation of the PR-A isoform. This has been consistent with the findings that the introduction of additional PR-B isoform prematurely arrests ductal growth without altering the potential for lobuloalveolar growth (Shyamala et al., 2000). In addition, despite a robust lobuloalveolar growth in the transplants of PR-B transgene mammary glands, there was a limited lateral ductal branching and almost no functional differentiation (Shyamala et al., 2000). Thus, the increased ductal growth and lateral branching seen in virgin glands of MTA1-TG mice could be caused by downregulation of PR-B. Another interesting feature of mammary epithelium in MTA1-TG mice was its resemblance to that of PR-A TG mice. Both animal models showed excessive lateral branching in virgin mammary gland, loss in basement membrane integrity, characteristics commonly associated with transformed cells. Similar to the mammary epithelial cells of PR-A TG mice, the gland of adult MTA1-TG mice contained some very thick ducts resembling those seen in early pregnancy. Histological analysis revealed the glands of MTA1 transgenic mice contained ducts composed of multilayered luminal cells, in contrast to the monolayer associated with the normal ducts. This phenotype was also observed in PR-A transgenic mice (Shyamala et al., 2000). Furthermore, in the aberrant mammary epithelial structures in PR-A TG mice, there is an increase in cyclin D1 expression accompanied by an increase in cell proliferation (Chou et al., 2003). Therefore, it is likely that there is an increased responsiveness to progesterone in MTA1-TG mice due to the increase in total PR levels and that progesterone-induced growth may require the coordinated actions of PR-A and PR-B. Ligand-induced ER is also likely to be disrupted by the overexpression of MTA1. Together, these observations suggest that increased ductal growth and extensive ductal branching in MTA1-TG mice result from alterations in the ratios of PR isoforms.

We have demonstrated that MTA1 overexpression in the mammary glands of pregnant transgenic mice results in a reduced density of alveoli, a defect that is a consequence of reduced ductal and alveolar epithelial cell proliferation. It is possible that this phenotype was caused by a decrease in PR-B level and downregulation of cyclin D1 in pregnant MTA1-TG mice. These results are consistent with those from a recent study showing that the selective activation of PR-A in PR-B knockout mice can impair progesterone-dependent ductal branching and alveolar morphogenesis during pregnancy (Mukherjee-Jericevich et al., 2003). Thus, upregualtion of PR-A and downregulation of PR-B in MTA1-TG mammary glands may partially account for the lack of hormonal dependency of growth in the virgin gland and for the delayed and retarded development of alveolar-lobular structures during pregnancy.

We have demonstrated that mammary glands of the MTA1-transgenic mice show a delay in involution. Although the mammary glands of the MTA1-TG mice eventually undergo involution, it appears that fewer epithelial cells are lost than in wild-type regressed mammary glands. Delayed involution seen in MTA1-TG mice correspond with a delay in the onset of apoptosis and upregulation of anti-apoptotic molecule Bcl-X_L, a target of PR-A.

We demonstrate for the first time that MTA1 play a role in tumorigenesis of the mammary gland in an MMTV-LTR driven mouse model. Histological analysis of the mammary tumors showed two adenocarcinoma and three lymphomas. In addition, 30% of transgenic mice developed hyperplastic nodules in the mammary gland. This observation raises the possibility that the presence of the MTA1 transgene in mammary glands may result in the retention of epithelial structures, particularly in uniparous and multiparous mice, which could lead to the development of hyperplasia and tumors over time. The fact that MTA1 overexpression upregulate PR-A isoform in the mammary gland could explain in part the mechanism of tumor formation in MTA1 transgenic mice. These finding are consistent with previous studies indicating that overexpression of PR-A in PR-positive tumors may be associated with a more aggressive state. Although the ratios of PR-A and PR-B appear to be equivalent in the normal mammary gland, a subset of PR invasive tumors show an imbalance of PR-A and PR-B in favor of PR-A (Mote et al., 2001; Mote et al., 2002; Graham et al., 1995). MTA1 overexpression in human breast cancer cells also promotes an aggressive phenotype to the cells and induces tumorigenicity in nude mice (Mazumdar et al., 2001) (R.B.-Y. and R.K., unpublished). In addition, the ratio of PR isoforms was also deregulated in MTA1 overexpressing breast cancer cells. The fact that cyclin D1 was also upregulated in MTA1-deregulate breast cancer cells as well as in virgin transgenic mice as well as MTA1-TG tumors suggested that MTA1 is not a universal repressor and that cyclin D1 upregulation could also contribute to tumorigenesis in mammary gland. It is interesting to note that co-repressors and co-activators (apparently molecules with opposite functions) could be found in the same complex because of a highly dynamic nature of the target gene chromatin **(**Perissi et al., 2004**)**. In this context, as MTA1 has been shown to interact with co-activators (Mishra et al., 2003; Talukder et al., 2003), it is possible that MTA1 may influence gene expression by multiple mechanisms.

The observation that Bcl-X_L was upregulated in MTA1-TG induced breast tumors is important as it raises the possibility of involvement of Bcl-X_L in to the formation of hyperplastic nodules and breast tumors in MTA1-TG mice. Indeed, overexpression of the anti-apoptotic protein Bcl-X_L has been implicated in the development, progression and drug-resistance in tumors (Strasser et al., 1997). Furthermore, Bcl-X_L also plays a crucial role in protecting cells from DNA damage, regardless of whether or not they have mutations in p53 pathway (Deverman et al., 2002; Klocke et al., 2002; Maclean et al., 2003), and Bcl-X_L expression in tumors is also considered a good predictor of response to therapy and prognosis (Sjostrom et al., 2002; Vilenchik et al., 2002). In addition, Bcl-X_L upregulation is widely associated with a higher tumor grade and increased number of nodal metastases, and, hence, implicated as an inhibitor of apoptosis during later stages of the disease (Olopade et al., 1997). Together, these findings establish that MTA1 plays an important role in mammary gland development and tumorigenesis.

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