A Single Targeted Ets2 Allele Restricts Development of Mammary Tumors in Transgenic Mice

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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 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 extracellular 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-Tg(MMTV-PyVT)634 Mul male transgenic mice (MMTV-PyMT mice), which were obtained from The Jackson Laboratory (Bar Harbor, ME), with Ets2<sup>+/−</sup> heterozygous mice bred in a Swiss/ Black outbred background (Ets2<sup>+/−</sup>). All tumors were derived from females of the F<sub>1</sub> generation of these crosses. Animals were inspected for visible tumors weekly. After tumors were first observed, the length and width of tumors were measured, and the excised tumors were weighed. A portion of each tumor was measured, and the excised tumors were weighed. A portion of each tumor was fixed in Bouin’s 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/liter) 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<sup>+/−</sup> 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/Ets2\(^{+/-}\)). 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. 1A). 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. 1C). The average weight of the largest 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. 1C, columns A) or the average weights of both tumors were statistically significantly different (Student’s t, \(P = 0.003\)). The excellent fit of the growth of both types of tumors to an exponential function of similar slope (Fig. 1B) 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 littersmates, as judged by mammary duct tree development in whole mounts (Fig. 2A 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. 2B). 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 mammmary duct 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
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+/+ tumors 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. 1B), 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).

**Discussion**

The targeted Ets2 allele is responsible for a dramatic difference in the size of mammary tumors initiated by the PyMT oncogene. This...
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 L32 RNAs. B, Ets2 signal measured by phosphor imaging normalized to the signal generated by the L32 RNA probe. C, signals for PyMT mRNA detected by phosphor image analysis and normalized to the L32 ribosomal protein RNA.

Ets2 RESTRICTS MOUSE MAMMARY TUMORS

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References


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