Impaired mammary gland and lymphoid development caused by inducible expression of Axin in transgenic mice

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Axin is a component of the canonical Wnt pathway that negatively regulates signal transduction by promoting degradation of β-catenin. To study the role of Axin in development, we developed strains of transgenic mice in which its expression can be manipulated by the administration of doxycycline (Dox). Animals carrying both mouse mammary tumor virus (MMTV)–reverse tetracycline transactivator and tetracycline response element (TRE)2–Axin–green fluorescent protein (GFP) transgenes exhibited Dox-dependent Axin expression and, when induced from birth, displayed abnormalities in the development of mammary glands and lymphoid tissues, both sites in which the MMTV promoter is active. The transgenic mammary glands underwent normal ductal elongation and side branching during sexual maturation and early pregnancy, but failed to develop lobulo-alveoli, resulting in a defect in lactation. Axin attenuated the expression of cyclin D1, a Wnt target that promotes the growth and differentiation of mammary lobulo-alveoli. Increased apoptosis occurred in the mammary epithelia, consistent with the inhibition of a Wnt/cyclin D1 survival signal by Axin. High levels of programmed cell death also occurred in the thymus and spleen. Immature thymocytes underwent massive apoptosis, indicating that the overexpression of Axin blocks the normal development of T lymphocytes. Our data imply that the Axin tumor suppressor controls cell survival, growth, and differentiation through the regulation of an apoptotic signaling pathway.

Introduction

Axin was first identified as a negative regulator of embryonic axis formation, exerting its effects by modulating the Wnt signal transduction pathway (Zeng et al., 1997). Substantial evidence from several laboratories has since revealed that Axin plays a central role in regulating the stability of β-catenin, a crucial event in cellular responses to Wnt signaling (reviewed in Miller et al., 1999; Kikuchi, 2000; Peifer and Polakis, 2000). Distinct functional domains of Axin mediate its interaction with several Wnt signaling molecules, including disheveled, the serine/threonine kinase GSK-3β, β-catenin,

adenomatous polyposis coli (APC),* and the serine/threonine phosphatase PP2A_c (Behrens et al., 1998; Fagotto et al., 1999; Hsu et al., 1999; Kikuchi, 2000). In the absence of a Wnt signal, β-catenin forms a complex including Axin, APC, and GSK-3β, leading to its phosphorylation by GSK-3β and degradation through the ubiquitin-dependent proteolysis system (Aberle et al., 1997; Hart et al., 1998; Farr et al., 2000). Wnt signals perturb the formation of this complex by activating the upstream regulators, disheveled, casein kinase Iε, and GBP/Frat1 (Li et al., 1999; Peters et al., 1999; Smalley et al., 1999; Willert et al., 1999; Julius et al., 2000; Kikuchi, 2000). Thus, β-catenin is protected from phosphorylation and consequent degradation. The downstream signaling events are triggered by the accumulation of nuclear β-catenin, which interacts with transcription factors of the lymphoid enhancer factor/T cell factor (LEF/TCF) family (Behrens et al., 1996; Molenaar et al., 1996) to regulate target genes, such as cyclin D1, c-myc, and c-jun (He et al., 1998; Mann et al., 1999; Shtutman et al., 1999; Tetsu and McCormick, 1999). In addition to its interaction with components of the Wnt pathway, Axin has been shown to activate the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling cascade through

domains distinct from those regulating Wnt/β-catenin (Zhang et al., 1999).

Members of the Wnt family are important for diverse processes during embryonic, fetal, and postnatal development, including the development of the embryonic axis, central nervous system, limbs, reproductive tract, kidneys, and mammary glands (reviewed in Wodarz and Nusse, 1998). Furthermore, several Wnt signaling molecules have been implicated in the development of different forms of cancer (reviewed in Behrens, 2000; Polakis, 2000). For instance, mammary gland tumors develop in transgenic mice with elevated expression of Wnt-1, Wnt-10b, or the Wnt target gene cyclin D1 (Tsukamoto et al., 1988; Wang et al., 1994; Lane and Leder, 1997). The development of colorectal cancers is commonly initiated by mutations of APC in humans and mice (reviewed in Behrens, 2000; Polakis, 2000). Expression of a stabilized form of β-catenin can lead to the formation of hair follicle and mammary tumors in transgenic mice (Gat et al., 1998; Imbert et al., 2001), and mutations affecting the stability of β-catenin have been identified in human malignancies (reviewed in Polakis, 2000). Finally, the recent finding that Axin is mutated in hepatocellular carcinomas suggests that it acts as a tumor suppressor (Satoh et al., 2000).

Although some understanding of the role of Axin in early mouse embryogenesis has been gained through studying loss of function mutations (Gluecksohn-Schoenheimer, 1949; Jacobs-Cohen et al., 1984; Perry et al., 1995; Zeng et al., 1997), its role in later developmental events mediated by Wnt signals is unclear, in part because of the early embryonic lethality of Axin mutants. To further investigate the role of Axin in mammalian development and tumorigenesis, we have developed bi-transgenic strains of mice in which the overexpression of Axin can be induced in several tissues, using the tetracycline-dependent activation system (Gossen et al., 1995). We have used these mice to examine the consequences of Axin overexpression in the postnatal development of the mammary gland and lymphoid tissues. Our results imply that the Axin tumor suppressor plays a role in cell survival, growth, and differentiation through the regulation of an apoptotic signaling pathway.

Results

Regulated expression of Axin in transgenic mice

To investigate the potential ability of Axin to regulate Wnt signal transduction during mammary gland development and tumorigenesis, Axin was expressed in transgenic mice using the promoter/enhancer of mouse mammary tumor virus (MMTV). Initial attempts to establish lines of mice expressing an MMTV–Axin transgene were unsuccessful (unpublished data). We suspected this might be due to pre- or perinatal lethal effects of the transgene, since the MMTV promoter/enhancer is active in several tissues in addition to the mammary gland (Hennighausen et al., 1995). We therefore attempted to express Axin using a tetracycline-dependent activation system, which had been successfully used for conditional gene expression in eukaryotic cells and in various tissues of mice (reviewed in Saez et al., 1997). In this system, three components are required to achieve tetracy-
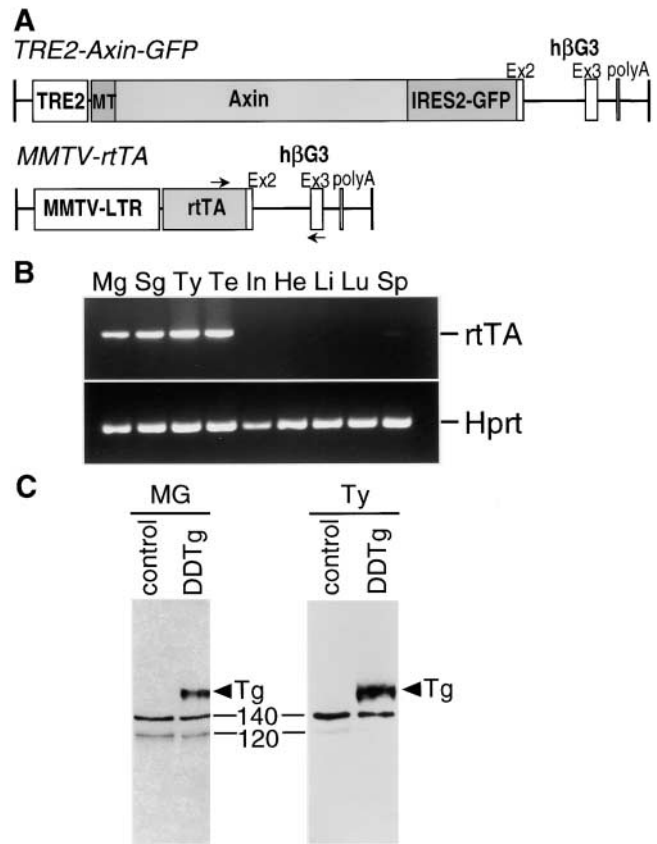

Figure 1. **DNA constructs used for Dox-inducible expression of Axin and analyses of rtTA and Axin expression in transgenic mice.** (A) TRE2–Axin–GFP consisted of a tetracycline responsive element (TRE2), the myc-tagged, full-length mouse Axin coding sequence, IRES2, and the enhanced GFP. MMTV–rtTA consisted of the MMTV LTR and rtTA coding sequence. Both constructs included noncoding exon2, intron, exon3, and polyadenylation sequences of hβG. (B) Expression of rtTA in tissues of MMTV–rtTA transgenic mice (line MTA4) was analyzed by RT-PCR using primers (arrows) within the rtTA coding region and noncoding exon 3 of hβG, which are specific for the spliced form of the mRNA. Strong signals were detected in Mg, Sg, Ty, and Te, whereas a weak signal was consistently observed in Sp. Expression of the housekeeping gene Hprt was used as a control. (C) Protein lysates from the mammary gland (16.5 dpc) and thymus (3 mo) of control and DDTg mice were immunostained with anti-Axin antibody. The endogenous Axin with an apparent molecular mass of 120 and 140 kD were detected in both control and DDTg lysates. Extracts from DDTg mice contained an additional transgenic MT–Axin protein (Tg). The increased size of MT–Axin is due to the myc-tag and to additional NH2-terminal sequences encoded by the full-length Axin cDNA that are not translated in the most abundant endogenous form of Axin. Mg, mammary gland; Sg, salivary gland; Ty, thymus; Te, testis; In, intestine; He, heart; Li, liver; Lu, lung; Sp, spleen.

cline-dependent expression. First, the reverse tetracycline transactivator (rtTA), a fusion of tet-repressor DNA binding domain and VP16 transcriptional activation domain, is expressed in a tissue- or cell-specific pattern, using appropriate regulatory sequences. Second, a tetracycline response element (TRE) is used to control expression of the target gene. Third, the drug doxycycline (Dox), which can be administered in a temporally specific fashion, induces rtTA to bind to the TRE, turning on the expression of the target gene (Gossen et al., 1995).

To express Axin in a tetracycline-dependent manner, the two DNA constructs MMTV–rtTA and TRE2–Axin–green fluorescent protein (GFP) (Fig. 1 A) were used separately to generate transgenic mice (see Materials and methods for details). For easier detection of transgene expression, a myc epitope tag (MT) was inserted at the amino terminus of Axin. The enhanced GFP was included under the control of an internal ribosomal entry sequence (IRES2) as an additional reporter for expression of the Axin transgene (Fig. 1 A). One MMTV–rtTA transgenic line (MTA4), which was used for the studies reported here, expressed rtTA mRNA strongly in the mammary gland, salivary gland, thymus, and testis, weakly but consistently in the spleen, and not detectably in the intestine, heart, liver, or lung, as determined by RT-PCR analysis (Fig. 1 B).

Two different TRE2–Axin–GFP lines, TA11 and TA32, were used in these studies. By crossing either of these lines with MTA4 (in the absence of Dox induction), we were able to obtain the double transgenic mice, which were born without any obvious defects. To induce the expression of MT–Axin and GFP in these offspring, their mothers were fed Dox in the drinking water starting at the time of parturition, and the offspring were maintained on Dox throughout their lives. Expression of MT–Axin and GFP, as well as phenotypic abnormalities, were only observed in the Dox-induced double transgenic offspring (hereafter referred to as DDTg mice), and never in single transgenic siblings treated with Dox, or double transgenics in the absence of Dox (unpublished data). DDTg mice derived from lines TA11 and TA32 expressed MT–Axin and GFP both in the testes and mammary glands, whereas only line TA32 showed expression in the thymus (see Fig. 6 A). Therefore, both TA lines were used for mammary gland analyses, whereas line TA32 was used to study the effects of Axin in lymphoid organs. Western blot analyses with anti-Axin antiserum detected a novel MT–Axin protein in the mammary gland and thymus of DDTg mice, in addition to the endogenous Axin proteins of 120 and 140 kD in control tissues (Fig. 1 C). The transgenic MT–Axin protein was expressed at 1.5 and 2.5 the level of endogenous Axin in mammary gland and thymus, respectively. Approximately 40% of DDTg mice derived from line TA32 died at ~3 wk of age (with Dox treatment starting at the day of birth), whereas those from line TA11 rarely died prematurely. The cause of death in these animals has not been determined, and most of the mice that survived this critical period went on to live to adulthood.

Dox-induced overexpression of Axin inhibits the development of lactating mammary gland

DDTg females, which had been induced with Dox since birth, were mated to wild-type males to assess the effects of Axin overexpression on the postnatal phases of mammary gland development. We studied mammary gland development in a total of 16 DDTg mice, including 12 from line TA11 and 4 from line TA32. An anomaly in mammary gland function was observed soon after they delivered their litters (Fig. 2). In the newborn pups of control females, including TA/MTA double transgenic mice without Dox induction (Fig. 2 A, animal on the left), the stomachs filled with milk could be seen clearly through the body wall.
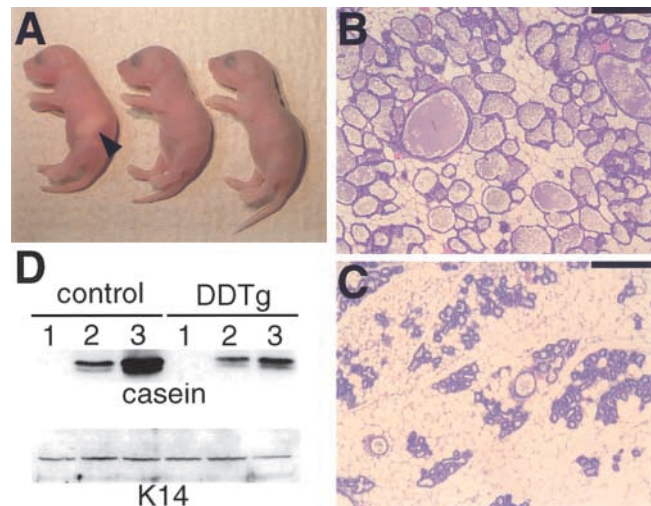

Figure 2. **Dox-induced expression of Axin inhibits maturation of the mammary gland.** (A) In a pup born to a control female (left) the stomach filled with milk is clearly visible (arrowhead). In two pups born to a female DDTg mouse (center and right) no milk was visible in the stomachs. (B and C) Hematoxylin- and eosin-stained sections of mouse mammary glands at 2 d postpartum. In a control mouse (B), the lobulo-alveoli were fully developed and lactation effects were seen. In a DDTg mouse at 2 d postpartum (C), there was hypoplastic development of lobulo-alveoli with very little lactation. (D) Immunoblot analyses showed the expression of casein, a marker for terminally differentiated mammary epithelium, in mammary glands at various developmental stages (lane 1, 2-mo-old virgin; lane 2, 9.5 dpc; lane 3, 16.5 dpc). K14 was also analyzed as a control for epithelial cell content of the mammary glands. Bars, 250 μm.

However, when DDTg females were maintained on Dox throughout their pregnancy the stomachs of all their pups appeared empty (Fig. 2 A, two pups on the right), suggesting a deficiency in lactation. The pups were otherwise normal, as they could be rescued by fostering to normal, lactating females.

The mammary epithelium develops during three phases: fetal development, puberty, and pregnancy (reviewed in Sakakura, 1991). In female fetuses, the mammary buds develop beginning at E12, they become mammary sprouts at E16, and by birth each has ramified into a small mammary gland tree with 15–20 branching ducts. The gland then remains static until puberty. During sexual maturation, the distal end of each duct grows and forms a terminal end bud, a specialized and highly proliferative epithelial structure. The end bud elongates and ramifies to form the mammary ductal tree system, which spreads throughout the fatty stroma. Finally, during pregnancy, the mammary epithelial ducts form side branches that develop into lobulo-alveolar structures, eventually filling the entire mammary fat pad. In a histological section of normal lactating mammary gland, the stroma is densely packed with alveolar lobules, which are well extended and filled with milk (Fig. 2 B). However, the lactating mammary glands of DDTg females showed severe hypoplasia of the lobulo-alveoli (Fig. 2 C; $n = 6$, four from line TA11 and two from line TA32). They were very small in diameter and contained little if any milk. This hypoplastic alveolar development was accompanied by a deficiency in terminal differentiation of mammary epithelia, as shown by

Figure 3. **Defective development of mammary gland at late pregnancy in DDTg mice.** Whole mount staining of the number four mammary glands revealed no significant differences in ductal elongation or side branching between control (A–C) and DDTg (D–F) mice at mid-pregnancy (8.5 dpc). However, at late pregnancy (16.5 dpc), although the DDTg mammary epithelia showed continued side branching, their alveolar development was severely hypoplastic (J–L) as compared with controls at the same stage (G–I). Bars: (A, D, G, and J) 3 mm; (B, E, H, and K) 1 mm; (C, F, I, and L) 250 μm.
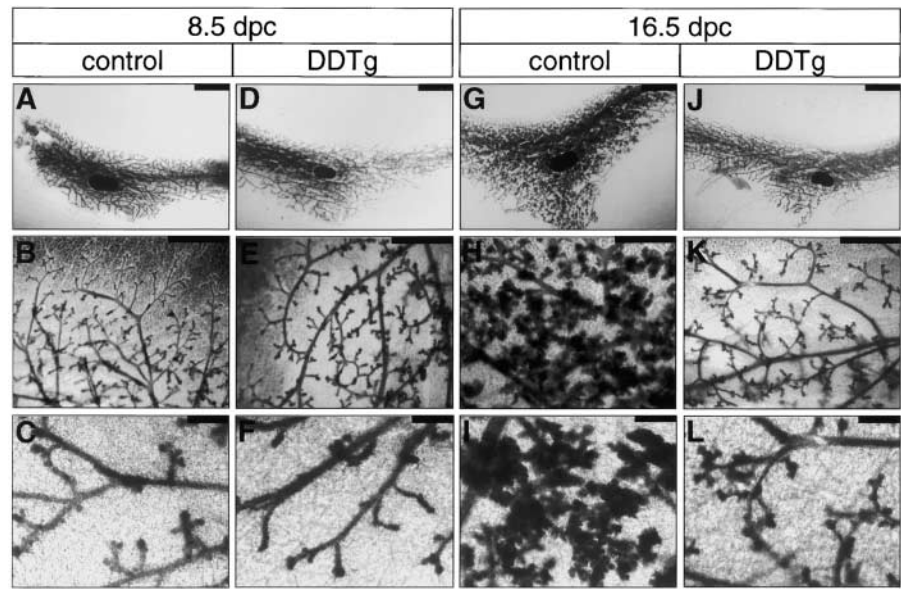
reduction in expression of the milk protein, casein, at late pregnancy (Fig. 2 D).

A more thorough examination of mammary gland development in DDTg females by whole mount staining showed that the mammary ductal tree system developed normally, and that side branching had occurred at mid-pregnancy (8.5 d postcoitum [dpc]) to an extent comparable to controls (Fig. 3, A–F; $n = 3$, line TA11). However, late in pregnancy (16.5 dpc), although the number of side branches had further increased in DDTg females, the maturation of mammary tissue was severely impaired, with an absence of lobulo-alveolar structures (Fig. 3, G–L; $n = 5$, three from line TA11 and two from line TA32). Although the DDTg mammary glands had developed small numbers of alveoli, they never matched the size and density of those in control mice. The same abnormalities were observed when Dox was administered starting at the beginning of pregnancy, ruling out the possibility that the defects were a result of Axin overexpression during prenatal development or sexual maturation (unpublished data; $n = 3$, two from line TA11 and one from line TA32) This phenotypic defect could also be reversed after the withdrawal of Dox, as shown by successful lactation with later litters, indicating that it was caused by the Dox-induced expression of Axin during pregnancy and lactation. The expression of the transgene-encoded MT–Axin protein in DDTg mammary gland epithelia was confirmed by immunostaining with anti-myc antibody (Fig. 4), and was consistent with the expression pattern expected for the MMTV promoter/enhancer (Hennighausen et al., 1995). Therefore, elevated expression of Axin in mammary epithelia appeared to inhibit mammary gland development at a late stage of pregnancy.

The defects in mammary gland development in the DDTg mice were remarkably similar to those in mice lacking cyclin D1, in which the mammary epithelium also undergoes normal elongation and side branching, but fails to form alveolar lobules (Fantl et al., 1995; Sicinski et al., 1995). As cyclin D1 has been identified as a bona fide target gene of the Wnt/β-catenin signaling pathway (Shtutman et

al., 1999; Tetsu and McCormick, 1999), we examined the possible inhibitory effects of Axin on Wnt signaling. Even though high levels of cytoplasmic β-catenin were consistently exhibited in normal epithelia of developing mammary alveoli (Fig. 5 A), the DDTg mice showed relatively lower levels of β-catenin at the same stage (Fig. 5 B, $n = 3$). In normal mammary epithelia undergoing alveolar development, cyclin D1 was uniformly and strongly expressed, as revealed by immunostaining (Fig. 5 C). However, its expression was significantly reduced in the mammary epithelia of DDTg mice (Fig. 5 D, $n = 2$). Immunoblot analyses showed an approximately ninefold reduction of the steady-state level of cyclin D1 in the mammary gland of DDTg mice (Fig. 5 E). Therefore, the inhibitory effects of MT–Axin on alveolar development in the lactating mammary
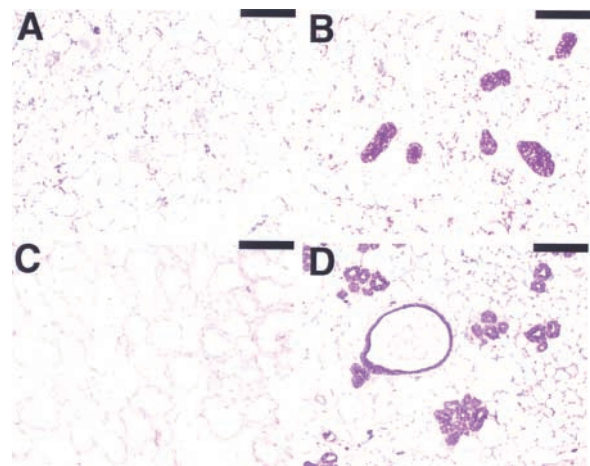

Figure 4. **Inducible expression of Axin in the mammary gland epithelia of DDTg mice.** Immunohistochemical staining with an anti-myc antibody localized MT–Axin to the epithelia of mammary glands in DDTg mice (B and D). The mammary glands of control mice showed only background staining (A and C). (A and B) 8.5 dpc; (C and D) 2 d postpartum. Bars, 125 μm.
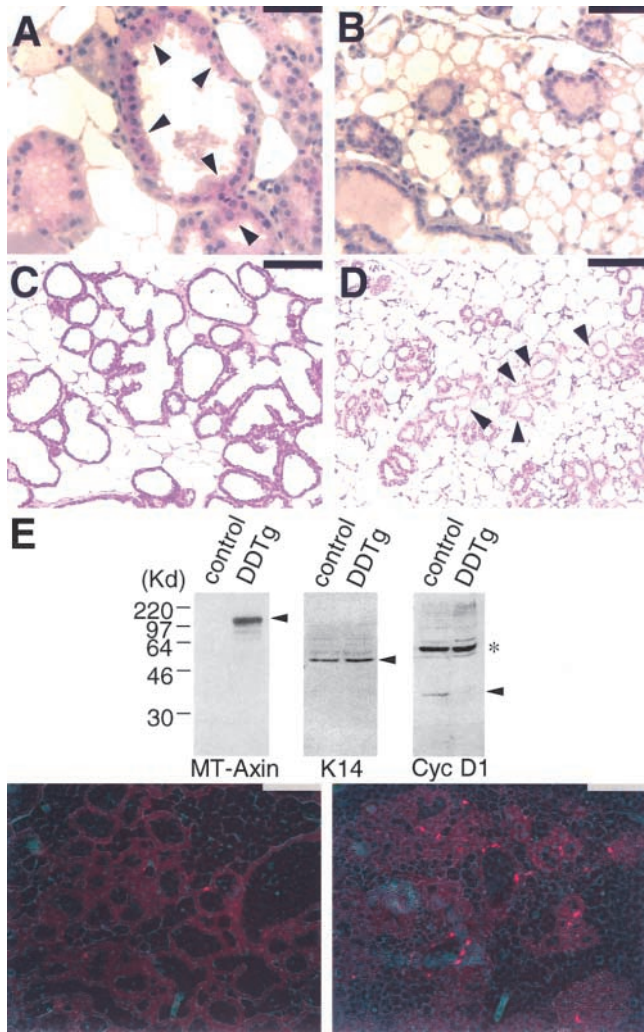
Figure 5. **Reduced β-catenin and cyclin D1 expression and increased apoptosis in lactating mammary gland epithelia of DDTg mice.** (A and B) Sections were immunostained with an anti–β–catenin antibody (red staining), and counterstained with hematoxylin (blue staining). (A) High levels of cytoplasmic β-catenin (arrowheads) were observed in normal epithelia of lactating mammary gland at 2 d postpartum. (B) The mammary gland of DDTg mice at 2 d postpartum showed reduced levels of β-catenin. (C and D) Immunohistochemical staining with an anti-cyclin D1 antibody revealed a reduced level of cyclin D1 expression in the epithelia of lactating mammary gland at 2 d postpartum. (C) The lactating mammary gland of a control mouse showed intense and uniform staining. (D) Mammary epithelia of DDTg mice showed reduced staining and, in some areas (indicated by arrowheads), lack of staining above background levels. (E) Protein lysates from control and DDTg mammary gland at 17.5 dpc were immunostained with (left) anti-myc antibody to detect transgenic expression of MT–Axin, (center) anti-K14 (50 kD) antibody, serving as a control of the epithelial cell content of the mammary gland, and (right) anti-cyclin D1 antibody. The level of cyclin D1 (36 kD) was ninefold reduced in the DDTg mammary epithelia compared with the control, after normalization for K14 levels. The positions of protein molecular weight markers are indicated at the left. Arrowheads indicate the presence of the specific protein bands and the asterisk indicates nonspecific bands. (F and G) TUNEL staining of control (F) and DDTg (G) mouse mammary glands at 2 d postpartum. The epithelia of DDTg lactating mammary gland displayed increased numbers of apoptotic cells. Bars: (A and B) 62.5 μm; (C, D, F, and G) 125 μm.

gland may be mediated, at least in part, by its effects on the Wnt-induced expression of cyclin D1.

In addition to the repression of mammary epithelial growth and differentiation, the inhibition of Wnt/cyclin D1 signaling by overexpression of Axin could also result in deficiencies in cell survival. TdT-mediated dUTP nick end labeling (TUNEL) analyses revealed a large increase in the number of apoptotic cells in the mammary epithelia of DDTg mice, indicating that programmed cell death contributed to the observed hypoplastic development of this organ (Fig. 5, F and G; $n = 2$).

Dox-induced overexpression of Axin interferes with normal thymic development

Histological analyses of the DDTg mice that died at about 3 wk of age revealed no abnormalities in the heart, intestine, kidney, liver, lung, pancreas, salivary gland, skin, or testes. However, all of the DDTg mice (derived from TRE2–Axin–GFP line TA32), even those that survived, shared a defect in the thymus, which was much smaller than in normal mice at the same age. We analyzed a total of 10 DDTg animals, 6 at 3 mo and 4 at 4 mo of age. The thymus was one of the tissues that expressed rtTA at high levels in MMTV–rtTA transgenic mice (Fig. 1 B), and the Dox-inducible TRE–Axin–GFP transgene was also expressed in this tissue, as indicated by whole mount fluorescence microscopy (Fig. 1 C and Fig. 6 A).

The progenitors of T cells originate in the bone marrow and arrive at the thymus where they develop into mature thymocytes (for review see Paul, 1999). A normal thymus has two lobes (left and right), each containing several lobules, which are further divided into cortical and medullary regions. The cortex contains many immature and resting thymocytes that express low levels of the T cell marker CD3. Mature thymocytes express high levels of CD3 and reside in medulla, an epithelial enriched environment (Paul, 1999).

Histological analyses of thymus from DDTg mice revealed a lack of normal organization. In normal 3-wk-old mice (Fig. 6 D), the thymic lobules are characterized by a darkly stained cortex and a lightly stained medulla. In contrast, the thymuses of 3-wk-old DDTg mice showed no separate lobules within a lobe and had no discernible cortex and medulla (Fig. 6 E; $n = 6$). In 4-wk-old DDTg mice, these defects were similar except that the thymus was even smaller in size (Fig. 6 F; $n = 4$).

The medulla and cortex could be easily distinguished by staining the epithelia with anticytokeratin antibodies and the thymocytes with methyl green (Fig. 6 G). The thymocyte-enriched cortical region was absent in the thymuses of 3- and 4-wk-old DDTg mice (Fig. 6, H and I), implying the loss of immature thymocytes that normally reside in the cortex. Because the expression of CD3 is low in immature thymocytes and high in mature thymocytes (Paul, 1999), CD3 staining was also used to differentiate these two cell types and regions. In control thymus, mature thymocytes in the medulla stained more darkly, whereas immature thymocytes in the cortex showed lighter staining (Fig. 6 B). This method confirmed that the immature thymocyte–enriched cortical region was lost in DDTg mice (Fig. 6 C).

Figure 6. **Elevated expression of Axin affects development of thymus.** (A) Whole mount fluorescence microscopy of control thymus (left) and thymus from a 3-wk-old DDTg mouse (right). GFP expression is visible only in the DDTg thymus. No fluorescence above background was observed in nontransgenic or single TRE2–Axin–GFP transgenic mice, or in double transgenic mice without Dox administration (unpublished data). (D–F) Hematoxylin- and eosin-stained sections of thymus from a 3-wk-old control mouse (D), a 3-wk-old DDTg mouse (E), and a 4-wk-old DDTg mouse (F). Immunohistological staining of thymus was performed with anti-CD3 (B and C) or anti-pan cytokeratin (G–I) antibody. (G–I) Sections were stained with anti-cytokeratin antibodies in brown color to mark the thymic epithelia, whereas thymocytes were counterstained with methyl green. In a control mouse (G), the medulla showed heavy brown staining, whereas the green stain indicated the many immature thymocytes in the cortex. In the thymus of a 3-wk-old (H) and a 4-wk-old (I) DDTg mouse, the thymocyte-enriched cortex had disappeared. The T cell marker CD3 is expressed highly in mature thymocytes and weakly in immature thymocytes. This marker distinguished the medulla (m) from cortex (c) in a normal, 4-wk-old thymus (B). However, in the thymus of 4-wk-old DDTg mice, no distinguishable regions could be observed, indicating a loss of the cortical region and a dramatic decrease in the population of thymocytes expressing low levels of CD3 (C). Bars: (A, G, H, and I) 250 μm; (B and C) 125 μm; (D–F) 1 mm.
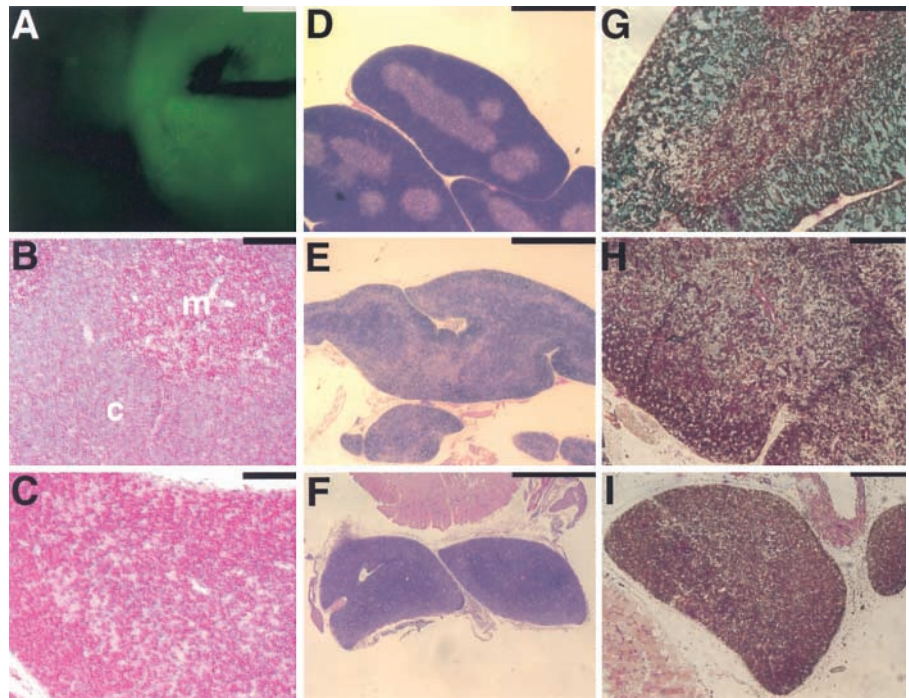
The paucity of immature thymocytes in the thymus suggested that expression of Axin in the DDTg mice might have induced apoptosis of these cells. Indeed, large numbers of apoptotic thymocytes with fragmented nuclei were observed in the thymic cortex of 3-wk-old DDTg mice (Fig. 7, A and B). Whereas programmed cell death occurs normally during the maturation of thymocytes (Sebzda et al., 1999), TUNEL staining confirmed that there was a massive increase in apoptosis in the thymuses of DDTg mice, especially in the cortical region (Fig. 7, C and D). We also examined other lymphoid organs for the presence of apoptotic cells, and observed increased apoptosis and decreased numbers of lymphocytes in the spleen (Fig. 7, E–H) and lymph nodes (unpublished data), although the effect was not as severe as in the thymus.

Discussion

An important role of Axin in early embryogenesis has been demonstrated through the analysis of loss of function mutants in mice (Zeng et al., 1997) and zebra fish (Heisenberg et al., 2001) as well as expression of wild-type and dominant-negative forms in frog embryos (Zeng et al., 1997; Itoh et al., 1998; Fagotto et al., 1999). However, little is known about the role of Axin in later developmental events, e.g., its capacity to negatively regulate many Wnt-mediated signaling events during fetal and postnatal development. Axin has also been implicated as a tumor suppressor, based on its mutation in human hepatocellular carcinoma (Satoh et al., 2000). However, the mechanism of its function as a tumor suppressor has not yet been investigated in an animal model.

One approach to these questions, which we describe in this paper, is to generate transgenic mice in which the expression of wild-type Axin (or, potentially, mutant forms) can be induced to high levels in a regulated manner. We have developed MMTV–rtTA and TRE2–Axin–GFP transgenic lines that, when crossed, produce bitransgenic animals in which Axin can be induced in several tissues by administering Dox. We have used this system to examine the consequences of Axin overexpression in two tissue types where the Wnt/β-catenin/LEF-Tcf signaling pathway is known to play important roles, the mammary gland and the lymphoid tissues.

The Wnt signal transduction pathway has been implicated in the pregnancy-dependent development, as well as neoplastic transformation, of the mouse mammary gland. The earliest evidence implicating Wnts in these processes was the finding that Wnt-1 and Wnt-3 were activated by the insertion of MMTV proviruses in mouse mammary carcinomas (Nusse and Varmus, 1992). Transgenic mice that constitutively expressed Wnt-1 or Wnt-10b under the control of the MMTV long terminal repeat (LTR) displayed hyperplastic mammary epithelia, with premature side branching and lobulo-alveolar development in virgin females (and estrogen-independent mammary ductal morphogenesis in males), eventually progressing to mammary adenocarcinomas (Tsukamoto et al., 1988; Lane and Leder, 1997). Wnt-1 is also able to induce branching morphogenesis of cultured mammary epithelial cells (Uyttendaele et al., 1998). These findings implied that Wnt signaling can stimulate the survival, growth, and differentiation of mammary epithelial cells, and that aberrant activation of this pathway results in hyperplastic development and oncogenic transformation. It should
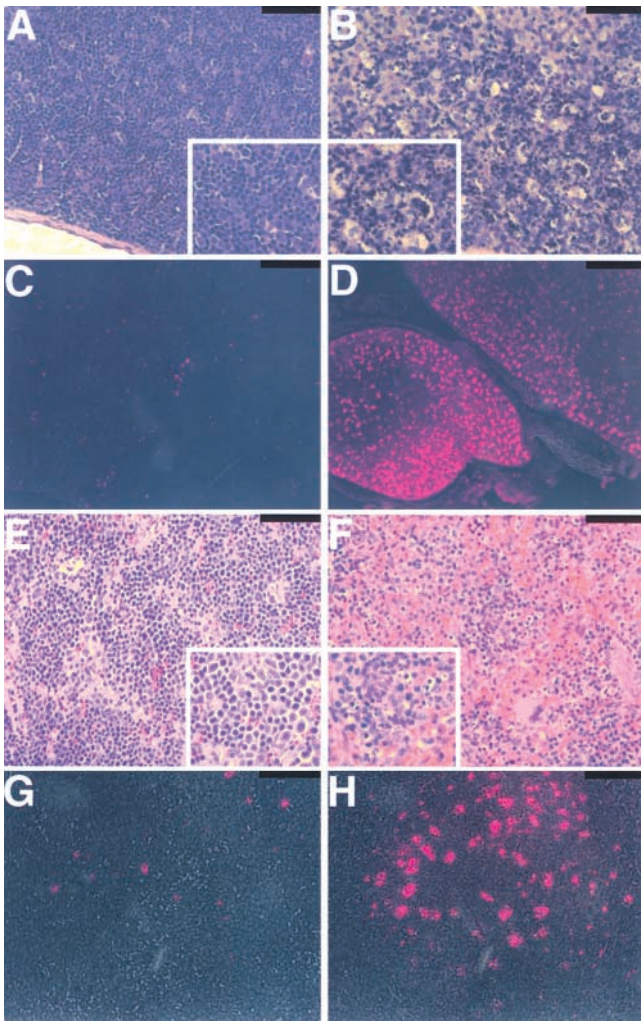
Figure 7.   **Axin induces extensive apoptosis in the lymphocytes of DDTg mice.** Hematoxylin and eosin staining (A, B, E, and F) and TUNEL staining (C, D, G, and H) were performed on sections of thymus (A–D) and spleen (E–H) of 3-wk-old mice. Lymphocytes were normally packed in the thymus (A) and spleen (E) of control mice. Fragmented nuclei were identified in the thymus (B) and spleen (F) of 3-wk-old DDTg mice. The insets display magnified images revealing the nuclear morphology. In the TUNEL assay, high levels of apoptotic signals were detected in the thymus (D) and spleen (H) of DDTg mice. Background levels of apoptosis were observed in the thymus (C) and spleen (G) of control mice. Bars: (A, B, and E–H) 62.5 μm; (C and D) 250 μm.

also be noted that this pathway plays an important role during the earliest fetal stages of mammary gland development, as shown by the absence of mammary glands in LEF-1 mutant mice (van Genderen et al., 1994).

Several members of the Wnt family are expressed at various stages of normal mammary gland development, including Wnt-4, -5b, -6, -7b, and -10b in the epithelium and Wnt-2, -5a, and -6 in the stromal compartment (for review see Robinson et al., 2000). This raises the possibility that some Wnts may mediate epithelial induction by the stroma, whereas others may serve in an autocrine pathway. To date, the only member of the Wnt family whose role in mammary gland development has been evaluated using a null mutation is Wnt-4, which is expressed in the mammary epithe-

lium during early to mid-pregnancy. In these experiments (Brisken et al., 2000), mammary buds from E14.5 Wnt4−/− fetuses (which die perinatally) were transplanted into wild-type mice, where their subsequent development could be assessed. The mutant mammary glands showed a specific defect in progesterone-induced ductal side branching early in pregnancy, indicating that Wnt-4 functions downstream of progesterone signaling in this process. However, at later stages of pregnancy, the mutant epithelia exhibited a more normal pattern of branching and alveolar development, suggesting that other members of the Wnt family expressed later in mammary gland development may eventually compensate for the lack of Wnt-4 (Brisken et al., 2000).

In the present study, we have shown that the induced overexpression of Axin in the mammary epithelium of DDTg mice interferes with the later phases of mammary gland development. The gland develops normally during the early stages of pregnancy, forming a normal ductal tree system and undergoing extensive side branching. Thus, Axin overexpression does not appear to interfere with the Wnt-4–mediated signaling events, which are required specifically for lateral branching during mid-pregnancy (Brisken et al., 2000). However, later in pregnancy the maturation of DDTg mammary tissue is severely impaired, with the absence of mature lobulo-alveolar structures and a failure of lactation. Given the established activity of Axin as a negative regulator of the canonical Wnt/β-catenin pathway, it is likely that Axin is interfering with a Wnt signaling event required for alveolar development. Interestingly, the lack of an effect of Axin on ductal side branching is consistent with evidence that Wnt-4 may signal through an alternative pathway (Kuhl et al., 2000) rather than the canonical Wnt/β-catenin pathway (Shimizu et al., 1997; Wong et al., 1994). If Wnt-4 signaled through the canonical Wnt/β-catenin pathway, it is likely that overexpression of Axin would have also had an effect during the stage of mammary gland development at which Wnt-4 is required.

Although the mechanisms by which various Wnts contribute to the control of mammary gland development are poorly understood, one downstream gene that appears to play an important role is cyclin D1. The cyclin D1 gene has been identified as a target of the canonical Wnt pathway, which is activated by β-catenin and TCF/LEF factors (Shtutman et al., 1999; Tetsu and McCormick, 1999). In mice with a targeted mutation in cyclin D1, the mammary ductal tree developed normally and formed many side branches early in pregnancy, however there was a dramatic impairment in the expansion of mammary epithelium and development of alveolar lobules in late pregnancy (Fantl et al., 1999). In the DDTg mice, we found that high levels of Axin appeared to reduce the accumulation of cytoplasmic β-catenin in the mammary epithelia. The expression of cyclin D1 in these cells was also greatly reduced, and the mammary gland phenotype was very similar to that of the cyclin D1 knockout mice. Thus, we propose that Axin can inhibit a Wnt-mediated signal required for the expression of cyclin D1 (Fig. 8). We also observed an increased number of apoptotic cells in the mammary epithelia of DDTg mice, suggesting that a Wnt signal inhibited by Axin normally provides a survival signal for these cells. This result is consistent with
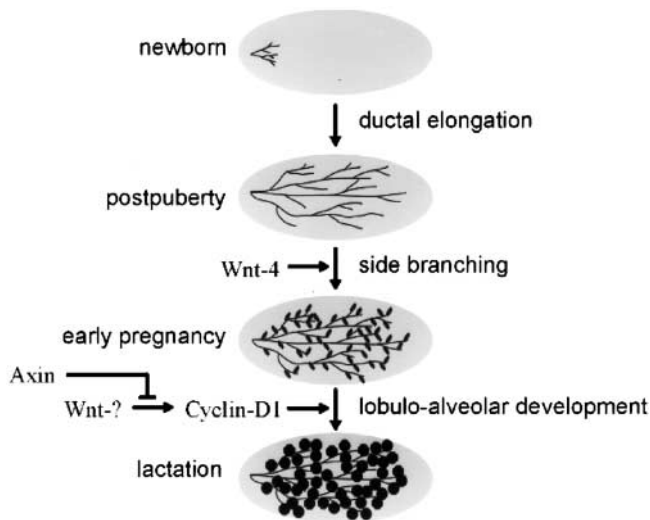
Figure 8. **Schematic diagram illustrating the proposed mechanism by which Axin may interfere with normal mammary gland development in DDTg mice.** Side branching, which requires Wnt-4, is not significantly affected by overexpression of Axin. However, lobulo-alveolar development is severely impaired. This may be due to the reduced expression of cyclin D1, a Wnt target gene, which is required for lobulo-alveolar development. See text for references. Diagram modified from Robinson et al. (2000).

recent reports that Wnt signaling may inhibit apoptosis through a β-catenin/Tcf-dependent mechanism in addition to promoting cell growth and proliferation (Bournat et al., 2000; Reya et al., 2000; Chen et al., 2001; Ioannidis et al., 2001). The ability of cyclin D1 to prevent programmed cell death may also play a role in Wnt-mediated, anti-apoptotic effects (Albanese et al., 1999).

Expression of Axin under the MMTV promoter/enhancer also caused abnormal development of the thymus, with a massive apoptosis of immature thymocytes. The progenitors of T cells are CD4-, CD8- double-negative thymocytes, which differentiate into double-positive cells, the largest cell population in the thymus. Double-positive thymocytes rest in the cortex and await signals to develop into single-positive, mature thymocytes bearing either CD4 or CD8. The maturation of thymocytes depends critically on the survival and death of immature thymocytes during a series of clonal selections, which ensure the generation of an effective immune response without autoimmune reactivity (for review see Sebzda et al., 1999). Although apoptosis is a normal mechanism of clonal selection, the level of apoptosis observed in the thymuses of DDTg mice was greatly elevated, suggesting that overexpression of Axin can interfere with a signaling pathway that regulates thymocyte survival. The spleens of these mice also contained a decreased number of lymphocytes. This might be due in part to the reduced number of thymocytes, which migrate to the spleen during their circulation, although increased apoptosis was also observed in the spleen itself.

It is possible that Axin promotes the apoptosis of lymphoid cells through its ability to negatively regulate the canonical Wnt signaling pathway. Although little is known about the participation of Wnts in the development of lymphocytes, the LEF/TCF family of transcription factors was originally identified through its role in the regulation of lymphocyte-specific genes (Travis et al., 1991; van de Wetering et al., 1991; Waterman et al., 1991). Furthermore, recent evidence suggests that Wnt signaling regulates the survival and differentiation of double-positive thymocytes through a β-catenin and LEF/TCF-dependent mechanism (Gounari et al., 2001; Ioannidis et al., 2001). TCF-1–deficient mice show a drastic decrease in the number of double-positive thymocytes (Verbeek et al., 1995), a phenomenon similar to that observed in DDTg mice. Thus, Axin might influence the function of LEF/TCF factors in the development and survival of lymphocytes.

Axin also has a pro-apoptotic activity that appears to be independent of its effects on the Wnt pathway, as shown by the induction of apoptosis in several types of cultured cells expressing elevated levels of Axin (Neo et al., 2000; Satoh et al., 2000; unpublished data). Besides regulating the Wnt/β-catenin pathway, Axin induces JNK/SAPK activity through its MEKK1 binding and self-binding domains (Zhang et al., 1999). Members of the JNK/SAPK family activate an apoptotic pathway in various cultured cells (Ip and Davis, 1998) and are required for the induction of neuron-specific apoptosis in the development of mouse brain (Yang et al., 1997; Kuan et al., 1999). It is therefore possible that Axin induces programmed cell death through a mechanism associated with its activation of the JNK/SAPK pathway. However, although programmed cell death occurred in the mammary glands and lymphoid tissues of DDTg mice, no apoptosis or phenotypic defects were observed in the testes, where strong expression of the transgene was also observed (unpublished data). Furthermore, the lack of any effect on the development and proliferation of the mammary epithelium until the late stages of pregnancy in DDTg mice argues against a generalized apoptotic activity of Axin. Thus, it appears more likely that the apoptotic effects of Axin are due to the inhibition of Wnt signals that specifically promote cell survival in certain tissues.

Materials and methods

Transgene constructs

Plasmid pTRE2–Axin–GFP was constructed by first inserting the 1.4-kb SacII-NotI fragment of IRES and enhanced GFP (pIRES2-GFP; CLONTECH Laboratories, Inc.) in the same restriction sites upstream of human β-globin BamHI-PstI 3′ fragment (hβG3′). The 3.3-kb NheI-AvrII fragment of myc-tagged, full-length Axin cDNA (Zeng et al., 1997) was added to the NheI site located 5′ to the IRES2–GFP–hβG3′. The 6.4-kb NheI-EcoRV fragment of MT–Axin–IRES2–GFP–hβG3′ was then inserted into the same restriction sites downstream of TRE2, which consisted of seven copies of the tet operator and a minimal CMV promoter (CLONTECH Laboratories, Inc.).

To create plasmid pMMTV–rtTA, a 1.0-kb blunt-ended EcoRI and BamHI fragment, which encoded rtTA, was inserted into the blunted NheI and BamHI sites that are downstream of the LTR of mouse MMTV (1.5-kb HindIII-SmaI fragment of pMSG vector; Amersham Pharmacia Biotech) and upstream of hβG3′. Both plasmids contained noncoding exon2, exon3, and polyadenylation signal of hβG3′ at their 3′ regions to ensure proper processing of the transcripts.

Generation and breeding of transgenic mice

All animals were housed under pathogen-free conditions in accordance with institutional guidelines. Gel-purified 7.4-kb TRE2–Axin–GFP and 4.2-kb MMTV–rtTA DNA fragments at a concentration of 3 μg/ml were separately microinjected into the pronuclei of fertilized mouse eggs. The injected embryos were cultured in vitro to the two-cell stage and transferred into the oviducts of 0.5-d pseudopregnant female mice (Hogan et al., 1994). Mice were genotyped for the presence of the transgenes by PCR and Southern blot analyses. For PCR, the primers rtTA01, 5′-cattgagatgttagataggc-3′, and rtTA02, 5′-aagttgttttcttaatccgc-3′, were used to identify the MMTV–rtTA transgene,

and GFP03, 5′-acggcaagctgaccctgaagt-3′, and GFP04, 5′-gcttctcgttgg-ggtctttgc-3′, primers were used to identify the TRE2–Axin–GFP transgene. The PCR reaction for TRE2–Axin–GFP was performed by denaturation at 94°C for 3 min and 35 cycles of amplification (94°C for 1 min, 60°C for 1 min 20 s, and 72°C for 2 min), followed by a 2-min extension at 72°C. To amplify the MMTV–rtTA transgene, 32 cycles of amplification (94°C for 50 s, 60°C for 1 min 20 s, and 72°C for 2 min) were performed. For Southern analyses, the full-length cDNA of rtTA and GFP were used as probes to detect the MMTV–rtTA and TRE2–Axin–GFP transgenic mice, respectively.

4 out of 15 MMTV–rtTA founders tested positive for integration of the transgene by PCR and Southern analyses. One of these lines, MTA4, which expressed the rtTA as determined by RT-PCR, was used in these studies. 3 out of 29 TRE2–Axin–GFP founders possessed the integrated transgene, and the two lines TA11 and TA32 were used in these studies. Dox (2 mg/ml plus 50 mg/ml sucrose) was administrated orally in the drinking water and replaced three times per week, by diluting a freshly prepared 10× Dox/sucrose stock solution.

RT-PCR analysis

Total RNA was isolated from tissues of 3-wk-old mice by a guanidine thiocyanate/cesium chloride gradient method (Hsu and Chen-Kiang, 1993). 5 µg of total RNA were subjected to first strand cDNA synthesis using the oligo dT primer (Ambion), followed by PCR amplification of either rtTA or Hprt. For rtTA, an upstream primer, 5′-ttacgggtctaccatcgagg-3′, within the rtTA coding sequence and a downstream primer, 5′-ggtggggtgaattctttgcc-3′, within the noncoding exon3 of hβG gene were used to amplify a 445-bp spliced form of rtTA mRNA transcript. PCR reactions were performed by denaturation at 94°C for 3 min and 40 cycles of amplification (94°C for 50 s, 58°C for 50 s, and 72°C for 50 s), followed by a 7-min extension at 72°C. For Hprt, a pair of primers (5′-cctgctggattacatataagcactg-3′ and 5′-gtcaagggcatatccaacaacaaac-3′) was used to amplify a 354-bp fragment of the Hprt mRNA. PCR reactions were performed by denaturation at 94°C for 3 min and 33 cycles of amplification (94°C for 30 s, 60°C for 50 s, and 72°C for 50 s), followed by a 7-min extension at 72°C.

Histology and immunohistochemistry

Mouse tissues were dissected in phosphate-buffered saline, fixed in 10% buffered formalin, and paraffin embedded. Tissues were sectioned, and stained with hematoxylin and eosin for histological evaluation. Tissue sections were subject to immunological staining by the use of avidin/biotinylated enzyme complex according to the manufacturer's protocol (Vector Laboratories). Mouse monoclonal anti-myc (Ab-1; Calbiochem), anti-β–catenin (H102; Santa Cruz Biotechnology, Inc.), and anti-CD3ε (M-20; Santa Cruz Biotechnology, Inc.) antibody, and rabbit polyclonal anti-cyc D1 (Ab-3; NeoMarkers) and anti-pan cytokeratin (Sigma-Aldrich) antibody were used in these analyses.

Immunoblot analysis

Protein extracts were subject to immunoblotting as previously described (Hsu et al., 1999). Anti-myc (Ab-1; Calbiochem), anti-cyclin D1 (Ab-3; NeoMarkers), anti-keratin 14 (K14) (Ab-1; NeoMarkers), anti-Axin (a gift of Dr. Roel Nusse, Stanford University, Stanford, CA), and anti-casein (a gift of Dr. Margaret Neville, University of Colorado, Denver, CO) were used as primary antibodies. Bound antibodies were detected with horseradish peroxidase–conjugated secondary antibodies, followed by enhanced chemiluminescence–mediated visualization (Amersham Pharmacia Biotech) and autoradiography.

Whole mount mammary gland staining

The number 4 mammary glands were dissected at the indicated times of development and spread on glass slides. After fixation in Carnoy's fixative for at least 4 h at room temperature, the tissues were hydrated and stained in carmine alum solution overnight as described previously (Robinson et al., 1995). Samples were then dehydrated, cleared in Histoclear, and photographed.

Apoptosis analysis

In situ TUNEL staining of apoptotic cells in tissue sections was performed based on the manufacturer's protocol (Intergen). The staining results were evaluated by fluorescence microscopy with appropriate excitation and emission filters. The fluorescent and phase-contrast pictures were taken, and superimposed images were created using Corel PhotoPaint.

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