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Epimorphin Overexpression in the Mouse Mammary Gland Promotes Alveolar Hyperplasia and Mammary Adenocarcinoma

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Abstract

Epimorphin/syntaxin-2 (EPM) is a plasma membrane–anchored protein that has at least two distinct functions depending on its membrane topology: vesicle fusion when localized to the cytoplasmic surface and morphogenic signaling when localized to the extracellular surface. Transgenic mice that express full-length extracellular EPM fused to the NH_2-terminal signal sequence of interleukin-2, under the control of the whey acidic protein (*WAP*) gene promoter, exhibit aberrant mammary gland morphogenesis associated with increased expression of CCAAT enhancer binding protein β (C/EBPβ). Here we report that aged nulliparous and uniparous female WAP-EPM transgenic mice develop alveolar hyperplasias and well-differentiated adenocarcinomas that express high levels of C/EBPβ, keratin-14, matrix metalloproteinase-3, and β-catenin. This study reveals another pathway in which overexpression and alteration of a normal morphogenic process promote the development of cancer in the mammary gland. (Cancer Res 2005; 65(19): 8617-21)

Introduction

Epimorphin/syntaxin-2 (EPM) represents a unique class of plasma membrane–anchored molecules that possess distinct functions depending on their membrane topology (1). EPM was originally discovered because an antibody raised against whole cells blocked hair follicle morphogenesis in dermal epithelia (2) and was subsequently rediscovered as syntaxin-2, a member of the syntaxin family of vesicle fusion proteins (3). As a morphogen, EPM stimulates luminal morphogenesis in gall bladder epithelial cells, hepatocytes, pancreatic epithelial cells, intestinal epithelial cells, and mammary epithelial cells (1). EPM also induces lung, hair follicle, and mammary branching morphogenesis, and induces functional differentiation of hepatocytes (1). EPM expression has been localized to stromal fibroblasts and myoepithelial cells in the mammary glands of virgin mice (4), but it appears to be present also in milk-producing epithelial cells of pregnant mice, and a cleaved form appears in milk (5). Extracellular EPM stimulates both branching morphogenesis and lumen formation in collagen gel assays depending on the mode of presentation to the cells (4, 5). Transgenic expression of EPM in mammary epithelial cells induces larger lumen size (5), precocious alveolar development, increased numbers of branches in young nulliparous mice, and delayed involution after weaning.[4] Here we report that transgenic mice expressing the extracellular form of EPM in mammary epithelial cells develop alveolar hyperplasias and a high incidence of mammary neoplasia. To our knowledge, this is the first report of a causal relationship between increased expression of EPM and mammary cancer. A number of other molecules that are involved in stromal-epithelial cross-talk, such as matrix metalloproteinase-3/stromelysin-1 (MMP-3), insulin-like growth factor-1, transforming growth factor β, and epidermal growth factor, are required for normal mammary gland development, and have been shown to cause mammary cancer when their signaling pathways are deregulated (6). This study reveals that EPM, a morphogen that does not directly stimulate proliferation (4), also has the capacity to cause mammary cancer when chronically overexpressed in the mouse mammary gland.

Materials and Methods

WAP-EPM transgenic mice. Engineering of WAP-EPM transgenic mice was described previously (5). Two transgenic founder lines, EP6 and EP4, on an FVB genetic background were analyzed. WAP-EPM males were crossed with wild-type (WT) females to generate transgene positive females and WT littermate controls. All animals were cared for in accordance with the Lawrence Berkeley National Laboratory Animal Welfare Regulatory Committee.

Tissue collection. The thoracic 2nd and 3rd mammary glands and inguinal 4th mammary glands were excised and either snap frozen on dry ice for RNA, DNA, and protein isolation, whole mounted for morphologic analysis, or formalin fixed and paraffin embedded for histologic analysis. For whole-mount analysis, one 4th inguinal mammary gland from each mouse was spread onto a glass slide, immersed in Carnoy's fixative overnight, stained with carmine alum, and destained with acidic alcohol.

Immunohistochemistry. Tissue sections (5 μm) were deparaffinized and rehydrated in a series of graded alcohol to 1× PBS. Endogenous peroxidase activity was blocked for 10 minutes by treating slides with 3% H_2O_2 in 70% ethanol. Antigen retrieval was done by microwaving samples on high for 10 minutes in sodium citrate buffer (pH 6). Slides were treated overnight in a humidified chamber with anti-EPM (MC-1 antibody, 1:50 dilution; ref. 2), anti–β-catenin (1:200 dilution; BD Biosciences PharMingen, San Diego, CA), anti–keratin-14 (clone AF64, 1:100 dilution; Covance, Princeton, NJ), and anti–CCAAT enhancer binding protein β (C/EBPβ) antibodies (clone SC-150, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Slides were subsequently washed in 1× PBS and treated with biotinylated anti-rat, anti-mouse, or anti-rabbit secondary antibodies. Signal amplification was done using an avidin-biotin horseradish peroxidase conjugate followed by visualization using 3,3′-diaminobenzidine (DAB) color substrate (Vector Laboratories, Burlingame, CA). DAB color development on normal mammary glands for all antibodies was 5 minutes, and for WAP-EPM mammary tumors it was 30 seconds in duration.

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Protein isolation and Western blot analysis. Frozen mammary glands or tumors were homogenized in 500 μL of extraction buffer [1% Triton X-100, 500 mmol/L Tris-HCl (pH 7.6), 200 mmol/L NaCl, 10 mmol/L $CaCl_2$, and 1× protease inhibitor cocktail from Calbiochem, EMD Biosciences, San Diego, CA]. The homogenate was centrifuged at 12,000 × *g* for 20 minutes at 4°C. The protein concentrations of supernatants were determined by Bio-Rad detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA). Mammary or tumor protein (10 μg) was loaded onto 12% polyacrylamide gels for SDS-PAGE. Equal protein loading was determined by Ponceau S staining. Membranes were blocked in 5% milk in 1× TBS-Tween 20 buffer for 30 minutes followed by primary antibody incubation overnight at 4°C in blocking buffer. Primary antibodies used were the same as those described above at the following dilutions: anti–β-catenin 1:20,000, anti–keratin-14 1:20,000, anti-C/EBPβ 1:10,000, and anti–MMP-3 (clone AB811, 1:10,000; Chemicon, Temecula, CA). After washing membranes in 1× TBS-Tween 20, horseradish peroxidase–conjugated secondary antibodies were incubated on membranes in 5% 1× TBS-Tween 20 for 1 hour at room temperature. Chemiluminescent detection of bands was achieved using Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

Results

Chronic overexpression of epimorphin/syntaxin-2 promotes mammary cancer. We previously used the WAP promoter to deliver transgenes to the mammary gland (7, 8), and thus transgenic mice would show a more pronounced phenotype with increased parity. The WAP promoter is regulated by glucocorticoids, prolactin, and insulin (9); as mice age, they secrete higher levels of glucocorticoids (10) and the lifetime exposure to a transgene obviously increases with age. We therefore hypothesized that a more pronounced phenotype would result also as a function of increasing age in WAP-EPM transgenics. Indeed, the mammary duct enlargement (ectasia) that we previously described in young pregnant WAP-EPM mice (5) was much more severe in aged transgenic mice, whereas age-matched WT mice had ducts of normal diameter (Fig. 1*B* and *D*).

As mice aged, an increasing percentage of WAP-EPM transgenic mice developed mammary cancers. Palpable mammary tumors were found in 2 of 17 (12%) nulliparous and in 3 of 9 (33%) parous WAP-EPM mice with an average latency of 18 months, whereas no palpable tumors were detected in 14 nulliparous or 8 parous WT littermate controls (Fig. 1*A* and *C*). Palpable tumors were most often found in the thoracic 2nd or 3rd mammary glands and averaged 107 ± 95 mm^2 in size ($L \times W$, mean ± SD). An even higher tumor incidence was revealed by histologic analysis. Focal neoplastic lesions were thus identified in 4 of 17 (24%) nulliparous and 8 of 9 (89%) parous WAP-EPM mice (Fig. 1*B* and *C*), whereas only 1 of 14 nulliparous WT controls and none of eight parous controls had a solitary microscopic mammary neoplasm (Fig. 1*C*).

We confirmed by immunohistochemistry that EPM expression is up-regulated in WAP-EPM mammary tumor epithelium compared with WT mammary glands (Fig. 2*A*). This suggests that transgenic expression of EPM is the driving force for formation of these tumors. The tumors that developed in transgenic mice were identified as adenosquamous carcinomas based on their glandular architecture and the presence of squamous differentiation (metaplasia). In general, the WAP-EPM tumors exhibited histologic features characteristic of Wnt pathway mammary tumors (11): branched ductules, glandular differentiation, squamous metaplasia,

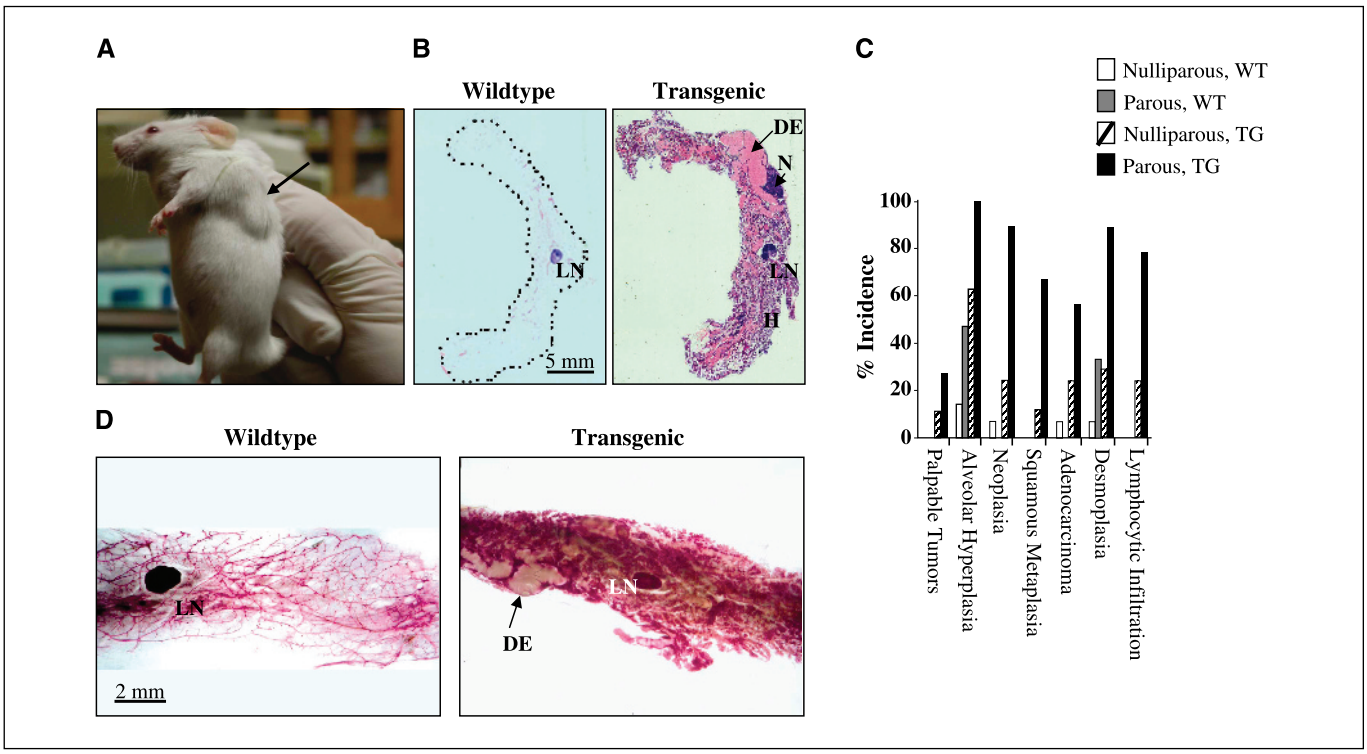

Figure 1. Mammary tumors found in WAP-EPM transgenic mice. *A,* tumor in 2nd to 3rd thoracic mammary glands of WAP-EPM mouse. *B,* H&E-stained sections showing mammary gland histology of aged WT and transgenic mice. By H&E analysis, WT mammary glands show predominately adipose and some simple ductal structures. In contrast, transgenic mammary glands show ductal ectasia, neoplasia, and florid hyperplasia of lactational alveoli. *D,* differences in mammary ductal structure are seen by carmine alum–stained whole mounts of aged WT and transgenic mice mammary glands. Transgenic mammary glands appear differentiated and mammary ducts are distended compared with WT. *C,* the percent incidence of both palpable tumors and type of progression in WT and transgenic, nulliparous, and parous aged mammary glands. *LN,* lymph node; *DE,* ductal ectasia; *N,* neoplastic lesion; *H,* hyperplasia.

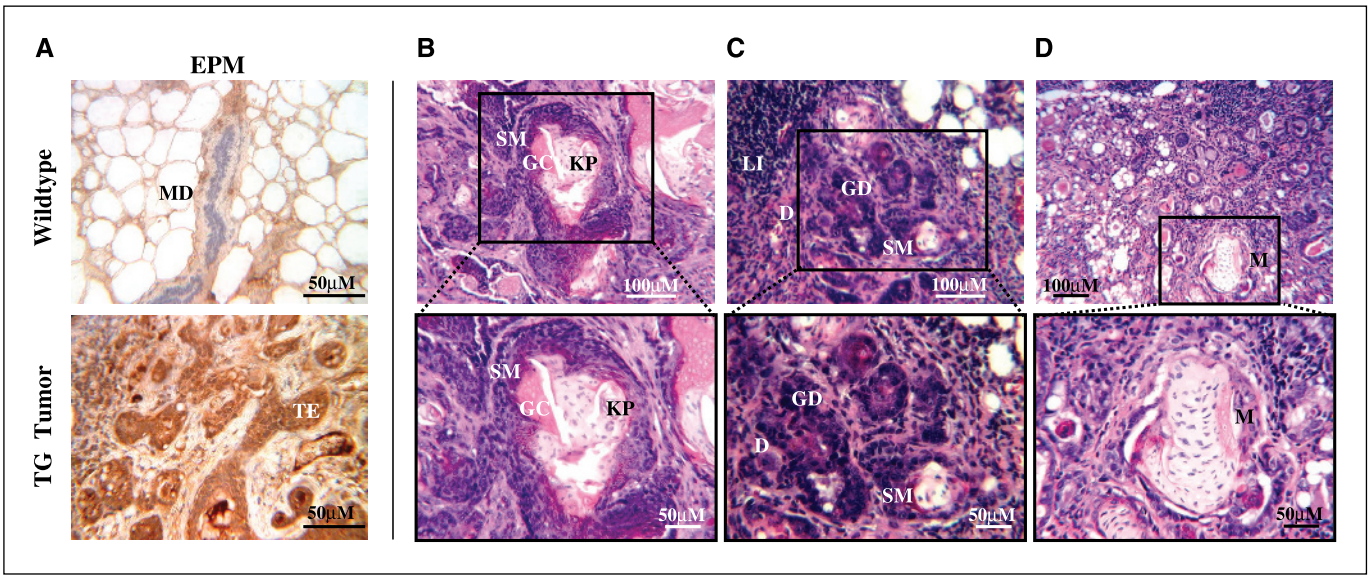

Figure 2. Histopathologic analysis of WAP-EPM transgenic mammary tumors reveals glandular differentiation and squamous metaplasia. *A,* immunohistochemistry staining for EPM on a normal mammary duct in a WT mouse and on a WAP-EPM transgenic mammary tumor. *B* and *D,* squamous metaplasia within WAP-EPM mammary tumors is marked by ductule structures containing keratin pearls and ghost cells that resemble hair follicle differentiation. *C,* glandular differentiation within WAP-EPM mammary tumors is marked by lobular structures with multiple layers of epithelial cells with large nuclei. Lymphocytic infiltrates and desmoplasia (dense stroma) are also evident surrounding these lobular structures. *D,* metaplastic structure resembling tooth development inside WAP-EPM tumor. *GC,* ghost cells; *GD,* glandular differentiation; *D,* desmoplasia; *LI,* leukocytic infiltration; *KP,* keratin pearl; *M,* metaplasia; *SM,* squamous metaplasia.

a dense (desmoplastic) stroma, significant lymphocytic infiltration, and high nuclear-to-cytoplasmic ratios (Fig. 2*B-D*). Many of the tumors also exhibited infiltrative behavior with invasion of the malignant cells between neural strands and skeletal muscle fibers (Figs. 2*C* and 3*B*). Areas of squamous metaplasia were marked by collections of horned cysts, keratin pearls, and ghost cells, as well as areas that resembled abortive hair shafts (Fig. 2*B*) and tooth formation (Fig. 2*D*).

The most common feature of the transgenic mammary glands was the frequent presence of diffuse lobuloalveolar hyperplasias with lactational differentiation in both tumor-free and tumor-bearing glands. Histologic analysis confirmed the presence of lactational hyperplasias with fully differentiated alveoli containing lipid droplets and proteinaceous secretions within their lumina (Fig. 3*A*). Compared with the hyperplastic lesions found diffusely in the mammary glands, the glandular structures found inside of tumors were less differentiated, were architecturally disorganized, and had atypical nuclei, fewer lipid droplets, and substantial lymphocytic infiltration (Fig. 3*B*). Hyperplasias were thus identified in 6 of 17 nulliparous (35%) and in 9 of 9 parous (100%) transgenic mammary glands (Fig. 1*D*). By comparison, only 2 of 14 nulliparous (14%) and 5 of 8 parous (63%) WT mice had lobular hyperplasias that were much less dense and less extensive than the hyperplastic lesions of their transgenic littermates (Fig. 1*D*).

Overexpression of Wnt pathway tumor markers in WAP-EPM mammary tumors. Tumor histopathology can often suggest the molecular pathways downstream of the transgene. For example, mammary tumors that develop in transgenic mice that have increased ErbB/Ras activity can be distinguished from mammary tumors that arise in transgenic mice that up-regulate the Wnt pathway by their histologic pattern (11).

Because many of the histologic features in WAP-EPM mammary tumors are shared by mouse mammary tumors induced by Wnt (11–13), we did immunohistochemical analysis to determine if tumors from these mice expressed Wnt-specific tumor markers. We analyzed two markers of Wnt pathway tumors, keratin-14 and β-catenin. Keratin-14 expression is a marker of myoepithelial differentiation and is expressed in a majority of Wnt pathway tumors but not in ErbB/Ras pathway tumors (11). β-Catenin is a downstream transcriptional effector of the Wnt pathway (13). Using immunohistochemistry and Western blot analysis, we found that both keratin-14 and β-catenin were overexpressed in the tumors of WAP-EPM mice compared with mammary glands from WT mice (Fig. 4*A*, *B*, and *D*). This was not the case for hyperplastic WAP-EPM mammary glands compared with WT mammary glands (Fig. 4*D*), nor were differences observed in young WAP-EPM mice at the midpregnant stage (data not shown). This suggests that this pathway is deregulated at later stages of mammary carcinogenesis. Localization of β-catenin was found to be nuclear in tumor epithelium (Fig. 4*A*), suggesting that the β-catenin transcriptional pathway is activated in WAP-EPM mammary tumors (12). Keratin-14 is localized to myoepithelial cells in the normal mammary gland (Fig. 4*B*). However, in the WAP-EPM tumors, keratin-14 is expressed throughout tumor epithelium and in ghost cells that are the products of epithelium-to-squamous transdifferentiation (Fig. 4*B*). This finding suggests that WAP-EPM mammary tumors show myoepithelial differentiation, which further supports that WAP-EPM mammary tumors should be classified with tumors of the Wnt pathway.

Overexpression of CCAAT enhancer binding protein β and stromelysin-1 in WAP-EPM mammary tumors. The WAP-EPM mammary tumors also expressed high levels of the transcription factor C/EBPβ compared with mammary glands from WT mice by immunohistochemistry (Fig. 4*C* and *D*). By Western blot analysis, we found that C/EBPβ and MMP-3 were up-regulated in the WAP-EPM tumor but not in hyperplastic mammary glands compared with WT age-matched mammary glands (Fig. 4*D*), again suggesting these are late changes.

Discussion

We found that WAP-EPM transgenic mice are highly prone to developing mammary cancers and lobular hyperplasias in the mammary gland with increased age. WAP-EPM mammary tumors are characterized as well-differentiated adenocarcinomas that express high levels of C/EBPβ, keratin-14, MMP-3, and β-catenin. Tumor formation and alveolar hyperplasias were greatly enhanced by parity, suggesting that increased stimulation of the WAP promoter, and thus EPM transgene, was the cause of increased severity. These findings suggest that, in addition to influencing developmental morphogenesis, EPM, when overexpressed, may play a role in cancer initiation and/or progression. Given that tumors appear only in aged animals, it is likely that EPM activates an oncogenic pathway as mice age.

We previously reported that mammary glands isolated from WAP-EPM midpregnant mice show substantially greater C/EBPβ expression compared with WT mammary glands (5). Moreover, in cultured mammary epithelial cells, EPM stimulated an increase of both the LIP isoform of C/EBPβ (5) and MMP-3 (14). We propose that EPM induces these pathologies partly through its ability to increase C/EBPβ expression and partly due to its ability to induce MMP-3. How EPM signals to stimulate the up-regulation of C/EBPβ, MMP-3, and the Wnt pathway and the connection among EPM, C/EBPβ, MMP-3, and Wnts in tumorigenesis are outstanding questions in need of further investigation.

To our knowledge, this is the first report of a causal relationship between increased expression of EPM and mammary cancer. There have been several findings suggesting this relationship may also function in humans. EPM has been reported to be up-regulated in human patients with activated ulcerative colitis, an autoimmune disease causing chronic inflammation of the colon that often results in cancer later in life (15). Furthermore, another member of the syntaxin family that is 70% homologous to EPM, syntaxin 1A, is thought to be deregulated in human small-cell lung carcinoma and has been associated with a more aggressive course of undifferentiated carcinoma of the colon and rectum (16, 17). Syntaxin 1A has been shown also to be highly expressed in pancreatic adenocarcinoma cell lines that contain an activated K-ras mutation (18). Together, these studies establish a clinical interest in syntaxin family proteins, such as EPM, for their potential role in inflammation and cancer progression.

In many oncogene-induced models of mammary cancer, transgenic mice develop tumors with very short latencies, making it difficult to pursue longitudinal studies of tumor initiation

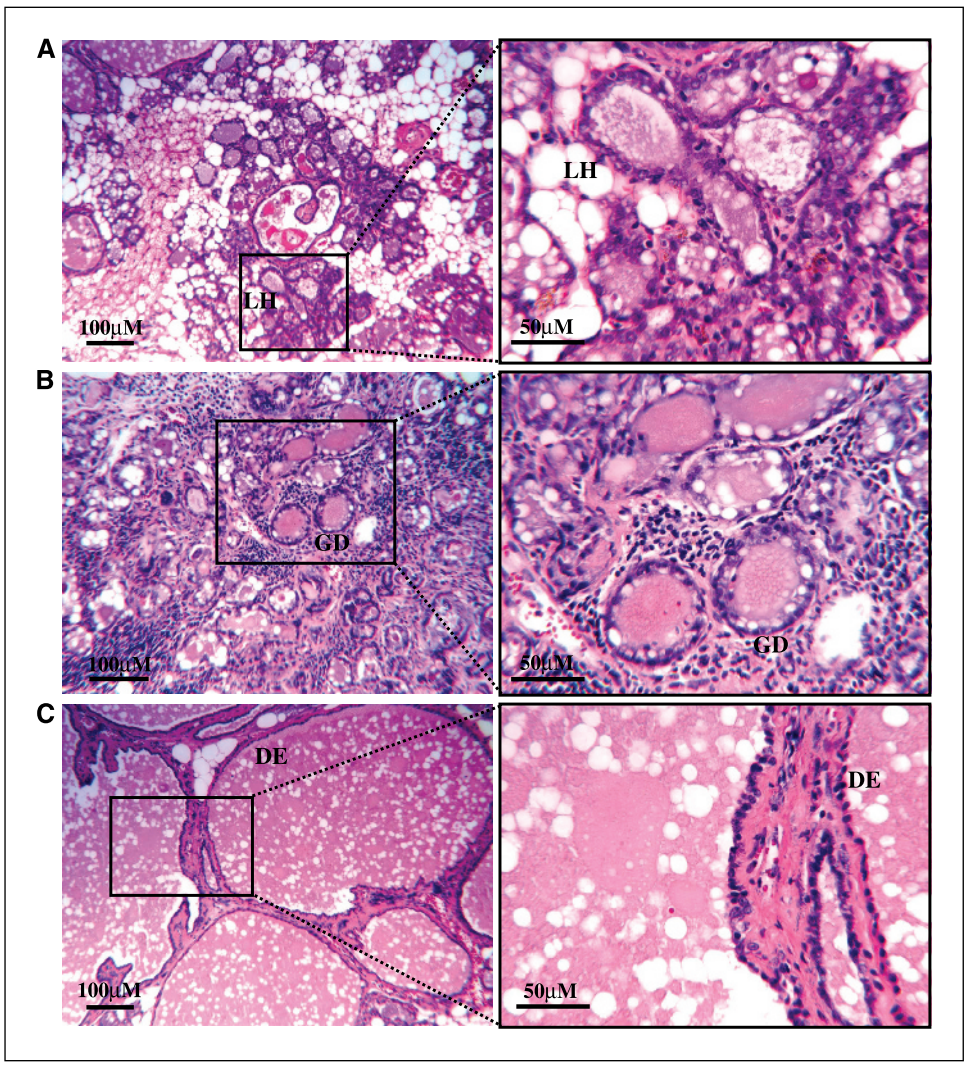

Figure 3. Histologic analysis of lobular hyperplasia in WAP-EPM transgenic mammary glands. *A,* diffuse lobular hyperplasia is observed throughout WAP-EPM mouse mammary glands marked by lipid droplets and proteinaceous secretions inside of ductule lumens. *B,* lobular hyperplasias within mammary tumors have atypical nuclei, lymphocytic infiltrates, and dark proteinaceous staining within ductule lumens. *C,* ductal ectasia in WAP-EPM transgenic mammary glands is more severe in aged mice but not in WT mice. *GD,* glandular differentiation; *LH,* lobular hyperplasia; *DE,* duct ectasia.

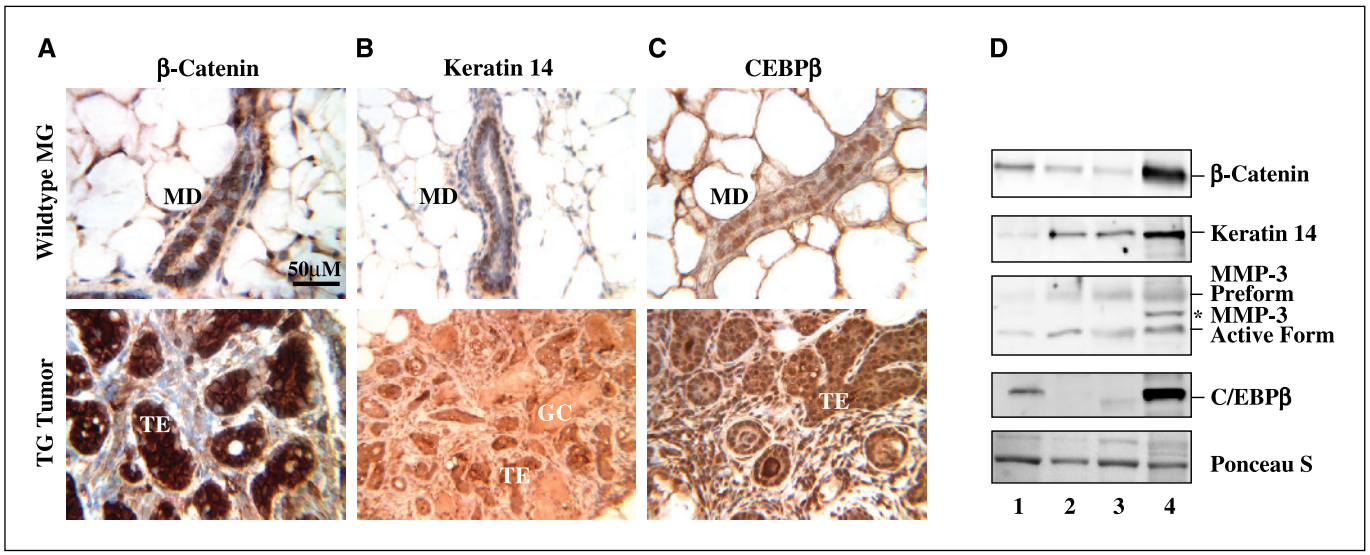

Figure 4. WAP-EPM tumors overexpress Wnt-pathway markers, C/EBPβ, and MMP-3. *A* to *C,* immunohistochemistry on WT mammary ducts and WAP-EPM transgenic mammary tumors for β-catenin (*A*), keratin-14 (*B*), and C/EBPβ(*C*). *A,* aberrant nuclear and cytoplasmic expression of β-catenin is observed in epithelium of WAP-EPM mammary tumors compared with the WT mammary gland. *A* to *C,* up-regulation of β-catenin, keratin-14, and C/EBPβ inside of WAP-EPM mammary tumors is observed compared with WT mammary glands. *D,* Western blot analysis comparing the immunoreactivity of β-catenin, keratin-14, MMP-3, and C/EBPβ in protein lysates from a parenchyma-free mammary fat pad (*1*), normal WT aged mammary gland (*2*), hyperplastic WAP-EPM mammary gland (*3*), and WAP-EPM mammary tumor (*4*). *GC,* ghost cells; *MD,* mammary duct; *TE,* tumor epithelium. *, possibly a glycosylated form of MMP-3.

and progression. Often, these aggressive tumors do not resemble the morphology of human breast cancers, are frequently hormone-independent, exhibit less stromal and inflammatory response, and rely solely on one genetic modification provided by the transgene itself. In contrast, human breast cancers rely on multiple genetic alterations over the human life span and take decades to develop (19). The more gradual progression towards tumorigenesis in the EPM transgenic mouse more closely resembles breast cancer formation in humans and therefore may be a useful additional model to study breast cancer progression and possibly prevention.

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