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Urethane-induced Mammary Carcinogenesis Susceptibility in Transgenic Mice Expressing a Dominant-negative TGF-β Type II Receptor

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Abstract. *Background/Aim: Transforming growth factor-β (TGF-β) plays dual suppressive and oncogenic roles in mammary carcinogenesis. Materials and Methods: To analyze whether TGF-β exerts suppressive or oncogenic actions on mammary carcinogenesis, transgenic mice overexpressing a dominant-negative mutant type II TGF-β receptor (TβRII-DNR) driven by the mouse mammary tumor virus (MMTV) promoter were treated with a low dose of urethane, a carcinogen present in fermented food products and alcoholic beverages. Results: Lobular proliferative lesions, showing high β-casein expression, developed in the mammary glands of TβRII-DNR$^{+/+}$ mice aged >61 weeks. Compared with wild-type mice, TβRII-DNR$^{+/+}$ mice administered with urethane showed significant increases in dysplastic hyperplasias and adenocarcinomas of the mammary glands. Conclusion: The functional decline of TGF-β signaling in mammary glands led to a high susceptibility to urethane-induced mammary carcinogenesis. TGF-β signaling may act as a tumor suppressor during mammary tumor development.*

Transforming growth factor-β (TGF-β) plays multiple roles in cancer progression (1). TGF-β signals through a cell surface complex of two types of transmembrane serine/threonine kinases, TGF-β type I (TβRI) and type II (TβRII). The formation of this complex results in the phosphorylation of downstream SMAD transcription factors (2, 3). SMADs exert both agonistic and antagonistic actions in TGF-β signaling (4). TGF-β inhibits the growth of both normal and transformed breast epithelial cells (5). In the early stages of breast cancer, TGF-β acts as a potent tumor suppressor by upregulating cell cycle arrest-related genes (6). Resistance to the TGF-β-mediated inhibitory effects on cell proliferation has been attributed to the loss of TβRII expression in breast cancer cells (7). Furthermore, transgenic mice expressing the active form of TGF-β1 are resistant to 7,12-dimethylbenz-[a]-anthracene (DMBA)-induced mammary carcinogenesis (8). Similar resistance to mammary tumorigenesis has been shown in TGF-β1 transgenic mice (9, 10). TGF-β1 function was specifically ablated in the mammary glands through the targeted overexpression of a dominant-negative TβRII (TβRII-DNR, a kinase-defective mutant) driven by a MMTV promoter. Virgin female TβRII-DNR transgenic mouse showed significant increases in lobulo-alveolar lesions (11, 12). Moreover, TβRII-DNR transgenic mice treated with DMBA showed a significant increase in mammary tumors (11). In contrast, TGF-β overexpression has been reported in advanced human breast cancers (13). Furthermore, cancer-associated fibroblasts (CAFs) promoted aggressive breast cancer cell phenotypes through paracrine TGF-β1-induced epithelial-mesenchymal transition (EMT) (14). Thus, TGF-β in breast cancer acts biphasically, *i.e.*, as both tumor suppressor and enhancer in the early and late stages, respectively (13). To analyze whether TGF-β exerts suppressive or oncogenic actions in mammary carcinogenesis, transgenic mice overexpressing TβRII-DNR driven by the MMTV promoter were developed and administered a low dose of a carcinogen, urethane (ethyl carbamate), which targets the mammary glands and lungs. Urethane is found in many fermented food products and alcoholic beverages, such as cheese, bread, yogurt, wine, whiskey, and soy sauce (15).

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Key Words: TGFβ signaling, functional loss, mammary cancer, environmental carcinogen.

Materials and Methods

Generation of transgenic mice. The pSKMMTV-SVPA vector (11) containing the human *TβRII* encoding extracellular and transmembrane domains (nucleotides 7 through 573) (TβRII-DNR)

was kindly provided by Dr. Lalage M. Wakefield (Center for Cancer Research, National Cancer Institute, MD, USA). The transgene is flanked by the MMTV-LTR promoter-enhancer and SV40 3' UTR and a polyadenylation signal on excision with BglII and SpeI. To generate MMTV-TβRII-DNR transgenic mice, the transgene was injected into the pronuclei of inbred FVB zygotes (16). Four founders were obtained, two of which expressed the transgene and were bred into lines A and B. Tail genomic DNA was prepared and screened using the PCR analysis. Because heterozygous TβRII-DNR mice up to 77 weeks of age did not develop any proliferative lesions in the mammary glands, they were bred with each other to produce homozygous offspring (TβRII-DNR$^{+/+}$) used in the present studies. Age-matched wild-type female FVB mice were used as controls. All manipulations of mice and animal experiments were approved by the Institutional Review Board of the Osaka Medical College (approval no. 17056) and were performed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals at Osaka Medical College.

Histopathology and immunohistochemistry. After euthanizing the animals, we removed their mammary glands from the axillary and femoral regions and lungs and also removed any other abnormal tissues. The tissues were immediately divided into portions, fixed in 10% phosphate-buffered formalin (PBF) solution, and processed for paraffin embedding. All paraffin-embedded tissues were cut in 4-μm slices and stained with hematoxylin and eosin (H&E) for histopathological examination.

The avidin-biotin complex method was used for immunohistochemistry. Unstained sections were immersed in citrate buffer (pH 6.4) and heated using the autoclave (110˚C for 20 min) for antigen retrieval before incubation with 1 μg/ml of an anti-PCNA mouse monoclonal antibody (clone PC10) or 0.5 μg/ml of anti-β-casein goat polyclonal antibody (clone M-14). These antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Cell proliferation and apoptosis. Formalin-fixed paraffin-embedded (FFPE) mammary glands were evaluated for cell proliferation as determined by PCNA-positive nuclear staining. The numbers of PCNA-positive cells in whole mammary glands were counted and expressed as percentage values. FFPE mammary glands were also used for quantitative analyses of apoptotic cell death using TUNEL reaction (Fujifilm Wako Pure Chemical Co., Osaka, Japan). The number of TUNEL-positive cells were counted in whole mammary glands and expressed as percentage values.

Quantitative Real-Time PCR (qPCR). FFPE tumor tissues were analyzed using a real-time PCR system (17). Total RNA was extracted from FFPE tissue samples using the RNeasy FFPE Kit (Qiagen, GmbH, Hilden, Germany) and cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, GmbH, Manheim, Germany). Real-time qPCR was performed using a Light Cycler (Roche Diagnostics) as follows: initial step at 95˚C for 30 sec, followed by 45 cycles for 10 sec at 95˚C, 10 sec at 62˚C, and 16 sec at 72˚C. The primer sequences for human *TβRII* were 5'-GCGTATCGCCAGCACGAT-3' and 5'-GCATGAGCAACTGCAGCATC-3'. The plasmid vector (0.1, 1.0, and 10 pg) containing the human *TβRII* gene was used for the quantitation of transgene expression. Using linear regression of the standard curve, the transcript copy numbers of the human *TβRII* gene were calculated as previously described (18).

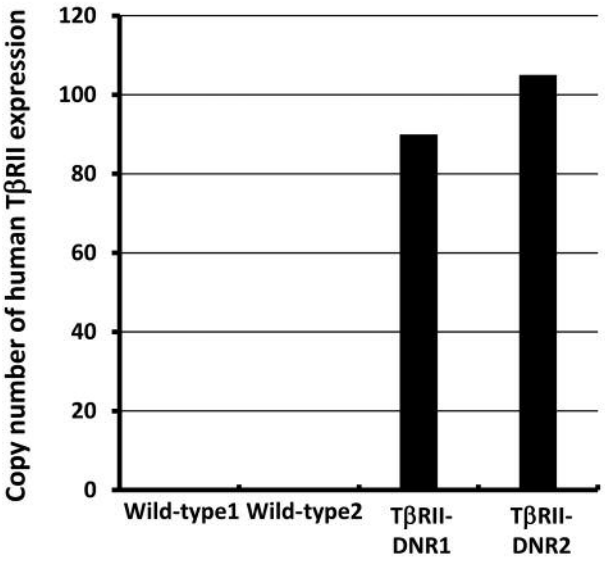


Figure 1. *Transcript copy numbers of the transgene in the mammary glands of dominant-negative mutant type II TGF-β receptor (TβRII-DNR$^{+/+}$) mice. Transgene expression was not detected in the mammary glands of wild-type mice. High copy numbers of the transgene were observed in the mammary glands of TβRII-DNR$^{+/+}$ mice.*

Chemical carcinogenesis study. A total of 23 wild-type FVB female mice (Japan SLC Inc., Hamamatsu, Japan) and 30 TβRII-DNR$^{+/+}$ transgenic female mice (A line) were used. The urethane dose was 500 ppm (0.05%) in potable water based on a previous report that the administration of 0.1% urethane in potable water for 15 months induced mammary tumors in 24% of mice (19). Wild-type and transgenic mice aged 6 weeks were provided with potable water containing 500 ppm urethane (ethyl carbamate; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) ad libitum in light-opaque bottles for 24 weeks and thereafter normal tap water until the termination of the experiment. The urethane solution was renewed twice weekly. At 25 to 36 weeks after commencement of the urethane treatment, mice found dead or sacrificed at a moribund condition were necropsied and their mammary glands and lungs were excised, fixed in 10% PBF solution, and processed for histopathological examination. The lungs were inflated with PBF solution before excision and immersion in the fixative. The mammary proliferative lesions were classified as previously reported (19).

Statistical analysis. The significance of differences in the incidences of lesions between groups was examined with Fisher's Exact probability test. The significance of differences between wild-type and transgenic mice was analyzed by the Mann–Whitney *U*-test (unpaired group and non-parametric analysis).

Results

Transgene expression in the mammary glands of TβRII-DNR mice. Real-time qPCR was performed to measure transgene expression in the mammary glands of TβRII-DNR$^{+/+}$ mice.

Table I. *Histopathological findings of mammary glands in MMTV-TβRII-DNR transgenic mice.*

	8-23 weeks old (%)		61-77 weeks old (%)			
Genotype	No. of mice examined	Normal	No. of mice examined	Normal	Lobular hyperplasia	Dysplastic hyperplasia
Wild type	13	13 (100)	11	11 (100)	0	0
TβRII-DNR$^{+/+}$-A line	20	20 (100)	33	29 (88)	6 (18)	2 (6)
TβRII-DNR$^{+/+}$-B line	13	13 (100)	13	12 (92)	1(8)	0

As shown in Figure 1, transgene expression was not detected in the mammary glands of the wild-type mice, whereas high copy numbers of the transgene were observed in the mammary glands of TβRII-DNR$^{+/+}$ mice.

Proliferative lesions of mammary glands in TβRII-DNR mice. Histopathological examination of the mammary glands of TβRII-DNR$^{+/+}$ mice (both A and B lines) up to 23 weeks of age showed no apparent abnormalities (Table I). However, although wild-type mice at 61-77 weeks of age did not have any proliferative lesions in their mammary glands (Figure 2A), the 18% of A line and 8% of B line TβRII-DNR$^{+/+}$ mice at the same age range showed lobular hyperplasia (Figure 2B), and some lesions in the A line were accompanied by dysplastic hyperplasia (Figure 2C) (Table I). The lobular and dysplastic hyperplasia were diffusely observed with dilatation of ducts containing eosinophilic secretion (Figure 2B, C). The secretion in TβRII-DNR$^{+/+}$ mice was strongly positive for β-casein expression using immunohistochemistry (Figure 2E) compared with that in wild-type mice (Figure 2D), suggesting a lactational phenotype, as previously described (12). Because TβRII-DNR$^{+/+}$ mice in B line showed a lower incidence of proliferative lesions than those observed in A line, the A line transgenic mice were used for subsequent analyses and the urethane treatment study.

Cell proliferation and apoptosis in mammary glands in TβRII-DNR mice. Cell proliferation in mammary glands, as assessed by PCNA-positive rates, was higher in the normal-appearing mammary glands (Figure 2G) and lobular hyperplasias of TβRII-DNR$^{+/+}$ mice (Figure 2H) than in those of wild-type mice (Figure 2F). As shown in Figure 3A, cell proliferation was significantly higher in whole mammary glands in TβRII-DNR$^{+/+}$ mice than in wild-type mice. Analysis of apoptotic cell death in the mammary glands by TUNEL assay showed many apoptotic cells in wild-type mice (Figure 2I) but decreasing trends of apoptotic cells in normal-appearing mammary glands (Figure 2J) and lobular hyperplasias (Figure 2K) in TβRII-DNR$^{+/+}$ mice. Quantitative analysis showed that the decreasing tendency was not statistically significant between TβRII-DNR$^{+/+}$ and wild-type mice (Figure 3B).

Enhanced mammary carcinogenesis in TβRII-DNR mice treated with urethane. To determine whether a local loss of TGF-β responsiveness might affect susceptibility to urethane-induced mammary carcinogenesis, wild-type and TβRII-DNR$^{+/+}$ mice were treated with a low dose of urethane in their potable water. Consumption of water containing urethane showed no differences between wild-type and TβRII-DNR$^{+/+}$ mice. The incidences of mammary lesions in mice treated with urethane are shown in Table II. Although mammary lobular hyperplasia tended to be higher in TβRII-DNR$^{+/+}$ mice, the incidence of mammary dysplastic hyperplasias (considered preneoplastic lesions) (Figure 4B) was significantly higher in urethane-treated TβRII-DNR$^{+/+}$ mice that in urethane-treated wild-type mice (Figure 4A). Furthermore, mammary adenocarcinomas were observed only in TβRII-DNR$^{+/+}$ mice (Figure 4C), with a statistically significant difference (Table II). Regardless of the transgenic status, the lungs of most of the mice treated with urethane showed hyperplasias, adenomas, and adenocarcinomas (Figure 4D).

Discussion

Homozygous TβRII-DNR$^{+/+}$ mice spontaneously developed mammary lobular and dysplastic hyperplasias with strong β-casein expression. Pathological analysis showed a significant increase in cell proliferation and a decrease in apoptotic cell death in both normal-appearing tissues and proliferative lesions of the mammary glands. Administration of 500 ppm urethane in potable water for 6 months resulted in a significant higher incidence of mammary preneoplastic lesions and adenocarcinomas in TβRII-DNR$^{+/+}$ mice than those in wild-type mice. Functional decline of TGF-β in the mammary glands showed high susceptibility to urethane-induced mammary carcinogenesis.

Transgenic mice overexpressing TGF-β1 in mammary glands showed resistance to DMBA-induced mammary carcinogenesis compared with that in MMTV-TGF-α transgenic mice (8). Furthermore, mice carrying both MMTV-TGF-β1 and MMTV-TGF-α (an epithelial mitogen) transgenes also showed resistance to DMBA-induced mammary tumor formation (8). Conversely, TGF-β1$^{+/-}$ (deletion of a single

Table II. *Incidences of preneoplastic and neoplastic lesions of mammary glands in MMTV-TβRII-DNR transgenic mice administered with urethane in drinking water.*

Genotype	No. of mice examined	Normal	Lobular hyperplasia	Dysplastic hyperplasia	Adenocarcinoma
Wild type	23	20 (87)	1 (4)	2 (9)	0
TβRII-DNR$^{+/+}$	30	9 (30)	4 (13)	11(37)*	6 (20)*

Parenthesis indicates % of lesion incidences. *$p<0.05$ as compared with those of the wild-type mice.

allele) mice treated with carcinogens showed enhanced liver (20) or lung (21) tumorigenesis compared to those in TGF-β1$^{+/+}$ mice. Furthermore, TGF-β1$^{+/-}$ mice also showed an increasing propensity for spontaneous colon tumorigenesis (22). The effect of endogenous TGF-β signaling on mammary tumorigenesis has been examined in transgenic mice by expression of TβRII-DNR driven by the MMTV promoter. Virgin female TβRII-DNR mice showed increased lobulo-alveolar development (11, 12) and enhanced DMBA-induced mammary tumorigenesis (11). Moreover, mammary carcinomas spontaneously developed in MMTV-TβRII-DNR mice with a long latency (23). The present experiment also showed the high susceptibility of MMTV-TβRII-DNR mice to urethane-induced mammary tumor development. These results suggested that TGF-β signaling protects mammary tumorigenesis. Although mutational inactivation of *TβRII* is common in human colon cancers (24), specific mutations of both *TβRI* and *TβRII* are rare in breast cancer (25). However, methylation is reportedly responsible for TβRII silencing in human breast cancers (26).

TGF-β-induced cell growth inhibition has been attributed to the induction of cyclin-dependent kinase (Cdk) inhibitors p15 or p21 by the SMAD signaling pathway (3). However, TβRI kinase inhibitor (SD-208) has been shown to suppress the invasiveness of pancreatic cancer cells via alterations in SMAD signaling (27). Furthermore, another TβRI kinase antagonist (SM16) inhibits growth of murine metastatic mammary cancer (28). In addition, TGF-β can both induce and suppress apoptosis. TGF-β may exhibit this reciprocal action because of the cross-talk between the PI3K-Akt and TGF-β pathways to determine whether cells undergo apoptosis or cell proliferation in response to TGF-β (3).

In contemporary medicine, TGF-β signaling is explained by microRNA (miRNA) actions. Certain miRNAs are involved in the post-transcriptional gene regulation of TGF-β signaling (29). Overexpression of miR-5590-3p induced down-regulation of TβRI, TβRII, SMAD3, and SMAD4 expression in a human colon cancer cell line, while miR-5590-3p was down-regulated in advanced breast cancer (30), indicating a dual role of TGF-β signaling. TGF-β induces EMT (14), which is crucial for embryonic development and tumor metastasis (31). Overexpression of the miR-200 family can prevent the TGF-β-induced mesenchymal-to-epithelial transition (MET) (32). miRNAs targeting TGF-βs, especially TβRII (30, 33), may be a potential tool in breast cancer therapy (29).

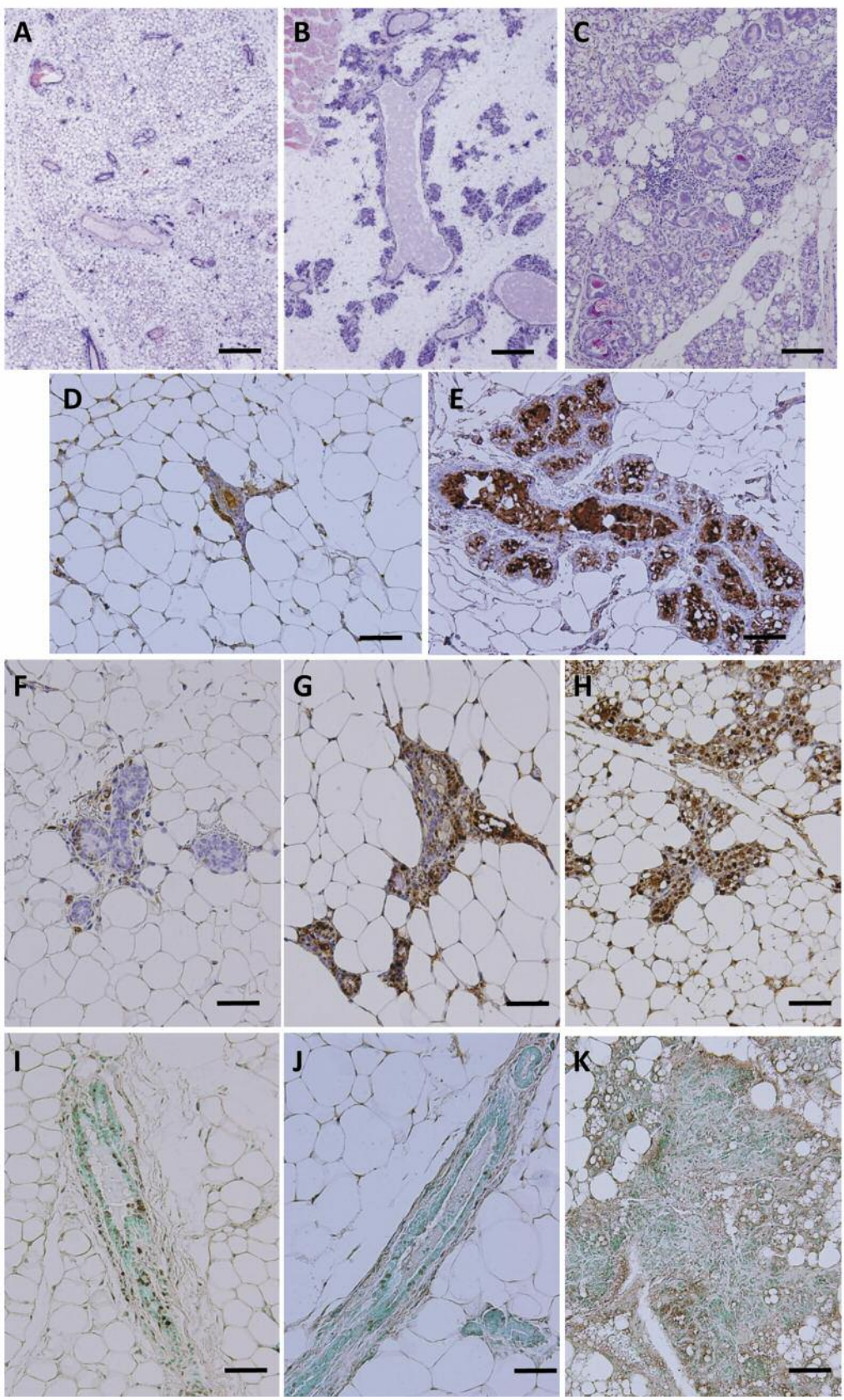
Previous studies have demonstrated the presence of urethane in fermented food products and alcoholic beverages (15). The maximum residue level of urethane in alcoholic beverages ranges by alcoholic species and country from 15 to 300 μg/l. However, summative or cumulative exposure to urethane should not be neglected (15).

In conclusion, down-regulation of TGF-β signaling by a TβRII-DNR transgene in the mammary glands induced mammary preneoplastic lesions that showed high susceptibility to urethane-induced mammary carcinogenesis. Although *TβRII* mutations are rare in human breast cancer (25), environmental factors such as urethane may influence breast cancer risk by inactivating the gene by methylation or specific miRNAs.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Figure 2. *Histopathology, cell proliferation, and apoptosis of mammary glands in TβRII-DNR$^{+/+}$ mice. A. Normal mammary glands in a wild-type mouse (85 weeks of age). B. Lobular hyperplasia of the mammary glands in a TβRII-DNR$^{+/+}$ mouse (61 weeks of age). Note the dilatation of mammary ducts containing eosinophilic secretion. C. Dysplastic hyperplasia of the mammary glands in a TβRII-DNR$^{+/+}$ mouse (71 weeks of age). D, E. β-Casein strongly expressed in the lumen of alveoli and ducts of the mammary glands of a TβRII-DNR mouse (E, 110 weeks of age), whereas a wild-type mouse shows only intracellular expression (D, 85 weeks of age). F-H. Although few PCNA-positive cells are visible in the mammary glands of a wild-type mouse (F, 57 weeks of age), many positive cells are seen in normal-appearing mammary glands (G, 70 weeks of age) and lobular hyperplasias (H, 71 weeks of age) of TβRII-DNR$^{+/+}$ mice. I-K. Many TUNEL-positive cells are present in normal mammary glands of a wild-type mouse (I, 57 weeks of age). However, only a few cells are positive by TUNEL in normal-appearing mammary glands (J, 66 weeks of age) and mammary proliferative lesions in a TβRII-DNR$^{+/+}$ mouse (K, 71 weeks of age). A-C: H&E stain; D, E: β-Casein immunohistochemistry; F-H: PCNA immunohistochemistry; I-K: TUNEL reaction. Scale bars: A-E, 100 μm; F-K, 50 μm.*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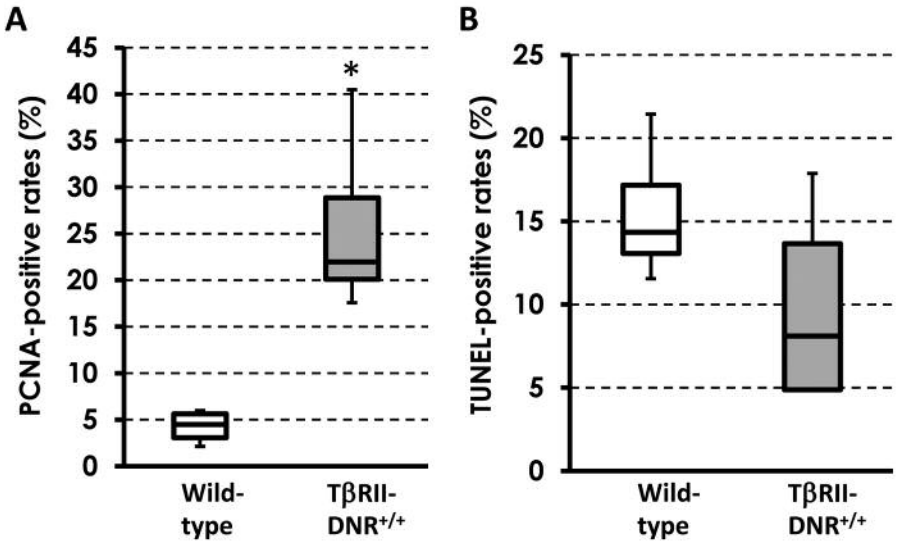

Figure 3. *Cell proliferation and apoptotic cell death in TβRII-DNR$^{+/+}$ mice. A. Cell proliferation, evaluated by PCNA-positive rates, is significantly higher in TβRII-DNR$^{+/+}$ mice than in wild-type mice. B. Apoptotic cell death, evaluated by TUNEL-positive rates, is lower in TβRII-DNR$^{+/+}$ mice than in wild-type mice; however, the difference is not statistically significant. *p<0.05 compared with wild-type mice. The boxes represent the 25th to 75th percentiles; horizontal lines within the box represent median values. The whiskers extend either to the 10th or 90th percentiles, respectively.*

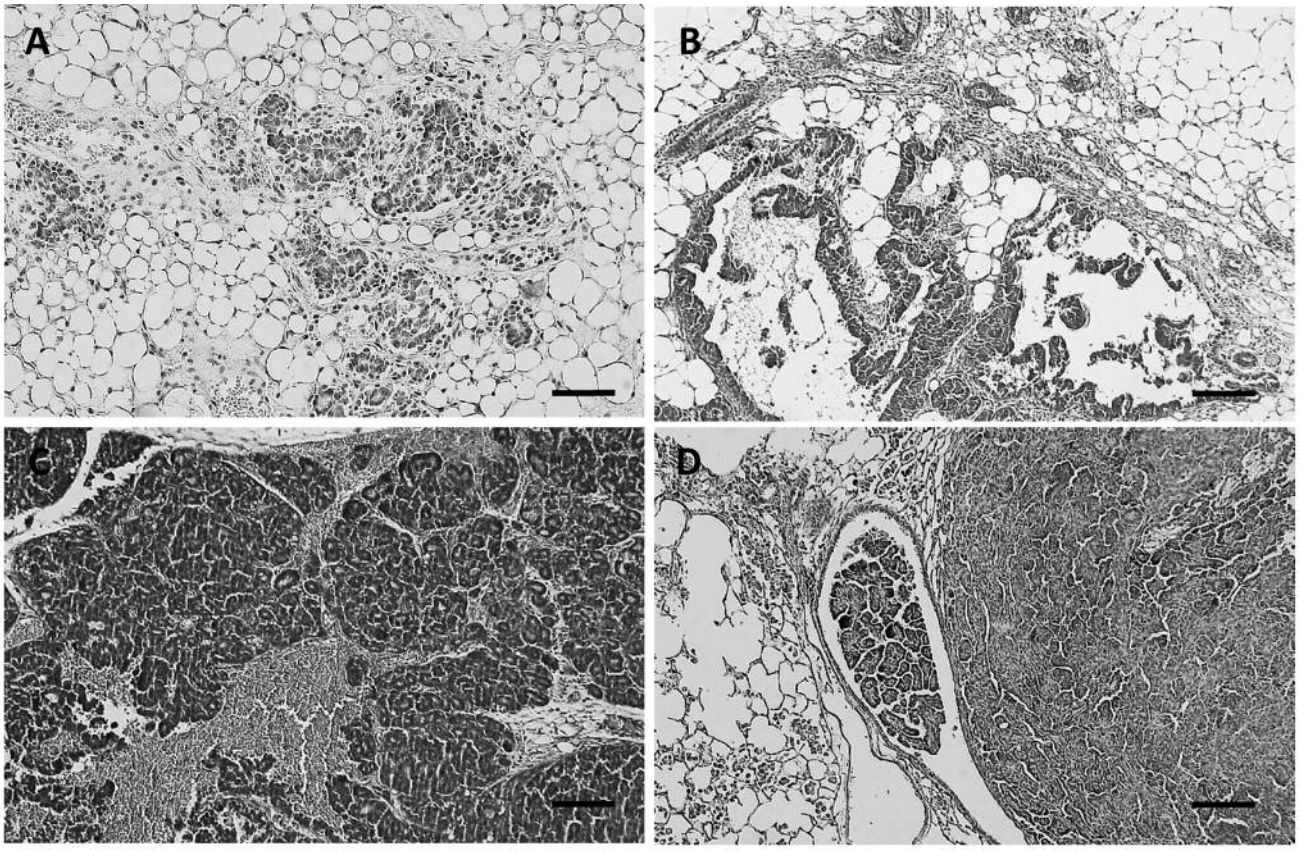

Figure 4. *Mammary and lung lesions of wild-type and TβRII-DNR$^{+/+}$ mice treated with urethane. A. Mammary hyperplasia of a wild-type mouse (37 weeks of age). B. Mammary dysplastic hyperplasia of a TβRII-DNR$^{+/+}$ mouse (34 weeks of age). C. Mammary adenocarcinoma of a TβRII-DNR$^{+/+}$ mouse (38 weeks of age). D. Lung adenocarcinoma of a TβRII-DNR$^{+/+}$ mouse (36 weeks of age). A-D: H&E stain; Scale bars: A-D, 100 μm.*

Authors' Contributions

M.A.S. conceptualized the experiments; performed all animal experiments, histopathological analyses, and real-time qPCR; and drafted the manuscript. E.S. generated the transgenic mice and performed breeding and histopathological staining. J.M. conducted the urethane experiment on mice. Y.K. discussed the whole project and data and contributed in improving the final manuscript. All Authors approved the final manuscript.

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