Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β1

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The transforming growth factors β (TGFs-β) are potent inhibitors of cell proliferation and are usually secreted in a latent form. TGF-β1, TGF-β2, and TGF-β3 are expressed in distinct but overlapping patterns in the developing mouse mammary gland. To study the role of transforming growth factor-β1 (TGF-β1) in normal mammary development and in mammary neoplasia, we have constructed three transgenic mouse lines that express a simian TGF-β1 5223/225 mutated to produce a constitutively active product under the control of the MMTV enhancer/promoter. Expression of the transgene, as confirmed by in situ hybridization, immunohistochemistry, and Northern blot analysis, was associated with marked suppression of the normal pattern of mammary ductal tree development in female transgenics. Reduction in total ductal tree volume was observed at 7 weeks, soon after estrous begins, and was most apparent at 13 weeks, as ductal growth in the normal mammary gland declines. This effect was seen in all three lines. However, during pregnancy, alveolar outgrowths developed from the hypoplastic ductal tree, and lactation occurred, therefore all transgenic females could feed full litters. Unlike many other transgenic mouse models in which expression of growth factors or oncogenes under control of the MMTV promoter leads to mammary epithelial hyperplasia and increased tumor formation, the MMTV–TGF-β1 5223/225 transgene causes conditional hypoplasia of the mammary ductal tree and no spontaneous tumors have been detected in the MMTV–TGF-β1 5223/225 transgenic animals.

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Transforming growth factor-β1 (TGF-β1) is the prototype of a family of polypeptides involved in growth control, extracellular matrix production, and development (for review, see Moses 1990). The TGFs-β can have marked stimulatory effects on connective tissue formation and may be angiogenic in vivo. They are chemotactic for fibroblasts, indirect mitogens for certain mesenchymal cells, and stimulators of extracellular matrix deposition. The TGFs-β are also potent inhibitors of proliferation of most cell types in culture, and in vivo studies have indicated that the predominant effect of the TGFs-β on cell proliferation is inhibition (Moses et al. 1990). TGF-β1, TGF-β2, and TGF-β3 show different patterns of expression in adult tissues (Miller et al. 1990) and different patterns of auto- and heterologous induction in cultured mouse embryo fibroblasts (Bascom et al. 1989). They also show different patterns of mRNA and protein expression during mouse embryogenesis as determined by in situ hybridization and immunohistochemistry (Pelton et al. 1990, 1991).

There is considerable evidence that the TGFs-β play an important role in mammary gland development, which is predominantly a postnatal event. The rudimentary gland in newborn pups proliferates under the influence of maternal hormones but is then quiescent until the pup is ~6 weeks old (Topper and Freeman 1989). A combination of systemic and local factors determines the rate and pattern of ductal growth into the fat pad (Haslam 1991). TGF-β1, TGF-β2, and TGF-β3 are expressed in the mouse mammary gland. They exhibit overlapping patterns of expression within the epithelium of the actively growing mammary end buds during branching morphogenesis, as well as within the epithelium of growth quiescent ducts (Robinson et al. 1991).
TGF-β3 is the only isoform detected in myoepithelial progenitor cells of the growing end buds and myoepithelial cells of the mature duct. Silberstein and Daniel (1987) demonstrated reversible inhibition of mammary gland epithelial cell growth by TGF-β1 administered in a slow-release pellet. Subsequent studies have shown that administration of exogenous TGF-β1, TGF-β2 or TGF-β3 into developing mammary glands results in the disappearance of the proliferating stem cell layer at the end buds and rapid involution of the ductal end buds (Robinson et al. 1991). The TGFs-β also cause increase in connective tissue deposition in front of treated end buds (Silverstein et al. 1990). Interestingly, exogenous TGFs-β do not inhibit alveolar morphogenesis during pregnancy (Daniel et al. 1989; Robinson et al. 1991).

In contrast to the TGFs-β, TGF-α is a mitogen for most cell types and may also play a significant role in mammary gland development. TGF-α is localized in vivo in the epithelial cap-cell layer of the advancing end buds and in stromal fibroblasts at the base of terminal buds (Snedeker et al. 1991). Furthermore, implantation of pellets containing TGF-α into regressed mammary glands of ovariectomized mice stimulated the reappearance of end buds (Snedeker et al. 1991). Significantly, overproduction of TGF-α in transgenic animals using either mouse mammary tumor virus (MMTV) promoters that cause autocrine stimulation of epithelial cells (Matsui et al. 1990) or a metallothionein promoter that gives paracrine stimulation of epithelial cells (Jhappan et al. 1990; Sandgren et al. 1990) leads to mammary epithelial hyperplasia and a marked increase in breast cancer development, particularly in multiparous animals. These results are similar to those obtained in transgenic mice carrying the c-myc (Stewart et al. 1984; Sinn et al. 1987), wnt-1 (Tsukamoto et al. 1988), int-2 (Muller et al. 1990), or neu (Bouchard et al. 1989; Guy et al. 1992) oncogenes under control of the MMTV promoter/enhancer.

We were interested in performing similar transgenic studies with the growth inhibitor TGF-β1. However, TGF-β1, similar to TGF-β2 and TGF-β3, is secreted in a latent form (Lawrence et al. 1984; Lioubin et al. 1991; Olofsson et al. 1992; Miyazono et al. 1993). Initial studies with a wild-type TGF-β1 cDNA linked to the MMTV long terminal repeat (LTR) gave no phenotype, perhaps because of lack of activation of the latent TGF-β1 (Y. Matsui, B.L.M. Hogan, and H.L. Moses, unpubl.). The latent form of TGF-β1 consists of the 25-kD mature TGF-β1 in a noncovalent association with the aminoterminal glycopeptide portion of the TGF-β1 precursor resulting in a 110-kD latent complex (Lyons and Moses 1990). Latent TGF-β1 released from platelets has an additional binding protein that is the product of a gene different from the TGF-β1 gene (Miyazono et al. 1993). The importance of this binding protein is not known as it is not necessary for maintenance of the latent state (Lyons et al. 1990). Latent TGF-β1 can be activated by treatment with acid, bases, chaotropic agents, or proteases [for review, see Lyons and Moses 1990]. Site-directed mutagenesis of cysteines in the pro region of the pre-pro form of TGF-β1 generates TGF-β1 S223/225, which encodes a TGF-β1 that is mostly in the active form when secreted (Brunner et al. 1989). This provided the appropriate construct for use in transgenic studies.

Three lines of mice carrying the MMTV–TGF-β1 S223/225 transgene have been generated and all show a similar suppression phenotype of mammary ductal development. Unlike many other transgenic mouse models in which expression of growth factors or oncogenes under control of the MMTV promoter leads to mammary epithelial hyperplasia and increased tumor formation, the MMTV–TGF-β1 S223/225 transgene causes conditional hypoplasia of the mammary ductal tree, and there is no increase in spontaneous mammary tumor formation.

Results

Construction of MMTV–TGF-β1 S223/225 transgenic mice

Transgenic mice expressing simian TGF-β1 under the control of the complete, hormone-inducible, MMTV LTR promoter/enhancer were generated by injecting one-cell embryos with the construct shown in Figure 1A. The mutant simian MMTV–TGF-β1 S223/225 has the cysteine codons at positions 223 and 225 [Fig. 1B] within the pro region of the precursor changed to serine codons leaving the region encoding mature, active TGF-β1 unchanged (Brunner et al. 1989). This mutation results in the production of an active TGF-β1. The vector pMTV–TGF-β1 S223/225 contains exons 2 and 3 of the rabbit β-globin gene and thus provides a splicing event upstream of the TGF-β1 S223/225 cDNA.

Founders were generated and used to establish three lines of transgenic mice (41, 46, and 66). Female mice in these three lines have been studied extensively, and all have similar but varying degrees of histological abnormalities, depending on the transgene copy number. Unlike MMTV–TGF-α female mice, which are unable to feed their young, MMTV–TGF-β1 S223/225 females can feed multiple successive litters successfully.

Identification of transgenic animals

Screening of transgenic mice was done by Southern blots of DNA isolated from mouse tissues. Copy numbers of the transgene were assessed by densitometer scanning of blots hybridized with a 32P-labeled rabbit β-globin exon 3 probe and a murine TGF-β1 probe (data not shown). Loading was normalized using a murine probe for TGF-β2, a single copy gene. The homozygous mice from lines 41 and 46 have two copies of the transgene, and line 41 mice have one copy per insertion site.

Expression of the transgene

Northern blot analysis of total RNA was carried out using a 974-bp murine TGF-β1 cDNA probe (Derynck et al. 1986) and a 986-bp simian TGF-β1 cDNA probe (Sharples et al. 1987). The regions selected were from the 5′ end of the cDNAs, where there is less similarity between
Figure 1. (A) Structure of the MMTV-TGF-β1 S223/225 construct. The stippled region corresponds to the 1.5-kb MMTV LTR. The solid regions correspond to exons 2 and 3 of the rabbit β-globin gene. The open region consists of the second intron and the 3' noncoding region of the gene. A 1.4-kb simian TGF-β1 S223/225 (hatched region) was inserted into the third intron of the β-globin gene. (B) The simian TGF-β1 S223/225 (Brunner et al. 1989) insert showing the sites of mutations at codons 223 and 225. Both encode cysteine residues in the pro region (open area) of the TGF-β1 cDNA and have been mutated to encode serines. The regions encoding mature, active TGF-β1 (hatched area) and signal peptide (stippled area) remain unchanged. These mutations result in production of a constitutively active TGF-β1 protein (Brunner et al. 1989).

sequences from the two different species (Sharples et al. 1987). Figure 2A demonstrates that the murine cDNA is specific for endogenous mouse sequences, whereas the simian probe is specific for sequences expressed from the transgene. Using the simian probe, transgene expression was demonstrated in mammary glands from 13-week virgin and 4-day lactating transgenic animals but not in the ovary from the 13-week virgin animal [Fig. 2B]. We have been unable to detect transgene expression in mammary glands from 5-week-old transgenic animals, whereas expression is observed consistently in mammary glands from 13-week transgenic females [Fig. 2C]. No hybridization was detected in kidney RNA from wild-type or transgenic animals (Fig. 2B,C) using the simian probe, whereas endogenous sequences were detected with the murine probe [data not shown]. Transgene expression at high levels was apparent in the salivary gland [Fig. 2A,C] in agreement with previous reports (Sinn et al. 1987; Matsui et al. 1990). The MMTV promoter/enhancer has also been shown previously to cause

Figure 2. Northern blot analysis of total RNA from tissues of wild-type and transgenic mice using a murine or simian TGF-β1 cDNA probe. (A) The murine probe hybridizes to TGF-β1 mRNA from the BALB/MK cell line and mammary gland from a wild-type animal [left] known to express TGF-β1 normally [Bascom et al. 1989; Robinson et al. 1991]. The simian probe hybridizes to a band present in RNA from the salivary gland of a transgenic animal and not the RNA from BALB/MK cells or tissues from wild-type mice [right], indicating that the simian probe is specific for the transgene. (B) Transgene expression is demonstrated in mammary glands from 13-week-old virgin and 4-day postpartum transgenic mice but not from kidney and ovary of transgenic mice. (C) Transgene expression is apparent in RNA from mammary and salivary glands from 13-week-old virgin transgenic mice but not from the mammary glands of a 5-week-old virgin transgenic mouse. RNA from a kidney of a wild-type mouse was included as a negative control. Loading in all lanes was monitored by using a probe to the ubiquitously expressed cyclophilin (1B15).
transgene expression in the mammary gland of virgin adult, pregnant, and postpartum mice in agreement with the present study as well as in male reproductive organs (Sinn et al. 1987; Bouchard et al. 1989; Matsui et al. 1990).

In situ hybridization using a single-stranded $^{35}$S-labeled rabbit $\beta$-globin exon 3 anti-sense riboprobe demonstrated expression of the transgene localized to the ductal epithelium of the MMTV-TGF-β1$^{5223/225}$ mice [Fig. 3A], the sense riboprobe used as a control showed only background grains [Fig. 3B]. Wild-type controls were negative, as expected [data not shown].

To test for TGF-β1 protein expression, immunohistochemical analysis was carried out using a TGF-β1-specific antibody [Pelton et al. 1991]. The mammary glands of 13-week wild-type littermates and [C57BL/6xDBA]F$_1$ (B6D2) age-matched controls revealed minimal staining in the periductal stroma, with occasional light staining in the ductal epithelial cells [Fig. 4A]. Transgenic females at 13-weeks had strong reactivity within the mammary ductal epithelial cells and little immunostaining detectable in the periductal stroma [Fig. 4B]. Greater TGF-β1 immunoreactivity was also observed in mammary ductal epithelium of 7-week-old transgenic animals in comparison with age-matched wild-type glands. In general, end buds had lower levels of reactivity than terminal ducts [Fig. 4C,D]. High levels of expression were apparent in developing mammary lobuloalveolar units of transgenic mice during early pregnancy [Fig. 4E]. Evidence of transgene expression in mammary epithelium was obtained in all three lines.

Consequences of MMTV–TGF-β1$^{5223/225}$ transgene expression

Whole-mount preparations from nontransgenic and transgenic virgin female mice revealed that transgenic mice had markedly suppressed development of the mammary ductal tree. In 7-week-old transgenic mice and matched nontransgenic controls, significant differences in the degree of ductal tree extension were seen. The wild-type D6B2 female [Fig. 5A] has numerous ductal branches extending beyond the central lymph node in the inguinal glands, as well as more numerous end buds than do transgenic mice at this stage [Fig. 5B]. Previous studies with exogenous administration of TGFs-β in slow-release pellets have shown TGFβ-induced involution of end buds (Silberstein and Daniel 1987; Daniel et al. 1989). Histological analysis was used to examine these changes in the MMTV–TGF-β1$^{5223/225}$ animals. In general, although a greater number of end buds existed in the wild-type glands, the histologic appearance of the end buds at 7 weeks was similar in transgenic and wild-type animals [Fig. 5C,D] and was in agreement with the appearance of end buds in whole-mount preparations [Fig. 5A,B]. No consistent difference was observed in the amount or character of periductal connective tissue in wild-type [Fig. 4F] versus transgenic [Fig. 4G] specimens.

By 13 weeks the effect of transgene expression was more pronounced. Virgin wild-type females exhibited extensive dichotomous branching, complete ductal arborization, extension of the mammary ductal tree to the limit of the mammary fat pad, and dwindling numbers of end buds [Fig. 6A,C]. In contrast, virgin transgenic mice typically had markedly less developed ductal trees that did not approach the fat pad borders and numerous end buds [Fig. 6B,D]. However, lateral branching, which occurs to a greater extent in D6B2 mice [Fig. 6C] than in C57 mice [Daniel et al. 1989], is markedly suppressed in transgenic animals [Fig. 6D]. This general phenotype of retarded mammary ductal development was observed to varying degrees in heterozygous animals from all three lines and the homozygous animals examined [lines 46 and 66]. The phenotype was more severe in homozygous animals that carried four copies of the transgene.

Effect of transgene expression on labeling index of mammary epithelial cells

The diminished ductal development in MMTV–TGF-β1$^{5223/225}$ transgenic animals indicated that the transgene was inhibiting end bud or ductal epithelial proliferation as TGF-β1 does in cultured mammary epithelial cells [Knabbe et al. 1987]. To estimate proliferation rates, animals were treated with bromodeoxyuridine (BrdU) and formalin-fixed, and paraffin sections were stained with anti-BrdU antibodies. Color photomicrographs were taken of end buds and terminal ducts of nonserial sections from 7-week-old animals. Approximately 150–500 cells, depending on the number present, were scored as being positive or negative for BrdU incorporation in end buds or ducts in each animal. As shown in Table 1, 7-week-old transgenic animals exhibited minimal [approximately twofold] reduction in labeling index in both end bud and terminal duct epithelial cells. In 13-week animals, only terminal ducts were scored for comparison in transgenic and wild-type animals because end buds...
had largely disappeared in the wild-type mammary gland (Fig. 6A, C). The degree of suppression of labeling index was much more marked in the ductal epithelium of transgenic animals at 13-weeks (~10-fold) [Table 1]. Interestingly, the labeling index in end buds of transgenic animals at 13-weeks was very low in comparison with end buds of transgenic animals at 7 weeks [Table 1]. This could be attributable to greater expression of the transgene at 13 weeks causing greater inhibition of epithelial proliferation.
The virtual absence of lateral branching in transgenic animals at 13-weeks (Fig. 6D) suggested that cells contributing to lateral branching may be more sensitive to inhibition by the transgene product. To test this possibility, the third glands of 12-week C57BL/6 mice were implanted with ELVAX pellets containing either bovine serum albumin or TGF-β1 [150 ng] for 48 hr, a time and dose that gave maximal inhibition in 5-week animals. The animals were then injected with 100 μCi of [3H]thymidine 1 hr before sacrifice, and the tissue was processed for autoradiography. Only epithelial cells in alveolar-like structures adjacent to the implant were counted. The results are shown in Table 2. TGF-β1 had no effect on the labeling index of the alveolar-like outgrowths comprising the lateral branching. The alveolar outgrowths occurring during pregnancy were also not inhibited in transgenic animals, even though these outgrowths showed high levels of transgene expression by immunohistochemistry (Fig. 4E). Whole-mount preparations of pregnant and lactating glands showed fully developed lobules in both transgenic and wild-type animals, but the transgenic animals had fewer lobules [data not shown]. Nevertheless, sufficient mammary tissue was present

Table 1. **Labeling index as determined by BrdU incorporation in mammary epithelium of wild-type and transgenic (line 46) animals at 7 and 13 weeks**

| Weeks | nontransgenic | transgenic |
|-------|---------------|------------|
|       | Mean ± S.D. (no. of animals) |             |
| 7     |               |            |
| end buds | 0.29 ± 0.05 (4) | 0.17 ± 0.05 (5) |
| terminal ducts | 0.11 ± 0.08 (3) | 0.05 ± 0.03 (6) |
| 13    |               |            |
| end buds | not done      | 0.02 ± 0.02 (4) |
| terminal ducts | 0.05 ± 0.02(3) | 0.005 ± 0.005(3) |
even in homozygous animals for the mothers to feed full litters of 9–11 pups. The histologic appearance of lactating mammary tissue from transgenic animals appeared very similar to that of wild type, although ductal and alveolar epithelial cells in transgenic animals frequently appeared more flattened and somewhat atrophic (data not shown).

Finally, no tumors have been found in either virgin or multiparous TGF-β1 female MMTV-TGF-β1s223/225 transgenic mice followed for > 300 days, indicating that misexpression of TGF-β1 probably does not accelerate mammary tumor formation.

**Discussion**

Overproduction of TGF-β1 in mouse mammary epithelial cells under control of the MMTV promoter/enhancer markedly retards ductal development. The TGFs-β are known to be potent inhibitors of proliferation of most nontransformed cell types in culture, including most epithelial cells, and also inhibit proliferation in vivo when administered exogenously (for review, see Moses et al. 1990). Thus, the retarded ductal development observed in this study may be the result of inhibition of cell proliferation by TGF-β1 encoded by the transgene. This view is supported by determinations of BrdU nuclear labeling indices in transgenic and control mammary epithelium (Table 1). The fact that ductal development is not suppressed completely could be attributable to low levels of transgene expression. Alternatively, specific populations of mammary epithelial cells may be resistant to the growth inhibitory effects of TGF-β1.

Previous studies involving administration of TGF-β1, TGF-β2, or TGF-β3 to mouse mammary glands using slow release-pellets have demonstrated inhibition of ductal elongation and involution of end buds, associated with localized fibrosis at the bud tips (Silverstein and Daniel 1987; Daniel et al. 1989; Robinson et al. 1991). This inhibition was observed only when implants were placed in stroma directly in front of advancing end buds, where TGF-β is not normally found. When implants were placed along differentiated mammary ducts, no excess accumulation of the periductal matrix was observed (C.W. Daniel and S.D. Robinson, unpubl.).

The findings obtained with exogenous TGFs-β are in general agreement with the present study using MMTV-TGF-β1s223/225 transgenic animals. Partial suppression of ductal elongation during puberty in the transgenics is consistent with the observation that elevation of TGF-β levels are generally inhibitory to ductal buds. That the end buds in transgenic mice were only partially inhibited may be attributed to higher levels of TGF-β released by the implants and that this release was into the stromal rather than the epithelial compartment. Interestingly, neither excess production of TGF-β1 by transgenic mice nor release of exogenous TGF-β1 by slow-release implants caused accumulation of excess matrix around the differentiated mammary ducts. This suggests the existence of regulatory mechanisms limiting the accumulation of matrix even in the presence of abundant TGF-β.

One of the more interesting aspects of these studies is the resistance of mammary epithelial cells in pregnant animals to TGF-β1-induced growth inhibition. Cell culture studies have indicated that growth of virtually all nontransformed epithelial cells is inhibited by the TGFs-β whereas resistance to this growth inhibitory effect develops frequently in neoplastically transformed cells [Moses et al. 1985; Masui et al. 1986]. The alveolar outgrowth during pregnancy in MMTV-TGF-β1s223/225 transgenic animals is almost certainly attributable to physiological resistance instead of lack of transgene expression, because immunohistochemistry indicates high levels of TGF-β1 expression in developing alveoli. In addition, exogenously administered TGF-β1 does not inhibit development of alveoli [Daniel et al. 1989].

The lateral branches observed in the D6B2 wild-type mice may be similar to the pregnancy-associated alveolar outgrowths in terms of lack of sensitivity to inhibition by TGF-β1. Exogenously administered TGF-β1 did not decrease nuclear labeling indices of cells of lateral branches of wild-type animals (Table 2). Yet, one of the more striking features of the MMTV-TGF-β1 transgenic phenotype is the virtual absence of lateral branching. There are at least two possible explanations for this discrepancy: First, there is a difference in biological effectiveness of exogenously administered TGF-β1 versus that produced endogenously with the latter being more effective; and second, expression of the transgene does not specifically affect one population (lateral branches) but causes a general retardation of ductal tree development such that lateral branching does not occur. The ability of transgenic animals to feed full litters routinely was unexpected, particularly in view of the extreme retardation of ductal development observed in 13-week animals. However, one of the functions of TGFs-β in mammary development may be to prevent extensive ductal branching to leave space for alveoli to form during pregnancy [Robinson et al. 1991]. Whole-mount preparations of pregnant and lactating glands showed fully developed lobules in both transgenic and wild-type animals, but the transgenic animals had fewer lobules [data not shown]. Nevertheless, sufficient mammary tissue was present even in homozygous animals for the mothers to feed full litters of 9–11 pups. The histologic appearance of lactating mammary tissue from transgenic animals was very similar to that of wild-type, although duct and alveolar epithelial cells in transgenic animals frequently were more flattened and somewhat atrophic [data not shown]. The data suggest that the wild-type mammary gland has a substantial tissue re-

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**Table 2.** Effect of TGFβ1 implants on labeling index in lateral outgrowth in 12-week wild-type animals as determined by [3H]thymidine incorporation

| Treatment | Mean ± s.d. [no. of animals] |
|-----------|-----------------------------|
| BSA       | 0.074 ± 0.033 (4)           |
| TGF-β1    | 0.084 ± 0.048 (4)           |

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serve, as a marked reduction in ductal volume is tolerated so well. Previous studies have demonstrated that treatment of mammary explants with TGF-β1 suppresses casein synthesis (Robinson et al. 1993). The possibility that transgene expression alters the composition of milk produced is currently being investigated.

Two reports of TGF-β1 transgenic experiments have been published. One targeted expression to the skin using a keratin promoter (Sellheyer et al. 1993), resulting in a lethal phenotype with death occurring immediately after birth and characterized by hypoplasia of the epidermis and tautness of the skin. The second study used the whey acidic protein (WAP) promoter to obtain expression in mammary epithelium during pregnancy and lactation (Jhappan et al. 1993). The phenotype obtained in this study was inconsistent. There was sufficient inhibition of lobuloalveolar development in two of four transgenic lines to prevent lactation. Expression of the transgene under control of the WAP promoter was low in virgin glands and greatly induced at mid-pregnancy. The MMTV promoter/enhancer is expressed in the virgin gland as well as in alveolar outgrowths occurring during pregnancy. As demonstrated in the present study, MMTV-driven expression of TGF-β1 does not result in inhibition of alveolar outgrowths in agreement with previous studies with exogenously administered TGF-β1 (Daniel et al. 1989). The reasons for the different results using the WAP and MMTV promoters to drive TGF-β1 expression are not known. One possibility is higher levels of TGF-β1 transgene expression with the WAP promoter than with MMTV. Interestingly, Burdon et al. (1991) found that overexpression of the WAP gene in transgenic mice resulted in a failure to lactate and a histologic appearance of the postpartum mammary gland similar to that observed in the WAP–TGF-β1 transgenic mice (Jhappan et al. 1993).

The MMTV–TGF-β1 transgenic model has major advantages over the two TGF-β1 transgenic models described by others because the MMTV–TGF-β1 mice can be crossed with animals expressing growth factors or oncogenes that cause hyperplasia and increased tumor formation. The keratin–TGF-β1 transgenic mice die immediately after birth and therefore cannot be crossed (Sellheyer et al. 1993). The WAP–TGF-β1 transgenic phenotype (Jhappan et al. 1993) is inconsistent and results in expression of TGF-β1 at a stage of mammary development when the mammary epithelium is largely resistant to the growth inhibitory effects of TGFs–β, as demonstrated by our results. The MMTV promoter/enhancer has been used in numerous transgenic experiments to drive misexpression of growth factors and oncogenes (for review, see Groner 1992). These experiments include the transcription factor c-myc (Stewart et al. 1984, Sinn et al. 1987), growth factors TGF-α (Matsui et al. 1990) and int-2 (Muller et al. 1990), receptors ErbB2/neu (Muller et al. 1988; Bouchard et al. 1989; Guy et al. 1992), and the signal-transducing ras proteins (Tremblay et al. 1989). Common features of the phenotypes observed in these studies have been mammary ductal or alveolar hyperplasia, or both, and a marked increase in the frequency of mammary carcinomas (Groner 1992). Thus, the phenotype observed in the present study with MMTV–TGF-β1 transgenic mice is unique in that it involves mammary ductal hyperplasia. The effect of the MMTV–TGF-β1 transgene on mammary tumor formation remains to be established; but thus far, no tumors have been observed in transgenic animals at 1 year of age when neoplasia was apparent in studies with MMTV–TGF-α and MMTV–oncogene constructs. Because the early phenotype of the MMTV–TGF-β1 transgenic mice is opposite that observed with growth factors and oncogenes, we hypothesize that the presence and expression of the MMTV–TGF-β1 transgene may suppress tumor formation. Thus, crossing the MMTV–TGF-β1 mice with MMTV–TGF-α or MMTV–oncogene mice will be of particular interest.

Materials and methods

Generation of transgenic mice

The MMTV–TGF-β1 construct was prepared by first replacing the SV40 early promoter in the pKCR vector with the complete MMTV–LTR. A 1.4-kb simian TGF-β1 cDNA (Brunner et al. 1989) was inserted into the EcoRI site of B-globin (1991) found that overexpression of the WAP gene in transgenic mice resulted in a failure to lactate and a histologic appearance of the postpartum mammary gland similar to that observed in the WAP–TGF-β1 transgenic mice (Jhappan et al. 1993). The WAP–TGF-β1 transgenic phenotype (Jhappan et al. 1993) is inconsistent and results in expression of TGF-β1 at a stage of mammary development when the mammary epithelium is largely resistant to the growth inhibitory effects of TGFs–β, as demonstrated by our results. The MMTV promoter/enhancer has been used in numerous transgenic experiments to drive misexpression of growth factors and oncogenes (for review, see Groner 1992). These experiments include the transcription factor c-myc (Stewart et al. 1984, Sinn et al. 1987), growth factors TGF-α (Matsui et al. 1990) and int-2 (Muller et al. 1990), receptors ErbB2/neu (Muller et al. 1988; Bouchard et al. 1989; Guy et al. 1992), and the signal-transducing ras proteins (Tremblay et al. 1989). Common features of the phenotypes observed in these studies have been mammary ductal or alveolar hyperplasia, or both, and a marked increase in the frequency of mammary carcinomas [Groner 1992]. Thus, the phenotype observed in the present study with MMTV–TGF-β1 transgenic mice is unique in that it involves mammary ductal hyperplasia. The effect of the MMTV–TGF-β1 transgene on mammary tumor formation remains to be established; but thus far, no tumors have been observed in transgenic animals at 1 year of age when neoplasia was apparent in studies with MMTV–TGF-α and MMTV–oncogene constructs. Because the early phenotype of the MMTV–TGF-β1 transgenic mice is opposite that observed with growth factors and oncogenes, we hypothesize that the presence and expression of the MMTV–TGF-β1 transgene may suppress tumor formation. Thus, crossing the MMTV–TGF-β1 mice with MMTV–TGF-α or MMTV–oncogene mice will be of particular interest.

DNA isolation and detection

DNA was isolated by proteinase K digestion from a 0.5-cm piece of tail clipped from briefly anesthetized 12-day-old mice. DNA was purified by phenol/chloroform/isoamyl extractions. Transgenics were identified by Southern blot analysis of DNA restricted with either BamHI or EcoRI, separated on a 1.0% agarose gel, and then transferred to a Nylon filter. The blot was hybridized with a 32P-labeled probe made from either mouse TGF-β1 or an EcoRI–XhoI fragment of the transgene construct containing a 526-bp rabbit β-globin exon 3 (Fig. 2). After autoradiography, bands were scanned by densitometer and copy numbers of the transgene were determined for each line. The 41 line has one copy per transgene allele and the 46 and 66 lines each have two copies per allele.

Morphologic assessment of the mammary glands

The inguinal mammary glands were resected with the overlying skin and fixed en bloc. The thoracic glands were dissected away from subcutaneous tissues and fixed in tissue cassettes. All glands were fixed in freshly prepared 4% paraformaldehyde for 16 hr. The inguinal glands were then dissected from the skin and mammary glands and processed for whole-mounts, as described [Silberstein and Daniel 1987]. Whole-mounts were stained with hematoxylin and photographed on an Olympus Research Stereo microscope.
In situ hybridization
A 526-bp EcoRI-XhoI restriction fragment, containing a section of the rabbit β-globin exon 3, was inserted into PSP73, and a single-stranded antisense probe was labeled with 35S-labeled UTP. In situ hybridizations were performed for 18 hr at 60°C as described (Pelton et al. 1990). After hybridization, sections were washed for 30 min in 50% formamide at 65°C, treated with RNase at a concentration of 20 µg/ml for 30 min at 37°C, and washed twice for 30 min at 65°C in 2x SSC and for 30 min in 0.1x SSC.

TGF-β1 immunohistochemistry
Localization of TGF-β1 was carried out using a rabbit polyclonal antibody (10 µg/ml) to TGF-β1 (Pelton et al. 1991). Controls for immunohistochemistry were done using normal rabbit serum. TGF-β1 protein was then identified using avidin–biotin complex immunohistochemistry as described previously (Johnson et al. 1992).

BrdU immunohistochemistry
5-Bromo 2-deoxyuridine was dissolved in 10 mg/ml of 0.01 M PBS (pH 7.2). A total dose of 60 mg/kg was injected intraperitoneally into female mice 2 hr before sacrifice. Sections were denatured in 2 N HCl for 20 min at 37°C, neutralized in boric acid-borate buffer, and exposed to 20 ng/ml of trypsin at 37°C for 3 min. BrdU was detected using a 1:400 dilution of a rat monoclonal antibody specific for BrdU (Sera Labs, West Sinai, NY) and avidin–biotin complex immunohistochemistry (Repka and Adler 1992). To facilitate BrdU detection, sections were denatured in 2 N HCl for 20 min at 37°C, neutralized in boric acid–borate buffer, and exposed to 20 ng/ml of trypsin at 37°C for 3 min.

Northern blot analysis
Total RNA was isolated from mammary tissue of nonpregnant, pregnant, and lactating transgenic females by denaturation of tissue with guanidium isothiocyanate, ultracentrifugation through CsCl (Johnson et al. 1992), electrophoresis through 1% agarose, and transfer to nitrocellulose. Filters were probed with either the 974-bp SmaI–SmaI murine cDNA (Derynck et al. 1986) or the 986-bp PstI–BamHI simian cDNA (Sharplees et al. 1987) and cyclophilin (1B15) cDNA, as described previously (Johnson et al. 1992).

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References
Bascom, C.C., J.R. Wolfshohl, R.J. Coffey Jr., L. Madisen, N.R. Webb, A.F. Purchio, R. Derynck, and H.L. Moses. 1989. Complex regulation of transforming growth factor β1, β2, and β3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors β1 and β2. Mol. Cell. Biol. 9: 5508-5515.

Bouchard, L., L. Lamarre, P.J. Tremblay, and P. Jolicoeur. 1989. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV–c-neu oncogene. Cell 57: 931-936.

Brunner, A.M., H. Marquardt, A.R. Malacko, M.N. Lioubin, and A.F. Purchio. 1989. Site-directed mutagenesis of cysteine residues in the pro region of the transforming growth factor β1 precursor. J. Biol. Chem. 264: 13660-13664.

Burdon, T., R.J. Wall, A. Shamay, G.H. Smith, and L. Hennighausen. 1991. Over-expression of an endogenous milk protein gene in transgenic mice is associated with impaired mammary alveolar development and a milchlos phenotype. Mech. Dev. 36: 67-74.

Daniel, C.W., G.B. Silberstein, K. Van Horn, P. Strickland, and S. Robinson. 1989. TGF-β1-induced inhibition of mouse mammary ductal growth: Developmental specificity and characterization. Dev. Biol. 135: 20-30.

Derynck, R., J.A. Jarrett, E.Y. Chen, and D.V. Goeddel. 1986. The murine transforming growth factor-β1 precursor. J. Biol. Chem. 261: 4377-4379.

Groner, B. 1992. Oncogene expression in mammary epithelial cells. J. Cell. Biochem. 49: 128-136.

Guy, C.T., M.A. Webster, M. Schaller, T.J. Parsons, R.D. Cardiff, and W.J. Muller. 1992. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. 89: 10578-10582.

Haslam, S.Z. 1991. Stromal–epithelial interactions in normal and neoplastic mammary gland. Cancer Treat. Res. 53: 401-420.

Hogan, B.L.M., G. Horsburgh, J. Cohen, C.M. Hetherington, G. Fisher, and M.F. Lyon. 1986. Small eyes (Sey): A homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. J. Embryol. Exp. Morphol. 97: 95-110.

Hjappan, C., C. Stahle, R.N. Harkins, N. Fausto, G.M. Smith, and G.T. Merlino. 1990. TGF-α overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61: 1137-1146.

Hjappan, C., A.G. Geiser, E.C. Kordon, D. Bagheri, L. Hennighausen, A.B. Roberts, G.H. Smith, and G. Merlino. 1993. Targeting of a transforming growth factor β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J. 12: 1835-1845.

Johnson, M.D., L.I. Gold, and H.L. Moses. 1992. Evidence for transforming growth factor-β expression in human leptomeningeal cells and transforming growth factor-β-like activity in human cerebrospinal fluid. Lab. Invest. 67: 360-368.

Johnson, M.D., C.F. Federspiel, L.I. Gold, and H.L. Moses. 1992. Transforming growth factor-β and transforming growth factor β1-receptor expression in human meningioma cells. Am. J. Pathol. 141: 633-642.

Krabbe, C., M.E. Lippman, L.M. Wakefield, K.C. Flanders, A. Kasid, R. Derynck, and R.B. Dickson. 1987. Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417-428.

Lawrence, D.A., R. Pincher, C. Kryceve-Martinerie, and P. Julian. 1984. Normal embryo fibroblasts release transforming growth factors in a latent form. J. Cell. Physiol. 121: 184-188.

Lioubin, M.N., L. Madisen, R.A. Roth, and A.F. Purchio. 1991. Characterization of latent transforming growth factor-β 2
from monkey kidney cells. *Endocrinology* **128:** 2291–2296.

Lyons, R.M. and H.L. Moses. 1990. Transforming growth factors and the regulation of cell proliferation. *Eur. J. Biochem.* **187:** 467–473.

Lyons, R.M., L.E. Centry, A.F. Purchio, and H.L. Moses. 1990. Mechanism of activation of latent recombiant transforming growth factor β1 by plasmin. *J. Cell Biol.* **110:** 1361–1367.

Masui, T., L.M. Wakefield, J.F. Lechner, M.A. LaVeck, M.B. Sporn, and C.C. Harris. 1986. Type β transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. *Proc. Natl. Acad. Sci.* **83:** 2438–2442.

Matsui, Y., S.A. Halter, J.T. Holt, B.L.M. Hogan, and R.J. Coffey Jr. 1990. Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. *Cell* **61:** 1147–1156.

Miller, D.A., R.W. Pelton, R. Derynck, and H.L. Moses. 1990. Transforming growth factor β: A family of growth regulatory peptides. *Ann. N.Y. Acad. Sci.* **593:** 208–217.

Miyazono, K., H. Ichijo, and C.-H. Heldin. 1993. Transforming growth factor β: Latent forms, binding proteins and receptors. *Growth Factors* **8:** 11–22.

Moses, H.L. 1990. The biological actions of transforming growth factor β. In *Growth factors from genes to clinical application* (ed. V. Sara, K. Hall, and H. Low), pp. 141–155. Raven Press, New York.

Moses, H.L., R.F. Tucker, E.B. Leaf, R.J. Coffey Jr., J. Halper, and G.D. Shipley. 1985. Type β transforming growth factor is a growth stimulator and a growth inhibitor. In *Growth factors and transformation*. *Cancer Cells* **3:** 65–71.

Moses, H.L., E.Y. Yang, and J.A. Pietenpol. 1990. TGF-β stimulation and inhibition of cell proliferation: New mechanistic insights. *Cell* **63:** 245–247.

Muller, W.J., E. Sinn, P.K. Pattengale, R. Wallace, and P. Leder. 1988. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* **54:** 105–115.

Muller, W.J., F.S. Lee, C. Dickson, G. Peters, P. Pattengale, and P. Leder. 1990. The int-2 gene product acts as an epithelial growth factor in transgenic mice. *EMBO J.* **9:** 907–913.

Olofsson, A., K. Miyazono, T. Kanzaki, P. Colosetti, U. Engström, and C.-H. Heldin. 1992. Transforming growth factor β1, β2, and β3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. *J. Biol. Chem.* **267:** 19482–19488.

Pelton, R.W., M.E. Dickinson, H.L. Moses, and B.L.M. Hogan. 1990. In situ hybridization analysis of TGF-β3 RNA expression during mouse development: Comparative studies with TGF-β1 and β2. *Development* **110:** 600–620.

Pelton, R.W., B. Saxena, M. Jones, H.L. Moses, and L.I. Gold. 1991. Immunohistochemical localization of TGF-β1, TGF-β2, and TGF-β3 in the mouse embryo: Expression patterns suggest multiple roles during embryonic development. *J. Cell Biol.* **115:** 1091–1105.

Repka, A.M. and R. Adler 1992. Accurate determination of the time of cell birth using a sequential labeling technique with [3H]thyidine and bromodeoxyuridine ("window labeling"). *J. Histochem. Cytochem.* **40:** 947–953.

Robinson, S.D., G.B. Silberstein, A.B. Roberts, K.C. Flanders, and C.W. Daniel. 1991. Regulated expression and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development. *Development* **113:** 867–878.

Robinson, S.D., A.B. Roberts, and C.W. Daniel. 1993. TGFβ suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during preg-
Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1.

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