Transgenic Mice Expressing a Dominant-Negative Mutant Type II Transforming Growth Factor- β Receptor Exhibit Impaired Mammary Development and Enhanced Mammary Tumor Formation

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We have previously shown that expression of a dominant-negative type II transforming growth factor- β receptor (DNIIR) in mammary epithelium under control of the MMTV promoter/enhancer causes alveolar hyperplasia and differentiation in virgin mice. Here we show that MMTV-DNIIR female mice have accelerated mammary gland differentiation during early pregnancy with impaired development during late pregnancy and lactation followed by delayed postlactational involution. Mammary tumors, mostly carcinoma *in situ*, developed spontaneously in the MMTV-DNIIR mice with a long median latency (27.5 months). Crossbreeding to MMTV-transforming growth factor (TGF)- α mice to obtain mice expressing both transgenes resulted in mammary tumor formation with a much shorter latency more similar to those expressing only the MMTV-TGF- α transgene (<10 months median latency). The major difference in mammary tumors arising in MMTV-TGF- α compared to bigenic MMTV-DNIIR/MMTV-TGF- α was the marked suppression of tumor invasion by DNIIR transgene expression. Invading carcinoma cells in both MMTV-DNIIR and bigenic animals showed loss of DNIIR transgene expression as determined by *in situ* hybridization. The data indicate that signaling from endogenous TGF- β s not only plays an important role in normal mammary gland physiology but also can also suppress the early stage of tumor formation and contribute to tumor invasion once carcinomas have developed. (Am J Pathol 2003; 163:1539–1549)

A number of studies have implicated the transforming growth factor (TGF)- β family, TGF- β 1, TGF- β 2, and TGF- β 3, in the regulation of mammary gland morphogenesis during puberty, lactation, and involution. All three TGF- β ligands are expressed in various parts of the mammary

gland during different physiological states in both the epithelium and the stroma.¹ Silberstein and Daniel² provided the first direct evidence for TGF- β involvement in mammary gland development by demonstrating that experimentally implanted TGF- β 1 causes rapid and reversible inhibition of terminal end-bud growth. Subsequent evidence was derived from transgenic mouse studies in which a constitutively active mutant of TGF- β 1 was expressed under control of two different promoters. Studies from our laboratory showed that constitutively active TGF- β 1 driven by the mouse mammary tumor virus (MMTV) promoter/enhancer caused retardation of ductal development, but lobuloalveolar development was not inhibited during pregnancy and the mice sustained full litters until weaning.³ In contrast, expression of active TGF- β 1 during pregnancy driven by the whey acidic protein (WAP) promoter resulted in decreased lobuloalveolar development and failure of terminal differentiation, but did not affect branching morphogenesis during puberty.⁴ It was proposed that misexpression of TGF- β in these mice resulted in increased apoptosis of the alveolar progenitor cells.⁵ The difference in the nontumor phenotype mammary gland between MMTV- and WAP-TGF- β 1 transgenic mice is likely because of different spatial and temporal expression patterns with the two promoters. Notably, MMTV-TGF- β 1 expression caused suppression of mammary tumorigenesis induced by TGF- α or the chemical carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA).⁶ Similarly, recent studies have shown that WAP-TGF- β 1 transgenic mice are resistant to induction of mammary tumors by injection of the mouse mammary tumor virus^{7,8} in agreement with the previous studies with MMTV-TGF- β 1 mice.⁶

The effect of endogenous TGF- β signaling on mammary gland development has been examined in transgenic mice by expression of a truncated, kinase-defective, dominant-negative type II TGF- β receptor (DNIIR)

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regulated by the MMTV promoter/enhancer. The resultant phenotype includes increased lobuloalveolar development in virgin female mice.^{9,10} MMTV-DNIIR mice developed by one group did not develop mammary tumors spontaneously, but did demonstrate enhanced mammary tumorigenesis in response to administration of the chemical carcinogen, 7,12-dimethylbenz[a]anthracene. Enhancement by DNIIR expression of tumor formation in response to carcinogen administration has also been observed in the lung¹¹ and skin.¹²

In the present study, we have extended studies with the previously reported MMTV-DNIIR mice¹⁰ to show that inhibition of TGF- β signaling in mammary epithelial cells impairs full lactational differentiation and retards postlactational involution. Because impaired involution has been associated with enhanced mammary tumor formation in other transgenic mouse studies,^{13–15} tumor formation in the MMTV-DNIIR mice was examined. Multiparous MMTV-DNIIR female mice were found to spontaneously develop mammary carcinomas with a long median latency (>2 years). Co-expression of the MMTV-DNIIR and MMTV-TGF- α transgenes resulted in mammary tumor formation similar to that observed in MMTV-TGF- α mice except that DNIIR expression suppressed stromal invasion. The data support the hypotheses that signaling from endogenous TGF- β not only plays an important role in normal mammary gland physiology but also can suppress the early stage of tumor formation and contribute to tumor invasion once carcinomas have developed.

Materials and Methods

Transgene Detection

DNIIR transgene was detected by polymerase chain reaction analysis using primers for 526-bp β -globin exon 3 sequence as described.¹⁰ TGF- α transgene was detected by polymerase chain reaction analysis using primers for TGF- α as described.¹³

Transgenic Mice

The MMTV-DNIIR transgenic animals were generated by microinjection of the DNIIR from pRHC102(17) into (C57BL/6xDBA)F2 fertilized eggs, then bred to (C57BL/6) as described.¹⁰ Female mice homozygous for DNIIR transgene were mated two to six times to produce litters. C57BL/6 female wild-type (WT) mice were also bred to produce two to six litters and both of the above (31 DNIIR) and (28 WT) mice were used in the study. Male MMTV TGF- α were mated to female MMTV-DNIIR mice, to obtain bigenic MMTV TGF- α /DNIIR transgenic mice. Seventeen MMTV-TGF- α and 19 MMTV-TGF- α /DNIIR multiparous transgenic mice were also used in the study. Mice were housed in 12-hour dark/light cycles and fed standard chow and water. All mice were carefully examined by palpation for tumors weekly for 2.5 years. Mammary tumors were removed 3 to 4 weeks after detection, other organs were also removed for detection of metastasis if desired. Tissues were fixed in 4% formaldehyde

and sections were stained with hematoxylin and eosin (H&E) for histological diagnosis. Histological sections were analyzed and photographed using a Olympus BX41 Microscope (Melville, NY).

Extraction of RNA and Northern Blot Hybridization

Total RNA was isolated from tumors as described previously.¹⁰ Total RNA was resolved by electrophoresis and then transferred to nylon membrane (Hybond-N). The membranes were probed with ³²P-labeled cDNA: 0.6-kb *EcoRI*-*XhoI* fragment from plasmid RHC102 for DNIIR, 540-bp *HindIII*-*BamHI* fragment of pT7BC-1 for β -casein, 630-bp *SacI*-*PstI* fragment of mWAP+ for WAP, and 0.7-kb *BamHI*-*PstI* fragment from SP651B15 for cyclophilin.

In Situ Hybridization

The DNIIR 0.6-kb *EcoRI*-*XhoI* fragment from pRH-102 was inserted into SP72 and SP73 vectors to make the riboprobes in anti-sense and sense orientation, respectively. The DNIIR riboprobes were generated by transcribing the linearized plasmids with T7 polymerase. Single-stranded RNA was labeled with ³⁵S-labeled UTP (100 μ Ci; specific activity, 1400Ci/mmol; NEN, Boston, MA). *In situ* hybridization was performed for 18 hours at 55°C as described previously.¹⁰ Slides were exposed for 3.5 weeks, then developed and counterstained with 0.1% toluidine blue.

Histology and Immunostaining

For immunostaining and H&E staining, paraffin-embedded sections were used. Tumors and other tissues were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (5 μ m) were stained with H&E using standard procedures.

Immunostaining of β -casein was performed as previously described.¹⁰ Sections were dewaxed, rehydrated, and digested with 0.05% saponin for 30 minutes. Staining was performed using Vectastain ABC kit (Vector Laboratories, Hercules, CA), according to the manufacturer's instructions. Immunofluorescence reactions were performed using 1:1000 dilution of Streptavidin-C3 Conjugate (catalog no. S6402; Sigma Chemical Co., St. Louis, MO) for 30 minutes, then 1:10,000 dilution of Yo-Pro for 15 minutes, then sections were mounted with AquaPoly/Mount (catalog no. 18606; Polysciences, Inc., Warrington, PA).

Postlactational Involution

Homozygous MMTV-DNIIR female mice were bred to WT males. Pups were fed for 10 days and removed. The mothers were sacrificed at 4, 7, and 10 days after weaning. Paraffin sections of formaldehyde-fixed mammary glands were stained using the Apoptosis Kit (catalog no. S7100-6; Oncor, Gaithersburg, MD) to identify apoptotic

cells on day 4 glands. Slides were counterstained with 0.5% methyl green.

Statistical Analysis

Analyses of study results focused on estimating the time to tumor development for each of the study groups and comparing the incidence rates of each type of the tumor among different study groups. Because each mouse may develop one or more than one tumor in the same or different types of the tumor, tests of hypotheses concerning the significant differences of the incidence rates for each type of the tumor among different groups were performed using the generalized estimating equation method^{16,17} to adjust the intracorrelation effect for the mouse that had multiple tumors. For lifetime data analyses, the study groups were compared for survival with Kaplan-Meier estimates and log-rank tests. All tests of significance were two-sided, and differences were considered statistically significant when the *P* value was <0.05. All data were expressed as means $\pm$ SD. SAS version 8.02 and S-Plus 6 were used for all analyses.

Results

Effect of DNIIR Expression on Mammary Gland Differentiation During Pregnancy and Lactation

We have previously shown that expression of MMTV-DNIIR results in a precocious mammary development in virgin female mice.¹⁰ The phenotype was consistent only in mice having two transgene alleles (homozygous), and these mice were frequently unable to feed their pups. Thus, we examined the mammary phenotype of the homozygous MMTV-DNIIR mice during pregnancy. Five MMTV-DNIIR along with three nontransgenic mice were sacrificed at day 15 of their first pregnancy. The third thoracic mammary gland from each animal was removed for whole mount examination. Mammary glands from four of the five transgenic mice showed greater lobuloalveolar development than the control nontransgenic mice (Figure 1; A to D). The one transgenic mouse not showing a difference from controls did not have DNIIR transgene expression as determined by Northern blot analysis of RNA isolated from inguinal glands (data not shown). Histological examination of the MMTV-DNIIR glands showed more prominent vacuoles in the alveoli indicating a greater degree of differentiation in comparison to controls (Figure 1, C and D). Immunostaining for β -casein demonstrated positively staining material in the lumen of alveoli and ducts from the MMTV-DNIIR mammary glands, the control glands demonstrated only intracellular staining (Figure 1, E and F) indicating more advanced lactational differentiation as a result of transgene expression.

An examination of four MMTV-DNIIR mice in comparison to five control, nontransgenic mice after 1 day of lactation showed a striking reversal of the relative degree of functional development. The 1-day lactating MMTV-DNIIR mammary glands all exhibited a high level of transgene expression as determined by Northern blot analysis

Figure 1. Effect of MMTV-DNIIR transgene on pregnancy phenotype. **A:** Whole mount of mammary gland from WT 15-day pregnant female mice showing the typical degree of lobuloalveolar development. **B:** Typical appearance of MMTV-DNIIR mammary glands at 15 days of pregnancy demonstrating accelerated lobuloalveolar development. **C** and **D:** Histology of WT (**C**) and MMTV-DNIIR (**D**) as demonstrated by H&E-stained paraffin sections showing increased lobuloalveolar development and differentiation (vacuoles in alveolar cells) in the latter circumstance. **E** and **F:** β -casein immunostaining of WT (**E**) and MMTV-DNIIR (**F**) 15-day pregnant mammary glands. Scale bars: 500 μ m (**A** and **B**); 200 μ m (**C** and **D**); and 400 μ m (**E** and **F**).

and showed much less extensive alveolar development than the controls (Figure 2; A to D). β -Casein immunostaining also indicated little progression from the 15-day pregnant state (Figure 2, E and F). Northern blot analysis of β -casein mRNA from WT and DNIIR 15-day pregnant and 1-day lactating mammary glands showed variable expression in all groups with no significant differences among the groups (Figure 3). However, analysis of WAP mRNA expression demonstrated a fourfold greater expression in DNIIR *versus* WT glands during pregnancy (mean $\pm$ SD = 0.35 ± 0.17 for DNIIR and 0.08 ± 0.1 for WT, *P* = 0.0246) (Figure 3). In the DNIIR mammary glands, there was only a fourfold increase in WAP mRNA expression from 15-day pregnant to 1-day lactating while there was the expected 28-fold increase in the WT glands. The data indicate that interference with TGF- β -signaling mammary epithelial cells causes accelerated development and differentiation during early to mid pregnancy, but results in impaired mammary gland development during the last stages of pregnancy producing a lactating gland with many functional alveoli.

Figure 2. Effect of MMTV-DNIIR transgene on lactation phenotype. **A** and **B**: Whole-mount preparations of 1-day lactating mammary glands from WT (**A**) and MMTV-DNIIR (**B**) animals showing results of arrest of lobuloalveolar development in the latter stage of pregnancy. **C** and **D**: Histology of 1-day lactating mammary glands from WT (**C**) and MMTV-DNIIR (**D**) animals. **E** and **F**: β -casein immunostaining of WT (**E**) and MMTV-DNIIR (**F**) 1-day lactating mammary glands. Scale bars: 500 μ m (**A** and **B**); 200 μ m (**C** and **D**); and 400 μ m (**E** and **F**).

Effect of DNIIR Expression on Mammary Gland Differentiation During Postlactational Involution

To determine the effects of DNIIR expression on postlactational involution of the mammary gland, female mice that were able to have and feed full litters for 8 to 10 days were examined at 4, 7, and 10 days after weaning. For the 4-day involution time point, glands from four transgenic and five WT mice were examined histologically using H&E-stained paraffin sections and by Apoptag

Figure 3. Northern blot analysis of MMTV-DNIIR transgene, β -casein, and WAP expression in mammary glands of WT and MMTV-DNIIR 15-day pregnant and 1-day lactating mice. 1B15 is a probe for cyclophilin and is used as a loading control.

staining to compare rates of apoptosis. All glands from transgenic mice showed a phenotype reflective of the changes noted in the 1-day lactating glands with many fewer alveoli (Figure 4, A and B). Staining for apoptotic nuclei showed the high rate of apoptosis expected at this stage of involution in glands from both nontransgenic and transgenic animals. To determine whether there was a consistent difference in the rate of apoptosis in the two groups, 600 to 1000 epithelial nuclei from four transgenic and four nontransgenic mice were counted and scored positive or negative for Apoptag staining. The mean $\pm$ SD for the nontransgenic mice was 12.25 ± 4.27 and 8.8 ± 1.11 for the transgenic mice. Using a two-tailed, unpaired Student's *t*-test, a *P* value of 0.1692 was obtained indicating that the difference was not significant. However, an examination of mammary glands at 7 (Figure 4, E and F) and 10 (Figure 4, G and H) days after weaning demonstrated consistent retardation of involution in the transgenic animals.

Formation of Spontaneous Mammary Carcinoma in MMTV-DNIIR Mice

Previous studies demonstrated that another line of MMTV-DNIIR mice have increased mammary tumor formation only after administration of the chemical carcinogen, DMBA.¹¹ To determine whether abrogation of TGF- β signaling in mammary epithelial cells could result in spontaneous mammary tumor formation, DNIIR transgene was maximized by utilization of female mice homozygous for the transgene that had experienced two pregnancies. It is well known that hormonal stimulation during pregnancy drives high levels of MMTV transgene expression. Under these circumstances, we observed a high frequency of spontaneous mammary carcinoma development with a long latency. Fifteen of 32 (47%) MMTV-DNIIR female mice developed mammary tumors with a median latency of 27.5 months (Figure 5A and Table 1). A comparable number of WT multiparous female mice were followed for the same time interval and only one tumor was observed in one mouse, an intraductal papilloma. Thus, the difference in mammary tumor formation between the two populations was highly significant demonstrating that expression of the MMTV-DNIIR transgene alone can significantly accelerate the development of mammary carcinomas and supporting the hypothesis that signaling from endogenous TGF- β s can be tumor suppressive (Figure 5A).

Most of the mammary tumors arising in the MMTV-DNIIR mice were noninvasive and were diagnosed as carcinoma *in situ*/high-grade mammary intraepithelial neoplasia (CIS/HG-MIN) based on the consensus criteria developed by the Mouse Models of Human Cancer Consortium.¹⁸ Of the 18 mammary tumors that developed in 15 mice (four mice had two separate mammary tumors), 13 were CIS/HG-MIN and 5 were invasive; 2 were invasive adenocarcinomas, and 3 were invasive adenosquamous carcinomas (Table 1). It is of interest that adenocarcinomas tended to develop in older mice paralleling the human condition; three of the four adenocarcinomas

Figure 4. Effect of MMTV-DNIIR transgene expression on postlactational involution of the mammary gland. **A** and **B**: Histology of WT (**A**) and MMTV-DNIIR (**B**) mammary glands 4 days after weaning reflects the state of lobuloalveolar development in the lactating glands (see Figure 2) with less abundant alveoli in the DNIIR animals. **C** and **D**: Staining of apoptotic cells in mammary glands from WT (**C**) and DNIIR (**D**) animals 4 days after weaning showing slightly fewer apoptotic in the transgenic mammary glands. **E–H**: Representative histology of WT (**E** and **G**) and MMTV-DNIIR (**F** and **H**) mammary glands from animals 7 (**E** and **F**) and 10 (**G** and **H**) of postlactational involution showing retarded involution in the MMTV-DNIIR animals. Scale bar, 200 μ m.

Figure 5. Accelerated development of mammary carcinomas in MMTV-DNIIR female mice. **A:** Survival plot showing accelerated development of mammary tumors with a median latency of >2 years in the MMTV-DNIIR mice (**dashed line**) relative to control WT mice (**solid line**). **B** and **C:** Histology of mammary tumors from MMTV-DNIIR mice showing a CIS/HG-MIN pattern typical of >70% of the tumors observed. Note lack of invasion (**B**) and presence of tumor necrosis (**asterisk** in **C**). **D** and **E** show less differentiated mammary tumor (**D**) with areas of stromal invasion associated with a desmoplastic response (**E**). Scale bars: 100 μ m (**B–D**); 33 μ m (**E**).

developed in mice that were >2 years of age at the time of first detection of the tumor. Figure 5, B and C, shows the typical histological appearance of CIS/HG-MIN whereas Figure 5D illustrates the appearance of a poorly differentiated adenocarcinoma, and Figure 5E shows an area of stromal invasion.

Previous studies have indicated that TGF- β signaling in carcinoma plays an important role in the invasive ca-

pability of the cells.^{19–21} Thus, we were interested in determining the expression status of the DNIIR transgene in areas of carcinoma invasion. This was determined by Northern blot analysis of RNA extracted from tumors and by *in situ* hybridization using a transgene-specific probe as previously described.¹⁰ We were able to extract sufficient tumor RNA for Northern blotting from 10 of the 15 animals. All of the seven CIS/HG-MIN samples examined

Table 1. Mammary Tumors in Multiparous MMTV-DNIIR Female Mice

Animal no.	Age of tumor appearance (weeks)	Number of mammary tumors	Tumor type (no. of tumors)
1	73	1	CIS/HG-MIN*
2	73	1	CIS/HG-MIN
3	74	1	CIS/HG-MIN
4	78	1	CIS/HG-MIN
5	78	1	Invasive adenocarcinoma
6	78	2	CIS/HG-MIN (2)
7	82	1	CIS/HG-MIN
8	91	1	CIS/HG-MIN
9	91	1	CIS/HG-MIN
10	95	1	CIS/HG-MIN
11 [†]	99	1	CIS/HG-MIN
12	113	1	CIS/HG-MIN
13	117	1	Invasive adenosquamous carcinoma
14	121	2	Early CIS/HG-MIN Invasive adenocarcinoma
15	121	2	Invasive adenosquamous carcinoma (2)

*Carcinoma *in situ*/high-grade mammary intraepithelial neoplasia.[†]Metastatic carcinoma in mesentery and lung.

Fifteen of 32 mice (47%) developed 18 tumors.

Thirteen of 18 tumors were CIS/HG-MIN (72%).

showed transgene expression, whereas one (animal 14, Table 1) of three RNA samples from invasive carcinomas was negative by Northern analysis. By *in situ* hybridization, tumors exhibiting the CIS/HG-MIN pattern consistently showed transgene expression similar to that observed in nontumor ductal epithelial cells (Figure 6; A to D). However, poorly differentiated adenocarcinomas frequently showed no *in situ* hybridization evidence of transgene expression (Figure 6E), and all areas of tumor invasion showed no transgene expression (Figure 6F) even when other areas of the same tumor had evidence of transgene expression. The data are consistent with TGF- β signaling in carcinoma cells being involved in the processes of invasion.

Effect of DNIIR Expression on MMTV-TGF- α -Induced Mammary Tumors

We have previously demonstrated that overexpression of a constitutively active TGF- β 1 in mammary epithelial cells suppressed tumor induction by MMTV-TGF- α .⁶ Thus, we were interested in determining the effect of abrogation of endogenous TGF- β signaling on MMTV-TGF- α tumor induction and crossed female homozygous MMTV-DNIIR female mice with male heterozygous MMTV-TGF- α mice to obtain bigenic animals with one allele of each transgene. The development of mammary tumors in these bigenic animals were compared with that observed in mice expressing only the MMTV-TGF- α transgene. Similar to earlier reports,^{6,13} MMTV-TGF- α mice developed tumors with a median latency of ~9 months. However, we observed a higher frequency of tumor development (13 of 16 animals) with a higher percentage of animals having mammary tumors in more than one gland (11 of 13 mice with mammary tumors) (Table 2, Figure 7A). Of the tumor

group, all had at least one invasive adenocarcinoma, and 20 of the 43 mammary tumors (47%) were invasive adenocarcinomas with the remainder being CIS/HG-MIN.

In the bigenic animals expressing both the MMTV-TGF- α and MMTV-DNIIR transgenes, mammary tumors formed with approximately the same median latency and frequency as in the MMTV-TGF- α mice (Table 3, Figure 7A). However, the bigenic mice had a decreased incidence of invasive adenocarcinomas compared to tumors found in MMTV-TGF- α mice. Seventeen of 19 bigenic mice developed a total of 32 mammary carcinomas, but only 5 of the 17 animals with mammary tumors and 6 of the 32 tumors demonstrated invasion. The difference in invasion between the MMTV-TGF- α and bigenic mice was highly significant, $P = <0.001$. Figure 7B illustrates a typical area of invasion in a carcinoma arising in an MMTV-TGF- α mouse and Figure 7C shows a carcinoma *in situ* arising in a bigenic animal. As with the carcinomas arising in MMTV-DNIIR mammary glands, the areas of invasion in tumors arising in bigenic animals showed absence of MMTV-DNIIR transgene expression as determined by *in situ* hybridization (data not shown).

Discussion

Here we show that partial blockage of TGF- β signaling in mammary epithelial cells by expression of an MMTV-DNIIR transgene in female mice causes accelerated mammary gland differentiation during early pregnancy with impaired development during late pregnancy and lactation and delayed postlactational involution. It is not expected that TGF- β signaling would be completely blocked in all mammary epithelial cells with the MMTV-DNIIR transgene. First, it is clear that MMTV does not drive expression uniformly in all mammary epithelial cells.³ Second, in cell lines with uniform high-level expression of DNIIR, not all TGF- β signaling is blocked.²² The phenotypic changes observed in the mammary gland during the earlier stages of pregnancy are consistent with earlier studies indicating that both TGF- β 1 and - β 2 are expressed¹ and function to inhibit lactational differentiation.²³ The phenotypic changes observed in the last stage of pregnancy and early lactation showing lack of full lobuloalveolar development were not predicted based on current data concerning TGF- β function in the mammary gland. Expression of all isoforms of TGF- β is suppressed during the late stages of pregnancy and lactation;¹ however, activation of latent TGF- β in the stroma could still play a significant role in mammary gland physiology independent of changes in expression.²⁴

The question arises as to whether the altered differentiation during pregnancy and lactation and the retarded postlactational involution is causally related to the enhanced mammary tumor formation. Previous studies suggest that this may be the case. During postlactational involution, TGF- β 3 has been shown to be induced by milk stasis and to initiate apoptosis in the mammary gland epithelium.²⁵ Partial inhibition of TGF- β 3 signaling by the DNIIR transgene could explain the retardation of

Figure 6. MMTV-DNIIR transgene expression in noninvasive and invasive tumors. **A** and **B**: DNIIR transgene expression in transgenic mammary duct as determined by *in situ* hybridization using a transgene-specific anti-sense probe (**A**) and control sense probe (**B**). **C** and **D**: High levels of DNIIR transgene expression as shown by the dark autoradiographic grain in well differentiated CIS/HG-MIN. **E** and **F**: Lack of demonstrable DNIIR transgene expression in less-differentiated mammary carcinoma (**E**) and areas of stromal invasion (**F**). Bright-field images are shown; however, grain was also undetectable in dark-field images (not shown). Scale bars: 33 μm for (**A**, **B**, **D**, **F**); 100 μm (**C**, **E**).

involution in the MMTV-DNIIR mammary glands. TGF- β signaling has been implicated in the induction of senescence in mammary epithelial cells.^{4,5,7} Further, parity-induced increases in TGF- β 3 expression have been proposed as a mechanism for the protective effects of pregnancy for breast cancer development.²⁶ Thus, it is possible that the impaired TGF- β -induced senescence of mammary epithelial cells by the dominant-negative transgene leaves more cells in the proliferative pool enhancing the probability of further mutations leading to cancer.

The spontaneous development of mammary tumors in mice expressing the DNIIR transgene is a very important result because it indicates that endogenous TGF- β signaling suppresses mammary tumor formation initiated by endogenous events. This is the first demonstration that abrogation of signaling from endogenous TGF- β s can result in accelerated tumor formation. This observation in combination with our previous demonstration that constitutively active TGF- β 1 driven by the MMTV promoter/enhancer caused suppression of mammary tumorigenesis induced by TGF- α or the chemical carcinogen,

Table 2. Mammary Tumors in Multiparous MMTV-TGF- α Female Mice

Animal no.	Age of tumor appearance (weeks)	Number of mammary tumors	Tumor type (no. of tumors)
1	30	1	Invasive adenocarcinoma
2	30	3	CIS/HG-MIN (2)* Invasive adenocarcinoma (1)
3	32	7	CIS/HG-MIN (3) Invasive adenocarcinoma (4)
4	36	5	CIS/HG-MIN (3) Invasive adenocarcinoma (2)
5	35	2	CIS/HG-MIN (1) Invasive adenocarcinoma (1)
6	36	2	CIS/HG-MIN (1) Invasive adenocarcinoma (1)
7	38	4	CIS/HG-MIN (2) Invasive adenocarcinoma (2)
8	40	1	Invasive adenocarcinoma
9	38	2	Invasive adenocarcinoma (2)
10	41	2	CIS/HG-MIN (1) Invasive adenocarcinoma (1)
11	44	8	CIS/HG-MIN (7) Invasive adenocarcinoma (1)
12	46	3	CIS/HG-MIN (2) Invasive adenocarcinoma (1)
13	51	3	CIS/HG-MIN (1) Invasive adenocarcinoma (2)

*Carcinoma *in situ*/high-grade mammary intraepithelial neoplasia.
 Thirteen of 17 (76%) animals developed 43 mammary tumors.
 Twenty of 43 (47%) were invasive.

DMBA⁶ strongly support the hypothesis that TGF- β signaling is tumor suppressive in the mammary gland. There is considerable evidence for TGF- β signaling being tumor suppressive in other organs systems for both experimental animals and humans.²⁷

The results of the present study are somewhat different from those reported by another group with a line of MMTV-DNIIR mice developed in their laboratory⁹ where mammary tumors did not develop spontaneously. There are several potential reasons for the disparity, including strain differences (FVB/N strain in the previous study versus C57BL/6.DBA/2 in the present study) and level of transgene expression.

Cross breeding MMTV-DNIIR animals with MMTV-TGF- α mice to obtain mice expressing both transgenes resulted in mammary tumor formation with a much shorter

latency and was similar to those expressing only the MMTV-TGF- α transgene (<10 months median latency). The major difference in mammary tumors arising in MMTV-TGF- α compared to bigenic MMTV-DNIIR/MMTV-TGF- α was the marked suppression of tumor invasion by DNIIR transgene expression. These findings are consistent with evidence indicating that TGF- β signaling can enhance progression of carcinomas after they have developed. We have known for many years that carcinoma cells frequently lose the growth inhibitory response to TGF- β and increase production of one or more of the TGF- β isoforms (TGF- β 1, - β 2, and - β 3).^{27,28} High levels of TGF- β locally can have effects on host cells that favor tumor growth (suppression of immune surveillance, stimulation of connective tissue formation, and angiogenesis).²⁹ Some carcinoma cells that have become refrac-

Figure 7. Effect of MMTV-DNIIR transgene expression on mammary tumor initiation by MMTV-TGF- α transgene. **A:** Survival plot comparing development of mammary carcinomas in MMTV-TGF- α (solid line) and bigenic MMTV-TGF- α /MMTV-DNIIR (dashed line) multiparous female mice showing no significant difference in median latency ($P = 0.22$) or tumor incidence. **B** and **C:** A higher frequency of stromal invasion was observed in mammary tumors arising in MMTV-TGF- α mice (**B**) than in bigenic mice, which more frequently demonstrated a noninvasive CIS/HG-MIN pattern (**C**). Scale bar, 100 μ m.

Table 3. Mammary Tumors in Multiparous MMTV-TGF- α /MMTV-DNIIR Female Mice

Animal no.	Age of tumor appearance (months)	Number of mammary tumors	Tumor type (no. of tumors)
1	16	1	CIS/HG-MIN*
2	18	2	CIS/HG-MIN (2)
3	18	4	CIS/HG-MIN (3) Invasive adenocarcinoma (1)
4	21	1	CIS/HG-MIN
5	24	1	CIS/HG-MIN
6 [†]	24	3	CIS/HG-MIN (3)
7	25	1	CIS/HG-MIN
8	30	1	CIS/HG-MIN
9	30	2	CIS/HG-MIN (1) Invasive adenocarcinoma (1)
10	34	4	CIS/HG-MIN (4)
11	38	2	CIS/HG-MIN (2)
12	38	2	Invasive adenocarcinoma (2)
13	40	1	CIS/HG-MIN
14	43	2	CIS/HG-MIN (1) Invasive adenocarcinoma (1)
15	43	4	CIS/HG-MIN (3) Invasive adenocarcinoma (1)
16	45	1	CIS/HG-MIN
17	47	1	CIS/HG-MIN

*Carcinoma *in situ*/high-grade mammary intraepithelial neoplasia.

[†]Metastasis to lung.

Seventeen of 19 (89%) animals developed 32 mammary tumors.

Six of 32 (19%) were invasive.

tory to growth inhibition by TGF- β can still respond in an autocrine and paracrine manner to TGF- β , converting to a spindle morphology with increased plasticity and motility, changes that favor invasion and metastasis.¹⁹

Distant metastases are not observed as frequently in the MMTV-TGF- α mice^{13,30} as in the MMTV-*c-neu* model.^{21,31} This could be in part because of the cystic nature and large size of the mammary tumors in the MMTV-TGF- α mice precluding sufficient follow-up time for development of metastases. One pulmonary metastasis from a mammary tumor was observed in the bigenic mice expressing both the MMTV-TGF- α and MMTV-DNIIR transgenes (Table 3), and this tumor was observed to express the DNIIR transgene by *in situ* hybridization with a DNIIR transgene-specific probe (data not shown). On the surface, this seems contradictory to the data presented herein concerning suppression of invasion by DNIIR transgene expression. However, it is possible that microenvironmental factors can result in loss of MMTV-DNIIR transgene expression resulting in invasion and metastasis with re-establishment of transgene expression by the microenvironment of the metastatic site.

In summary, we have demonstrated that partial blockade of TGF- β signaling in mammary epithelial cells in female mice causes precocious mammary gland development during puberty and early pregnancy, with impaired development during late pregnancy and lactation and retarded postlactational involution. This is a common feature of transgenic mice expressing an oncogene in mammary epithelium^{13,15,32–34} and may contribute to the accelerated development of mammary tumors. Indeed, we show that abrogation of signaling from endogenous TGF- β s can result in accelerated spontaneous mammary tumor development. Although the effect of TGF- β signal-

ing in the mammary gland will likely be contextual depending on initiating events, these results indicate that signaling from endogenous TGF- β s suppresses formation of mammary tumors initiated by endogenous events. Further, we provide additional *in vivo* data supporting the hypothesis that, after tumors have developed, TGF- β signaling can enhance carcinoma cell invasion and metastasis. This provides a potentially important target for therapy.²¹

References

- Robinson SD, Silberstein GB, Roberts AB, Flanders KC, Daniel CW: Regulated expression and growth inhibitory effects of transforming growth factor- β isoforms in mouse mammary gland development. *Development* 1991, 113:867–878
- Silberstein GB, Daniel CW: Reversible inhibition of mammary gland growth by transforming growth factor- β . *Science* 1987, 237:291–293
- Pierce Jr DF, Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, Daniel CW, Hogan BL, Moses HL: Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF- β 1. *Genes Dev* 1993, 7:2308–2317
- Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen L, Roberts AB, Smith GH, Merlino G: Targeting expression of a transforming growth factor β 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *EMBO J* 1993, 12:1835–1845
- Kordon EC, McKnight RA, Jhappan C, Hennighausen L, Merlino G, Smith GH: Ectopic TGF β 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev Biol* 1995, 168:47–61
- Pierce Jr DF, Gorska AE, Chytil A, Meise KS, Page DL, Coffey Jr RJ, Moses HL: Mammary tumor suppression by transforming growth factor β 1 transgene expression. *Proc Natl Acad Sci USA* 1995, 92:4254–4258
- Boulanger CA, Smith GH: Reducing mammary cancer risk through premature stem cell senescence. *Oncogene* 2001, 20:2264–2272
- Buggiano V, Schere-Levy C, Abe K, Vanzulli S, Piazzon I, Smith GH,

- Kordon EC: Impairment of mammary lobular development induced by expression of TGF β 1 under the control of WAP promoter does not suppress tumorigenesis in MMTV-infected transgenic mice. *Int J Cancer* 2001, 92:568–576
9. Bottinger EP, Jakubczak JL, Roberts IS, Mumy M, Hemmati P, Bagnall K, Merlino G, Wakefield LM: Expression of a dominant-negative mutant TGF- β type II receptor in transgenic mice reveals essential roles for TGF- β in regulation of growth and differentiation in the exocrine pancreas. *EMBO J* 1997, 16:2621–2633
10. Gorska AE, Joseph H, Derynck R, Moses HL, Serra R: 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. *Cell Growth Differ* 1998, 9:229–238
11. Bottinger EP, Jakubczak JL, Haines DC, Bagnall K, Wakefield LM: Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor β receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz[*a*]anthracene. *Cancer Res* 1997, 57:5564–5570
12. Amendt C, Schirmacher P, Weber H, Blessing M: Expression of a dominant negative type II TGF- β receptor in mouse skin results in an increase in carcinoma incidence and an acceleration of carcinoma development. *Oncogene* 1998, 17:25–34
13. Matsui Y, Halter SA, Holt JT, Hogan BL, Coffey RJ: Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. *Cell* 1990, 61:1147–1155
14. Brandt R, Eisenbrandt R, Leenders F, Zschiesche W, Binas B, Jurgensen C, Theuring F: Mammary gland specific hEGF receptor transgene expression induces neoplasia and inhibits differentiation. *Oncogene* 2000, 19:2129–2137
15. Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF, Hla T: Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *J Biol Chem* 2001, 276:18563–18569
16. Liang KY, Zeger SL: Longitudinal data analysis using generalized linear models. *Biometrika* 1986, 73:13–22
17. Diggle P, Liang KY, Zeger SL: *Analysis of Longitudinal Data*. Oxford, Clarendon Press, 1994
18. Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, Rehm S, Russo J, Tavassoli FA, Wakefield LM, Ward JM, Green JE: The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. *Oncogene* 2000, 19:968–988
19. Dumont N, Arteaga CL: Transforming growth factor- β and breast cancer: tumor promoting effects of transforming growth factor- β . *Breast Cancer Res* 2000, 2:125–132
20. McEarchern JA, Kobie JJ, Mack V, Wu RS, Meade-Tollin L, Arteaga CL, Dumont N, Besselsen D, Seftor E, Hendrix MJ, Katsanis E, Akporiaye ET: Invasion and metastasis of a mammary tumor involves TGF- β signaling. *Int J Cancer* 2001, 91:76–82
21. Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, Easterly E, Roebuck LR, Ryan S, Gotwals PJ, Kotliansky V, Arteaga CL: Blockade of TGF- β inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002, 109:1551–1559
22. Bhowmick N, Zent R, Ghaissi M, McDonnell M, Moses HL: Integrin b1 signaling is necessary for transforming growth factor- β activation of p38MAPK and epithelial plasticity. *J Biol Chem* 2001, 276:46707–46713
23. Robinson SD, Roberts AB, Daniel CW: TGF β suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. *J Cell Biol* 1993, 120:245–251
24. Barcellos-Hoff MH, Ewan KB: Transforming growth factor- β and breast cancer: mammary gland development. *Breast Cancer Res* 2000, 2:92–99
25. Nguyen AV, Pollard JW: Transforming growth factor β 3 induces cell death during the first stage of mammary gland involution. *Development* 2000, 127:3107–3118
26. D'Cruz CM, Moody SE, Master SR, Hartman JL, Keiper EA, Imielinski MB, Cox JD, Wang JY, Ha SI, Keister BA, Chodosh LA: Persistent parity-induced changes in growth factors, TGF- β 3, and differentiation in the rodent mammary gland. *Mol Endocrinol* 2002, 16:2034–2051
27. Derynck R, Akhurst RJ, Balmain A: TGF- β signaling in tumor suppression and cancer progression. *Nat Genet* 2001, 29:117–129
28. Akhurst RJ, Derynck R: TGF- β signaling in cancer—a double-edged sword. *Trends Cell Biol* 2001, 11:S44–S51
29. Moses HL, Arteaga CL, Alexandrow MG, Dagnino L, Kawabata M, Pierce Jr DF, Serra R: TGF β regulation of cell proliferation. *Princess Takamatsu Symp* 1994, 24:250–263
30. Halter SA, Dempsey P, Matsui Y, Stokes MK, Graves-Deal R, Hogan BL, Coffey RJ: Distinctive patterns of hyperplasia in transgenic mice with mouse mammary tumor virus transforming growth factor-alpha. Characterization of mammary gland and skin proliferations. *Am J Pathol* 1992, 140:1131–1146
31. Muller WJ: Expression of activated oncogenes in the murine mammary gland: transgenic models for human breast cancer. *Cancer Metastasis Rev* 1991, 10:217–227
32. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P: Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell* 1987, 49:465–475
33. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ: Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* 1992, 89:10578–10582
34. Kwan H, Pecanka V, Tsukamoto A, Parslow TG, Guzman R, Lin TP, Muller WJ, Lee FS, Leder P, Varmus HE: Transgenes expressing the Wnt-1 and int-2 proto-oncogenes cooperate during mammary carcinogenesis in doubly transgenic mice. *Mol Cell Biol* 1992, 12:147–154