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Advances in Brief

A Single Targeted *Ets2* Allele Restricts Development of Mammary Tumors in Transgenic Mice[1]

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Abstract

Heterozygous female mice carrying a targeted mutation of the Ets2 transcription factor gene were mated with a mouse strain that develops mammary tumors due to the expression of the polyoma virus middle T oncogene. Tumors from females with only one wild-type *Ets2* gene were approximately one-half the size of tumors from controls. The smaller size of the tumors was correlated with a more differentiated state of early hyperplastic growths and not to differential growth of the frank tumors or to decreased middle T gene expression. Ets2 may regulate the progression of these aggressive mammary tumors.

Introduction

The Ets family of transcription factors represents >45 proteins (18 human proteins) that share a variant, winged helix-turn-helix DNA-binding domain (1). In both *Caenorhabditis elegans* and *Drosophila*, Ets factors mediate growth factor stimulation of the Ras-Raf-MAP[4] kinase signal transduction pathway to trigger specific developmental decisions (2). Activated ErbB2 (neu), Src, Ras, and Raf can stimulate Ets2 mediated transcriptional activation in cultured mammalian cells (1, 3, 4). The oncogene stimulation of this pathway activates the transcriptional activity of Ets1 and Ets2 by phosphorylation of a specific threonine residue in the *pointed* domain (3). Ets factors appear to be important mediators of transformation because dominant inhibitory Ets constructs can block transformation by Ras or ErbB2/Neu (5) and can partially reverse the transformed phenotype of a breast tumor cell line (6). Mice homozygous for a targeted mutation of Ets2 die during early development due to extraembryonic tissue deficiencies, which include low expression of MMP-9 (gelatinase B). Ets2-deficient animals, rescued from early embryonic lethality by aggregation with tetraploid wild-type embryos, develop normally but, as adults, resemble animals deficient in transforming growth factor-α, a member of the epidermal growth factor family of growth factors (7). The *PyMT* oncogene uses the same signal transduction pathways as do epidermal growth factor receptor members. Transgenic expression of PyMT in mammary gland results in early general hyperplasia and subsequent multifocal carcinomas, with 100% penetrance (8). The induction of tumors by PyMT is dependent on the c-Src tyrosine kinase (9). The potency of PyMT can be attributed, at least in part, to its ability to activate both the Shc adapter protein (and subsequently, Grb2-Sos, Ras, Raf, and MAP kinases) and PI 3′-kinase signaling pathways (10). Thus, whereas PyMT is not a cause of cancer in humans, transgenic mice expressing PyMT in mammary tissues provide an opportunity to identify mediators of signaling common to growth factor receptors and multiple activated oncogenes implicated in human disease. Here, we investigate the potential role of Ets2 in mediating biologically relevant signaling in mammary tumor cells *in vivo*.

Materials and Methods

RNA Analysis. Total RNA was purified from frozen tissues with acidic phenol (11). The levels of Ets2, MMP-3, L-32, and PyMT RNAs were determined by RNase protection assays, performed as described previously using antisense transcripts of mouse Ets2 cDNA (292-bp fragment), mouse L-32 cDNA (187-bp fragment; Ref. 7), and *PyMT* gene (368-bp fragment; Ref. 8). All these fragments were amplified by PCR and cloned into pGEM1 plasmid. The protected Ets2 and PyMT signals were normalized to the signals obtained from the mouse L-32 ribosomal protein RNA.

Tumor Formation and Analysis. Transgenic mammary tumor formation was achieved by mating FVB/N-TgN(MMTVPyVT)634 Mul male transgenic mice (MMTV-PyMT mice), which were obtained from The Jackson Laboratory (Bar Harbor, ME), with *Ets2*db1/+ heterozygous mice bred in a Swiss/Black outbred background (*Ets2*+/−). All tumors were derived from females of the F₁ generation of these crosses. Animals were inspected for visible tumors weekly. After tumors were first observed, the length and width of tumors were measured with a calipers every 3 days until 90–95 days or until the host was visibly affected by tumor burden. The volume of the tumor at 80, 85, 90, and 95 days was estimated by interpolating the growth curve of each tumor. Tumor volume was calculated by the formula: (length $\times$ width²)/2. For tumors from 80-day-old mice, the sizes of the two largest tumors of each animal were measured, and the excised tumors were weighed. A portion of each tumor was fixed in Bouins' fixative or 4% paraformaldehyde in PBS for histology, and the remainder was frozen in liquid nitrogen for RNA isolation.

Histology. Excised mammary glands were mounted on glass slides, fixed in acidic ethanol, and stained with carmine alum (12). Fixed tumors were processed for paraffin sections and subsequent staining with H&E. Photographic documentation was performed with SPOT digital camera and Adobe Photoshop software. Apoptotic tumor cells were identified in sections with the use of the Apotag commercial kit (Oncor) for visualizing nicked nuclear DNA. Mitotic activity was visualized by staining sections of tumors from animals injected with bromodeoxyuridine (1 mmol/100 g) 30 min before sacrifice. A commercial bromodeoxyuridine staining kit was used according to the manufacturer's instructions (Zymed Laboratories, Alameda, CA).

Results

Modification of Transgenic Mammary Tumor Growth by Ets2. To investigate the impact of altering the level of wild-type Ets2 on transgenic mammary tumors, we mated MMTV-PyMT males with *Ets2*+/− heterozygotes. We then compared tumor appearance and size

of PyMT-positive females having either one or two wild-type *Ets2* alleles (*PyMT/Ets2*$^{+/-}$ and *PyMT/Ets*$^{+/+}$). Only F$_1$ animals were compared to eliminate potential genetic background effects. The average size of mammary tumors that arise as a consequence of PyMT expression in wild-type and *Ets2*$^{+/-}$ heterozygotes is shown (Fig. 1). All PyMT-positive females developed tumors. However, the tumors from *PyMT/Ets2*$^{+/-}$ heterozygotes were smaller at all times of observation (Fig. 1*A*). To confirm that the significant difference in tumor size depended on the *Ets2* genotype, we weighed excised tumors from animals of the same age (Fig. 1*C*). The average weight of the largest
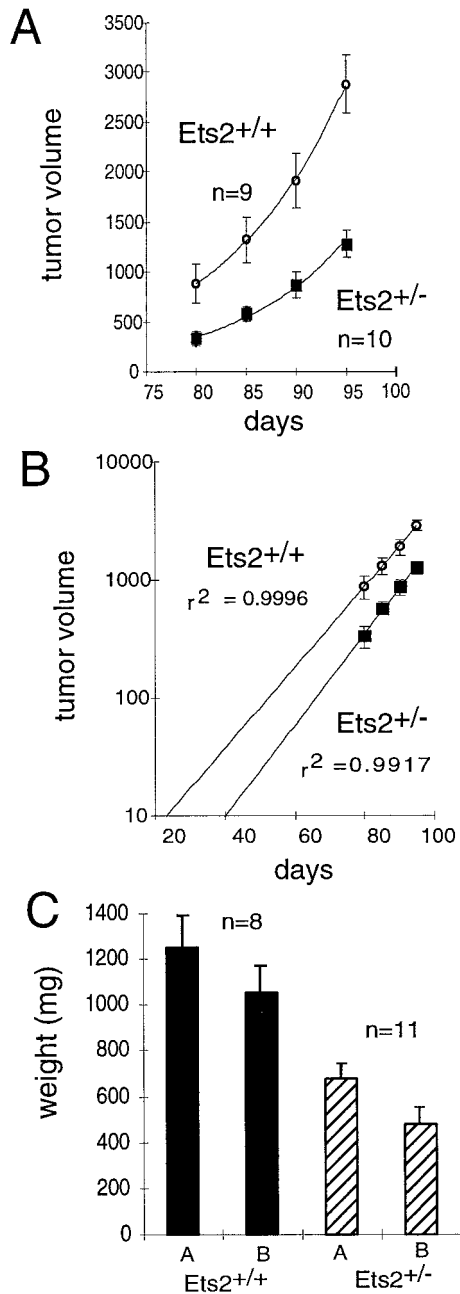

Fig. 1. Effect of Ets2 on the growth of mammary tumors in transgenic mice. *Ets2*$^{+/-}$ mice were mated with the MMTV-PyMT transgenic line. The sizes of the tumors arising in PyMT-positive females were measured at 3-day intervals. *A*, *data points*, means of tumor volumes of 10 PyMT-positive females of each genotype as a function of their *Ets2* genotype; *bars*, SE. *B*, the data in *A*, plotted on a logarithmic scale. The correlation coefficients of exponential curve fitting are shown. *C*, the two largest mammary tumors were excised and weighed in PyMT females 80 days of age. *Columns A*, largest tumor; *columns B*, smaller tumor. The differences among the larger and smaller tumor groups and the average of both tumors were significant ($P \leq 0.003$) by the Student's *t* test.

tumor of *Ets2*$^{+/-}$ heterozygotes was less than one-half of that of largest tumors of Ets2 wild-type animals. For each animal, comparisons of the average weights of either the largest tumors (Fig. 1*C*, *columns A*) or the average weights of both tumors were statistically significantly different (Student's *t* test, $P \leq 0.003$). The excellent fit of the growth of both types of tumors to an exponential function of similar slope (Fig. 1*B*) suggests that the difference in sizes of the tumors is related to a delay or difference in progression of the tumors, rather than a difference in growth rate of the mature tumors.

Mammary Gland and Tumor Development. The possible delayed onset of exponential tumor growth in *PyMT/Ets2*$^{+/-}$ heterozygotes suggested that mammary gland development might be delayed in *Ets2*$^{+/-}$ heterozygous females. However, no differences in mammary gland development was apparent in *Ets2*$^{+/-}$ females of ages 25, 35, or 47 days compared with wild-type littermates, as judged by mammary ductal tree development in whole mounts (Fig. 2*A* and data not shown). Furthermore, mammary development of a rescued, homozygous *Ets2*$^{-/-}$ 50-day-old female was normal and not distinguishable from a littermate. Thus, Ets2 is not essential for early mammary gland development, and delayed mammary-tree development is not the cause of the delayed tumor growth in bigenic *PyMT/Ets2*$^{+/-}$ females.

The development of mammary tumors was evaluated in whole mounts and histological sections of mammary tissue from PyMT females as a function of the presence or absence of the targeted *Ets2* allele. At 35 days, the mammary tissues of both *PyMT/Ets2*$^{+/+}$ and *PyMT/Ets2*$^{+/-}$ females revealed a focal, hyperplastic nodular mass beneath the nipple (Fig. 2*B*). Histological sections revealed that these were composed of small highly cellular nests of dysplastic cells separated by dense fibrous septae (data not shown). A relatively normal mammary ductal system emanated from the subareolar mass into the mammary fat pad.

At 47 days, multifocal cysts and solid nodules became evident in the peripheral mammary tree of both genotypes, as described previously for *PyMT/Ets2*$^{+/+}$ mice (8). Epithelial cysts were observed in both genotypes but were far more common in the *PyMT/Ets2*$^{+/-}$ mice (Fig. 2, *C* and *D*). Frequently, a series of cysts rather than solid nests of cells were found along the ducts of the heterozygous animals. These cysts appear as short side buds off of the duct, suggesting that they represent abortive attempts at alveolar differentiation. The fluid in the spaces indicate transepithelial transport and a level of functional differentiation. Histological sections confirmed that the hollow, fluid-filled cysts were more common to the *PyMT/Ets2*$^{+/-}$ animals (Fig. 2, *E* and *F*). The cysts were usually lined by multiple disorganized layers of epithelium. Sections of the solid masses revealed more disorganized epithelium that did not form well-organized, functional glands. In contrast, the *PyMT/Ets2*$^{+/+}$ mammary tree was dominated by the solid nodular masses of cells. Sections of the solid nests revealed a more disorganized epithelium composed of cells with large, hyperchromatic nuclei, scanty cytoplasm, and abundant mitotic figures. Although cystic and solid lesions were evident in specimens from both genotypes, the *PyMT/Ets2*$^{+/-}$ tissues were better differentiated, with more cysts and fewer solid dysplastic lesions.

Histological analysis of frank tumors from the animals of each genotype of 80 days and older revealed the previously described, typical phenotype for PyMT-induced mammary tumors (8, 13). The tumors were composed of poorly differentiated cords and nests of cells forming sheets, ill-defined, slit-like glandular spaces, or, occasionally, larger cystic spaces lined by a multilayered epithelium. Some foci appeared to be surrounded by a basement membrane. However, invasive foci were readily identified in older lesions. The invasive regions most commonly formed as cords of cells infiltrating a dense connective tissue. Whereas it was difficult to distinguish cytologically
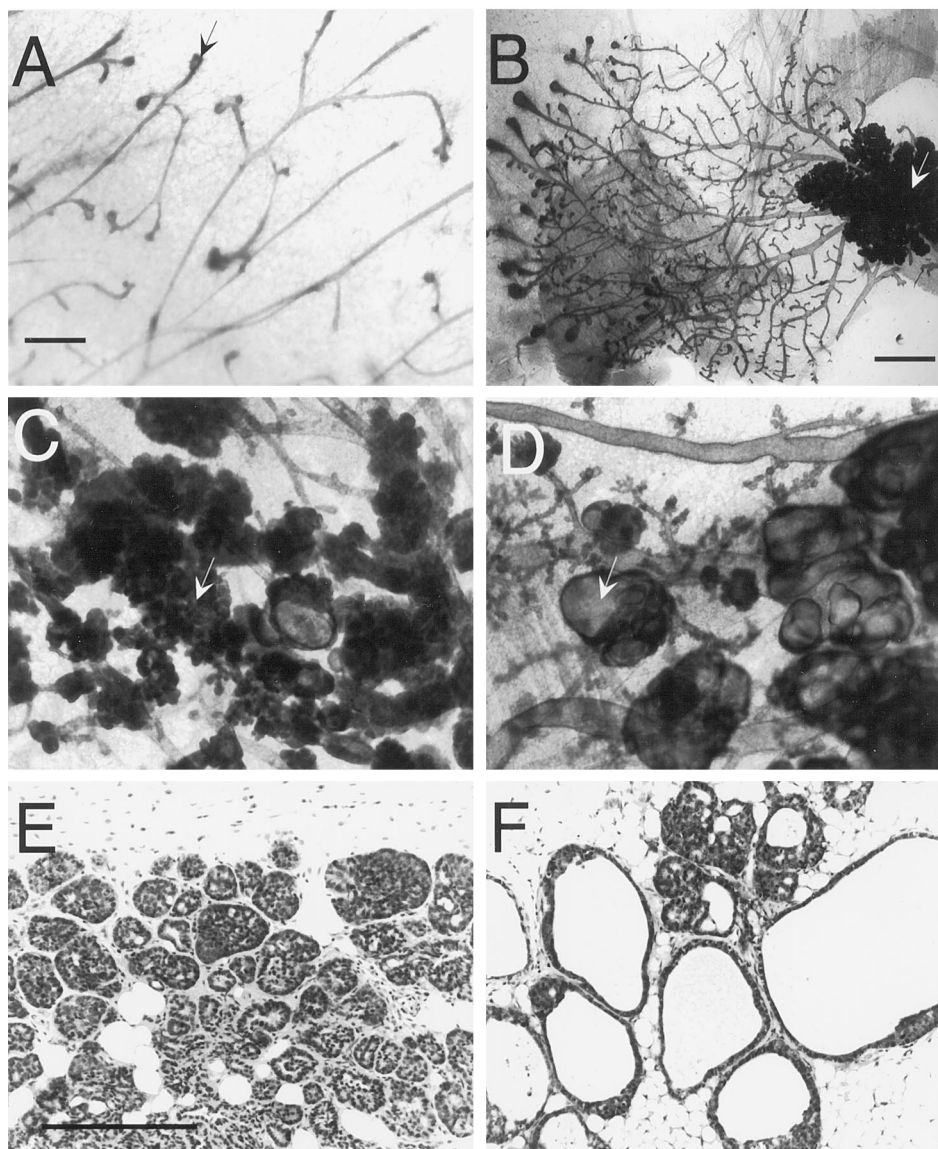
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Fig. 2. Mammary gland development and histopathology. *A–D*, the appearance of mammary tissues in whole mounts. *A*, normal ductule-tree development of mammary gland of 50-day-old $Ets2^{+/-}$ female. *Scale bar*, 200 μm (*A*, *C*, and *D*). *Arrow*, terminal end bud. *B*, whole mount of 35-day $PyMT/Ets2^{+/-}$ mammary gland. Note the focal cellular mass beneath the nipple area (*arrow*). *Scale bar*, 400 μm. *C* and *D*, multifocal mammary gland lesions arising in 47-day-old $PyMT/Ets2^{+/+}$ (*C*) and $PyMT/Ets2^{+/-}$ (*D*) females. *Arrows*, the common cystic structures of *D* and the smaller, less-differentiated masses of *C*. *E* and *F*, histological sections of contralateral mammary glands of the animals shown in *C* and *D*. Scale bar, 100 μm (*E* and *F*). Note the large cystic structures shown in *F*.

between the $PyMT/Ets2^{+/+}$ and the $PyMT/Ets2^{+/-}$ tumors, the tissues from the two groups had a consistent difference in the degree of differentiation. The invasive carcinomas of wild-type mice, in comparison to the bigenic carcinomas, tended to be less differentiated and have more tissue necrosis. Most of the bigenic carcinomas formed well-defined glands. Furthermore, the $PyMT/Ets2^{+/+}$ carcinomas generally had more obvious invasive foci than the $PyMT/Ets2^{+/-}$ tumors at earlier time points. Lung metastases were present in both groups at 79–81 days.

The degree of DNA synthesis by tumor cells from 80-day-old mice was judged by injecting bromodeoxyuridine 30 min before sacrifice and detecting its incorporation by immunohistochemistry. However, as expected from the similar growth rate of the tumors (Fig. 1*B*), the $PyMT/Ets2^{+/-}$ tumors could not be distinguished from $PyMT/Ets2^{+/+}$ by this method. Similarly, the degree of apoptosis, also judged immunohistochemically, was low and similar in 80-day tumors (data not shown).

Tumor Gene Expression. Mammary tumors arise rapidly in MMTV-PyMT transgenic mice because of the high level of expression of PyMT RNA (8). To determine whether a lower level of PyMT RNA might be responsible for the Ets2-dependent difference in tumor size, we measured Ets2 and PyMT RNAs by RNase protection. As expected, Ets2 mRNA was one-half as abundant in Ets2 heterozygotes as it was in wild-type tumors (Fig. 3, *A* and *B*). However, in the same tumors, the expression of PyMT RNA was unchanged (Fig. 3*C*). These results indicate that Ets2 did not limit PyMT expression. Furthermore, a survey of 20 potential targets or interactive partners of Ets2 did not reveal significant differences between $PyMT/Ets2^{+/+}$ and $PyMT/Ets2^{+/-}$ tumors. These RNAs included c-fos, fra1, fra2, Ets1, Fli1, GABPα, ERM, Net, Tel, p53, ErbB2, ErbB3, transforming growth factor-α, mK8, vascular endothelial growth factor-1, and fibroblast growth factor-2 (data not shown). Variable levels of p21 and Elf3 were noted but without any correlation with *Ets2* genotype. RNAs for MMP-3 and MMP-9 were either undetectable or at extremely low levels in tumors from 80-day-old mice. The identification of Ets2-sensitive target genes responsible for the slower development of $PyMT/Ets2^{+/-}$ tumors remains a future challenge.

Discussion

The targeted *Ets2* allele is responsible for a dramatic difference in the size of mammary tumors initiated by the *PyMT* oncogene. This
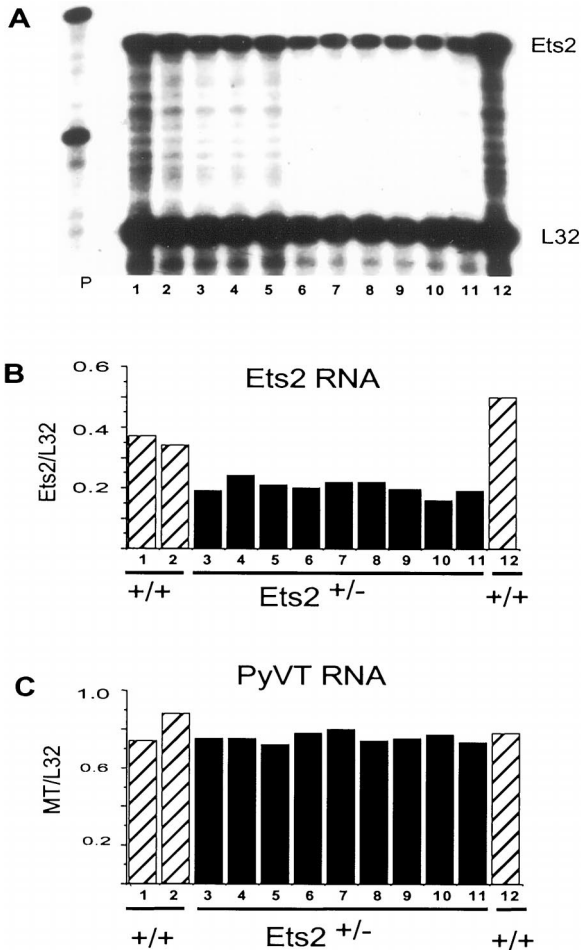
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Fig. 3. Expression of Ets2 and PyMT RNAs in mammary tumors. RNase protection analysis was performed on tumor RNA of the indicated *Ets2* genotypes. *A*, autoradiographic image of Ets2 and L-32 RNAs. *B*, Ets2 signal measured by phosphor imaging normalized to the signal generated by the L-32 rRNA probe. *C*, signals for PyMT mRNA detected by phosphor image analysis and normalized to the L-32 ribosomal protein RNA.

alterations resulting in the appearance of a single-hit model. The targeted *Ets2* allele may restrict this high-frequency event or events, resulting in a retardation of the progression to unrestricted growth.

A unique aspect of these experiments is the recognition that the *PyMT* transgene initially results in the formation of a dysplastic or hyperplastic subareolar mass. Although these cells appear undifferentiated, normal ductal structures emerge from the mass. Furthermore, the mass appears to be self-limiting, rarely growing to the size of the more peripheral tumors. This suggests that the subareolar region of the mammary gland, which is primarily the lactiferous ducts, has a unique biology and warrants further attention.

The effect of the targeted *Ets2* allele on tumor size stimulates consideration of Ets2 itself as a possible genetic modifier of human tumor development. Expression of Ets2 is elevated in prostate cancer (14). In humans, Ets2 is located on human chromosome 21. The neurocranial, viscerocranial, and cervical skeletal abnormalities induced by modestly elevated Ets2 expression in transgenic mice suggests a possible role for Ets2 in the phenotypic alterations and increased risk of leukemia associated with Down's syndrome (15). Genes that influence the level of expression of Ets2 could impact on tumor development in both mice and humans.

Ets2-sensitive target genes could be in either the epithelial or stromal cellular components, but their identity remains to be determined. In fibroblastic cells, MMP-3 is particularly sensitive to the level of Ets2 (7). MMPs are important for the vascularization, invasive behavior, and metastasis of tumors (16, 17). However, lung metastases were found in PyMT animals of both *Ets2* genotypes, and the similar exponential growth of the tumors suggests that, once established, the tumors do not appear to be restricted by blood vessel availability.

Several Ets transcription factors are capable of mediating transcriptional activation resulting from stimulation of the Ras-Raf-MAP kinase pathway by activated oncogenes or receptor-mediated tyrosine kinases. An example that is relevant to breast cancer is that oncogenic ErbB2 (Neu) activates Ets2-dependent reporter genes, and a dominant inhibitory Ets2 construct blocks the transforming activity of oncogenic Neu (5). The expression of dominant inhibitory mutant forms of Ets2 inhibits anchorage-independent growth of breast cancer cell lines (6) and ras-mediated cellular transformation (18). The importance of the Ras pathway for PyMT tumor formation has been demonstrated by the dependence of PyMT-initiated tumors on the Grb2 adapter protein, which functions upstream of Ras (19). Because PyMT and ErbB2 both activate Ras-MAP kinase pathways, Ets2 may also modulate the progression of tumors arising due to the expression of activated ErbB2 (20).

Acknowledgments

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effect may be due to a delay in the progression to invasive carcinoma. The similar exponential growth of the palpable tumors and the similar histology and gene expression profiles of both genotypes suggests the existence of an Ets2-dependent step in the early neoplastic progression of the tumors, rather than a limitation on tumor growth. The histological similarity of the tumors arising in both *PyMT*/*Ets2*^{+/+} and *PyMT*/*Ets2*^{+/−} mice and the absence of dramatic differences in apoptosis or mitotic indices are consistent with this suggestion. The normal mammary ductal tree development of *Ets2*^{+/−} and rescued *Ets2*^{−/−} females suggests that the early stages of mammary gland development are not significantly impacted by the mutant *Ets2* allele. Furthermore, it is unlikely that early PyMT expression is sensitive to Ets2 dosage because the earliest hyperplastic growths of *PyMT*/*Ets2*^{+/−} and *PyMT*/*Ets2*^{+/+} females were indistinguishable. However, at 47 days, the hyperplastic tissues of *PyMT*/*Ets2*^{+/−} mice were more differentiated. This difference in differentiation may reflect the limiting step between early hyperplastic growth and exponential tumor growth that is affected by the targeted *Ets2* allele. The multifocal lesions, complete penetrance, and bifunctional stimulation of both Src and PI 3′-kinase signal transduction pathways are consistent with the suggestion that PyMT can function in a single step to cause metastatic cancer (10). However, perhaps the high expression of PyMT combined with the stimulation of both Src and PI 3′ kinase signal transduction pathways ensures a high probability of additional progressive

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