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PRINCIPAL INVESTIGATOR: John Tynan
Fang Wen, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
LaJolla, CA 92037

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6. AUTHOR(S)John Tynan
Fang Wen, Md., Ph.D.**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**The Burnham Institute
LaJolla, CA 92037

E-Mail: jtynan@burnham.org or fwen@burnham.org

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Ets2 is a transcription factor that has been implicated in growth factor signaling and is essential for embryonic development. Reduced Ets2 activity restricts the appearance of transgenic mammary tumors caused by either Polyoma middle T antigen (PyMT) or activated Neu/ErbB2. In addition, the early growth of transplanted mammary tumors is limited by host Ets2 activity. Here we have tested genetically, with the use of a conditional Ets2^{fllox} allele and tissue specific Cre recombinase expression, whether Ets2 also functions within tumor cells by inactivating Ets2 within mammary luminal epithelial cells from which transgenic PyMT^{Y315/322F} tumors arise. We find that inactivation of Ets2 within tumor cells has no effect on tumor appearance or growth. By contrast, complete inactivation of Ets2 in both epithelial and stromal cells moderates the early hyperplastic phase of tumor development and the time of tumor appearance but does not prevent tumor occurrence and has no detectable effect on tumor growth. Thus Ets2 supports mammary tumors exclusively through their microenvironment.

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Introduction

Ets2 is a transcription factor that has been implicated in the Ras-Raf-MAPK signal transduction pathway and is essential for embryonic development[1]. Inactivation of Ets2 in mice by deletion of the conserved DNA binding domain (DBD) caused early embryonic lethality due to defective trophoblast function. When rescued by aggregation with tetraploid wild-type embryos, these mice are largely normal with the exception of a "waved" hair phenotype resembling that of TGF- α knockout mice[2, 3]. The inactivated Ets2 allele, denoted Ets2^{db1}, was found to restrict the development of mammary tumors caused by the expression of the polyoma virus middle T antigen (PyMT)[4]. To further analyze Ets2 regulation of mouse mammary tumorigenesis, we have used a conditional targeting approach to circumvent the embryonic lethality allowing generation of a mouse colony required for tumor study. The established conditional allele Ets2^{flox}, carrying the major part of the DBD flanked by two loxP sites ("floxed"), can be inactivated by Cre-mediated deletion of the "floxed" sequence. We proposed to generate trigenic mice that contain a mammary-specific Cre transgene (MMTV-Cre7)[5] and the Ets2^{flox} alleles in a mammary tumor model MMTV-NeuNDL that expresses an activated form of Neu[6]. Neu (ErbB2/HER2), a member of the EGF receptor family, uses the same signal transduction pathways as PyMT and is strongly implicated in human breast cancer[7]. I hypothesized that Ets2 deficiency in mammary epithelium would significantly suppress the development of tumors in this model.

BODY

As described in the 2003 annual report, the Ets2^{flox} mice were generated successfully, and they are homozygous viable and fertile as expected. When crossed with an epiblast-specific Cre transgenic line MORE [8], the bi-transgenic animals recapitulate the "waved" hair phenotype of rescued Ets2^{db1/db1} mice. The inactive allele generated after Cre-mediated deletion of exons 9 and 10 is designated Ets2^{db2} because of its similarity to the previous Ets2^{db1} allele. Due to mosaic expression of Cre, the overall recombination efficiency is 80-90% varying slightly among different mice and various tissues.

Additionally, a different tumor model utilizing a mutant form of PyMT (PyMT^{Y315/322F}) replaced the originally proposed MMTV-NeuNDL model as explained in the 2003 annual report. PyMT^{Y315/322F} is defective in activation of the phosphatidylinositol 3' (PI-3') kinase pathway, but retains signaling through Src and Shc [9]. MMTV driven expression of PyMT^{Y315/322F} causes development of focal mammary tumors with 100% penetrance in the transgenic mice.

In addition to the MMTV-Cre7 and Ets2^{flox} crossing, We added the breeding of the MORE line with the Ets2^{flox} mice to Task II of Year I in the original Statement of Work. As discussed in the original proposal, if Ets2 acts exclusively in the stroma during mammary tumorigenesis, the epithelium-restricted inactivation of Ets2 induced by MMTV-Cre7 will have no effect on tumor development. However, depleting functional Ets2 by 80-90% generally in the MORE;Ets2^{db2/flox} mice will demonstrate most dramatic effect of Ets2-dependent regulation of tumorigenesis. Therefore, setting up both MORE;Ets2^{flox} and MMTV-Cre;Ets2^{flox} breedings in the same tumor model system will help delineate the spatial pattern of Ets2 regulation of mammary tumorigenesis.

During the second year of the award, we obtained all the triple transgenic mice needed for the tumor study: 18 "waved" PyMT^{Y315/322F};MORE;Ets2^{flox/db2} mice and 24 control mice carrying PyMT^{Y315/322F};MORE;Ets2^{flox/+}, and 20 PyMT^{Y315/322F};MMTV-Cre7;Ets2^{flox/flox} mice as well as 16

control animals that are PyMT^{Y315/322F};MMTV-Cre7;Ets2^{fl^{ox}/+}. Tumor development in these animals was compared, and individual tumors from multiple mice were analyzed for genotyping, RNA expression and histopathology. We find that MMTV-Cre7 mediated inactivation of Ets2 within tumor cells has no effect on tumor appearance or growth. By contrast, complete inactivation of Ets2 in both epithelial and stromal cells in the MORE;Ets2^{fl^{ox}/db2} mice moderates the early hyperplastic phase of tumor development and the time of tumor appearance but does not prevent tumor occurrence and has no detectable effect on tumor growth. Thus Ets2 supports mammary tumors exclusively through their microenvironment.

The results of the study were submitted for publication in an article entitled "Ets2 Dependent Microenvironmental Support of Mouse Mammary Tumors", which is currently in press for Oncogene. The details of the study can be found in the paper [10].

KEY RESEARCH ACCOMPLISHMENTS

- generation of a conditional Ets2 allele that is inactivated by Cre recombinase
- Genetic evidence showing exclusively stromal support of Ets2 during PyMT^{Y315/322F}-induced mammary tumor formation at an early initiation phase

REPORTABLE OUTCOMES

Generation of the Ets2 conditional knockout mice provides a model system to study the potential role of Ets2 in related developmental and disease processes, which has been presented in the annual research symposium of the Molecular Pathology Program at University of California, San Diego. The results from these two parallel studies using Ets2^{fl^{ox}} combined with either the MORE mice or the MMTV-Cre7 mice, was presented at the 24th Congress of the International Association for Breast Cancer Research in November 2003. A journal article summarizing all the data was accepted for publication and is currently in press for Oncogene [10].

CONCLUSIONS

The Ets2 conditional knockout mice have been generated successfully, and the proposed tumor study investigating the regulatory role of Ets2 in mammary tumorigenesis has been completely finished. The results reveal that Ets2 deficiency, restricted to mammary epithelial cells, does not influence the appearance of mammary tumors. In contrast, complete inactivation of Ets2 in both epithelial and stromal cells significantly moderates the early hyperplastic phase of tumor development and tumor onset. Thus Ets2 supports mammary tumors exclusively via its function in the stroma.

REFERENCES

1. Yamamoto, H., et al., *Defective trophoblast function in mice with a targeted mutation of Ets2*. Genes and Development, 1998. **12**: p. 1315-1326.
2. Luetkeke, N.C., et al., *TGF α deficiency results in hair follicle and eye abnormalities in targeted*

- and *waved-1* mice. *Cell*, 1993. **73**: p. 263-278.
3. Mann, G.B., et al., *Mice with a null mutation of the TGF α gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation*. *Cell*, 1993. **73**: p. 249-261.
 4. Neznanov, N., et al., *A single targeted Ets2 allele restricts development of mammary tumors in transgenic mice*. *Cancer Research*, 1999. **59**: p. 4242-4246.
 5. Andrechek, E.R., et al., *Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis*. *Proc Natl Acad Sci U S A*, 2000. **97**(7): p. 3444-9.
 6. Siegel, P.M., et al., *Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer*. *Embo J*, 1999. **18**(8): p. 2149-64.
 7. Dankort, D.L. and W.J. Muller, *Signal transduction in mammary tumorigenesis: a transgenic perspective*. *Oncogene*, 2000. **19**(8): p. 1038-44.
 8. Tallquist, M.D. and P. Soriano, *Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function*. *Genesis*, 2000. **26**(2): p. 113-5.
 9. Dahl, J., et al., *Studies of partially transforming polyomavirus mutants establish a role for phosphatidylinositol 3-kinase in activation of pp70 S6 kinase*. *Mol Cell Biol*, 1996. **16**(6): p. 2728-35.
 10. John A. Tynan, F.W., William J. Muller and Robert G. Oshima, *Ets2 Dependent Microenvironmental Support of Mouse Mammary Tumors*. *Oncogene*, in press.

BIBLIOGRAPHY

I. Publications and meeting abstracts resulting from the funded research project:

1. Tynan JA, Wen F, Man AK, Young LT, Cardiff RD, Egeblad M, Werb Z, Muller WJ, and Oshima RG, *An Ets2 dependent stromal restriction of mouse mammary tumors*. Meeting abstract at the 24th Congress of the International Association for Breast Cancer Research.
2. Tynan JA, Wen F (co-first author), Muller WJ, and Oshima RG, *Ets2 dependent Microenvironmental support of mouse mammary tumors*. *Oncogene*, in press.

II. Personnel receiving funding from the award:

Fang Wen M.D. Ph.D

John A. Tynan Ph.D. candidate

Ets2 Dependent Microenvironmental Support of Mouse Mammary Tumors.

John A. Tynan^{1,4}, Fang Wen^{1,4}, William J. Muller² and Robert G. Oshima^{1,3}

¹Graduate Program in Molecular Pathology, University of California, San Diego, 9500 Gilman
Drive 0612, La Jolla, CA 92093

²Molecular Oncology Group, McGill University, 687, Pine Avenue West, Room H5.63A
Montreal PQ H3A 1A1, Canada

³Oncodevelopmental Biology Program, Cancer Research Center, The Burnham Institute, 10901
North Torrey Pines Road, La Jolla, CA 92037

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Correspondence to: R.G. Oshima, The Burnham Institute, 10901 North Torrey Pines Road, La
Jolla, CA 92037. Phone: (858) 646-3147. Fax: (858) 713-6268. E-mail:
rgoshima@burnham.org.

⁴These two authors contributed equally to this work.

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Abstract

Decreasing the amount of active mouse Ets2 transcription factor by half in mice or use of a MAP kinase insensitive hypomorphic targeted Ets2 allele restricts the appearance of transgenic mammary tumors caused by either Polyoma middle T antigen (PyMT) or activated Neu/ErbB2. In addition, the early growth of transplanted mammary tumors is limited by restricted Ets2 activity of the host. Here we have tested genetically, with the use of a conditional Ets2^{fllox} allele and tissue specific Cre recombinase expression, whether Ets2 also functions within tumor cells by inactivating Ets2 within mammary luminal epithelial cells from which transgenic PyMT^{Y315/322F} tumors arise. We find that inactivation of Ets2 within tumor cells has no effect on tumor appearance or growth. By contrast, complete inactivation of Ets2 in both epithelial and stromal cells moderates the early hyperplastic phase of tumor development and the time of tumor appearance but does not prevent tumor occurrence and has no detectable effect on tumor growth. Thus Ets2 supports mammary tumors exclusively through their microenvironment.

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Introduction

Genetic restrictions of the transcription factor Ets2 limits mouse mammary tumor development caused by transgenic expression of either Polyoma middle T antigen (PyMT) or activated Neu/ErbB2 (Neznanov et al., 1999). Targeted deletion of the Ets2 DNA binding domain (Ets2^{db1}) or mutation of the unique, activating MAPK phosphorylation site (Ets2^{A72}) revealed that Ets2 supports both autochthonous and transplanted mammary tumors (Man et al., 2003). The Ets2 dependent host effect on transplanted tumors showed that at least part of the function of Ets2 was mediated through the stroma (Man et al., 2003). However, a possible epithelial role for Ets2 is suggested by reversal of the transformed phenotype of BT20 breast tumor cells by a dominant negative Ets2 construct (Sapi et al., 1998), but such constructs also broadly inhibit Ets family transcription factor function (Hever et al., 2003). As Ets2 is regulated by oncogene activation of MAP kinase pathways, does it also function within tumor cells or only in stromal support? As previous studies only decreased Ets2 activity, we tested whether mammary tumors would develop in adult mice nearly devoid of Ets2 function. We have addressed these questions using a conditional Ets2 allele (Ets2^{flox}) that is inactivated by Cre recombinase (Cre). We show that Ets2 deficiency delays mammary tumor development, but tumors can form in the near absence of Ets2. We also show that mammary tumor development is not influenced by Ets2 deficiency in mammary epithelial cells. Thus, Ets2 supports mammary tumors exclusively through a stromal mechanism.

Results

Recombination of the Ets2^{flox} allele

To circumvent early embryonic lethality by inactivation of Ets2 (Yamamoto et al., 1998), a conditionally targeted Ets2 allele (Ets2^{flox}) was generated (Wen, Cecena and Oshima, in preparation). Ets2^{flox} (Fig. 1A) contains loxP sequences flanking exons 9 and 10, encoding the C-terminal portion of the Ets2 DNA binding domain. Cre converts Ets2^{flox} to Ets2^{db2} resulting in an inactive Ets2 protein lacking a functional DNA binding domain. Ets2^{flox/flox} mice were normal, while developmental studies confirmed the lethality of Ets2^{db2/db2} embryos and their similarity to the previously described Ets2^{db1/db1} embryos (Wen, Cecena and Oshima, in preparation).

To determine the degree to which complete loss of Ets2 function would impair mammary tumor formation, we used the Mox2-Cre mouse (MORE) to express Cre in an epiblast-restricted manner (Tallquist & Soriano, 2000). MORE mice were shown to express Cre in all primitive ectoderm derivatives by E7, but not in extraembryonic endoderm or trophoderm derivatives. This allows targeted recombination of loxP flanked alleles in all adult tissues of endodermal, ectodermal, and mesodermal lineages.

When Ets2^{flox/+};MORE mice were crossed to Ets2^{flox/flox} mice, the resulting Ets2^{flox/db2};MORE mice were viable. Analysis of the recombination status of the Ets2^{flox} allele by PCR showed less than 10-15% of the functional Ets2^{flox} allele remained in adult tissues (Fig. 1B). Mammary tissue was nearly completely recombined. This contrasted with the limited recombination of Ets2^{flox} in mammary tissues of Ets2^{flox/flox};MMTV-Cre7 mice (Fig. 1B) due to the epithelial specific Cre expression from MMTV-Cre7 mice (Andrechek et al., 2000) and the mixture of cell types found in the mammary gland.

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Restriction of mammary tumors by limited Ets2

MMTV mediated expression of the PyMT oncogene within the mammary epithelium has been used to generate mouse models of breast cancer (Guy et al., 1992). PyMT expression induced hyperplastic mammary epithelium capable of forming multifocal tumors with metastatic potential. Transgenic mice that express a mutant form of PyMT (PyMT^{Y315/322F}) limited the ability of PyMT to signal through the PI3K pathway and resulted in an extended hyperplastic phase (Webster et al., 1998). While delayed with respect to the original PyMT transgenic tumors, PyMT^{Y315/322F} mice develop focal mammary tumors with 100% penetrance.

Ets2^{flox/+};MORE;PyMT^{Y315/322F} mice developed tumors with a median onset of 137 days (Fig. 1C). By contrast, Ets2^{flox/db2};MORE;PyMT^{Y315/322F} mice developed tumors with median onset of 174 days, a significant delay of 37 days (Log-rank, p=0.007). However, once tumors appeared, the rate of tumor growth was not detectably altered by Ets2 deficiency (Fig. 1D). These results are similar to the effect of a single targeted Ets2 allele on MMTV-PyMT tumor appearance and growth (Neznanov et al., 1999).

Cre mediated Ets2^{flox} recombination was assessed by semi-quantitative PCR from individual tumors of Ets2^{flox/+};MORE;PyMT^{Y315/322F} and Ets2^{flox/db2};MORE;PyMT^{Y315/322F} mice (Fig. 2A). Most tumors from mice of both groups had significant levels of Ets2 recombination (Fig. 2A, lanes 1-3, 6, 7, 9). Unexpectedly, a number of tumors had non-recombined Ets2^{flox} alleles (Fig. 2A, lanes 4, 5 and 8). Figure 2C shows the degree of Ets2 gene inactivation of multiple single tumors from individual Ets2^{flox/+};MORE;PyMT^{Y315/322F} mice. About 75% or more of the Ets2^{flox} alleles of multiple tumors from ten Ets2^{flox/+} mice were recombined. However, three mice had multiple tumors with little recombination (Fig. 2C, mice 175, 182, 184) and five had mixtures of tumors with substantial or little recombination.

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The recombination of individual tumors from $Ets2^{flox/db2};MORE;PyMT^{Y315/322F}$ mice (Fig. 2D) also varied. Although the maximum residual Ets2 activity of any single tumor was still equal to the minimum Ets2 activity of $Ets2^{flox/+};MORE;PyMT^{Y315/322F}$ tumors (Fig. 2C), the incomplete recombination in some tumors was not sufficient to mask the role of Ets2 in support of $PyMT^{Y315/322F}$ tumors. These results indicate that Ets2 moderates $PyMT^{Y315/322F}$ tumor appearance but is not strictly required because multiple tumors arose in mice with nearly completely inactivated Ets2.

Equivalent levels of $PyMT^{Y315/322F}$ mRNA were found in tumors with different Ets2 activities (Fig. 2B). Thus the Ets2 dependent restriction of $PyMT^{Y315/322F}$ tumors is not due to differential oncogene expression. As expected, Ets2 mRNA encoding the loxP flanked region of exons 9 and 10 was 16-20 fold less in tumors of the $Ets2^{db2/db2}$ genotype than in tumors of the other 3 possible Ets2 genotype combinations (Fig. 2B). This confirmed the expected molecular consequence of the $Ets2^{db2}$ deletion in tumors.

Ets2 deficiency alters early $PyMT^{Y315/322F}$ hyperplastic growth

The effect of Ets2 deficiency on tumor appearance and the lack of effect on tumor growth are consistent with a function for Ets2 during the early progression to frank tumor formation. The $PyMT^{Y315/322F}$ mouse model causes a uniform and extended hyperplastic phase preceding tumor development that interferes with the glandular epithelium filling of the fat pad (Fig. 3C and E). This contrasts with both normal development (Fig. 3A) and the more rapid progression of tumors in the original wt $PyMT$ mammary tumor model (Fig. 3B) (Guy et al., 1992). The epithelial hyperplasia of $Ets2^{flox/db2};MORE;PyMT^{Y315/322F}$ mice filled less of the mammary fat pad than $Ets2^{flox/+};MORE;PyMT^{Y315/322F}$ mice (Fig. 3C-F). The average distance from the nipple area to

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the edge of the hyperplastic growth as a function of fat pad length was 30% less in the more severely Ets2 deficient animals (Fig. 3G). This restriction was specific for PyMT^{Y315/322F} mammary glands as the mammary epithelial tree of virgin Ets2^{fl^{ox}/db2};MORE females at 4 and 8 weeks was normal (data not shown).

Mammary epithelial Ets2 does not regulate tumor development

We used MMTV-Cre7 transgenic mice (Andrechek et al., 2000) to target the Ets2^{fl^{ox}} allele within the mammary epithelia and tumors of PyMT^{Y315/322F} mice. Tumor formation in female Ets2^{fl^{ox}/+};MMTV-Cre7;PyMT^{Y315/322F} and Ets2^{fl^{ox}/fl^{ox}};MMTV-Cre7;PyMT^{Y315/322F} mice was compared. No significant difference in tumor onset was found and median tumor onset of both groups of mice was 132 days (Fig. 4A) (Log-rank, p=0.62). This contrasts with the delay of tumor development between Ets2^{fl^{ox}/db2};MORE;PyMT^{Y315/322F} mice and Ets2^{fl^{ox}/+};MORE;PyMT^{Y315/322F} mice (Fig. 1C).

Epithelial Ets2 does not provide a selective advantage to mammary tumors

While the MMTV-Cre7 transgene is expected to express Cre in most luminal epithelial cells of the mammary gland, tumors might arise preferentially in cells without recombined Ets2 alleles. The genotypes of individual tumors were determined by PCR and revealed a significant degree of variation in Cre activity (Fig. 4B, C and E). Figure 4B shows the Ets2 genotypes of tumors derived from MMTV-Cre7;PyMT^{Y315/322F} mice of Ets2^{fl^{ox}/+} or Ets2^{fl^{ox}/fl^{ox}} genotype. In lanes 1 and 4, although the mouse is positive for the MMTV-Cre7 transgene, no detectable recombination has occurred in the tumors, while the tumors of lanes 2 and 5 show significant but

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incomplete level of Ets2^{flox} recombination. Lanes 3 and 6 show examples of near complete Ets2^{flox} recombination by the MMTV-Cre7 transgene.

We determined the genotype of tumor biopsies from multiple individual tumors of each mouse (Fig. 4C and E). In both the Ets2^{flox/+} (Fig. 4C) and Ets2^{flox/flox} (Fig. 4E) groups of mice there are four and six mice, respectively, with no detectable recombination in multiple tumors. Retrospectively, we found that MMTV-Cre7 parents of mice with no recombined tumors did not generate any mice with substantial recombination, suggesting the MMTV-Cre7 transgene was silent in the progeny of those particular parents. However, the majority of mice from each group developed multiple tumors with Ets2^{flox} alleles recombined at levels greater than 50%.

This mosaic recombination of Ets2^{flox} allowed us to determine if there was selective pressure on tumor formation with a functional Ets2^{flox} allele. Figure 4D summarizes the total number of tumors genotyped with greater than 50% recombination of the Ets2^{flox} allele. 48% (40/84) of Ets2^{flox/+};MMTV-Cre7;PyMT^{Y315/322F} mice had recombined the Ets2^{flox} allele in tumors. Similarly, 42% (36/85) of Ets2^{flox/flox};MMTV-Cre7;PyMT^{Y315/322F} mice had recombined Ets2^{flox} alleles (Chi-square, p=0.33). Thus, inactivation of Ets2^{flox} alleles occurred with the same frequency in both groups. If tumors from mice with no apparent MMTV-Cre7 function are excluded from the analysis, the frequency of Ets2^{flox} recombination is still equal in the two groups (Chi-square, p=0.36). These results indicate that recombination of both Ets2^{flox} alleles occurs as frequently in homozygous tumors as in heterozygous tumors. Thus, there is no apparent selection for Ets2 in mammary tumors in this model.

Discussion

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Nearly complete loss of Ets2 function in the adult mouse delays PyMT^{Y315/322F} induced tumor formation from a median time of 137 days to 174 days, but subsequent tumor growth was indistinguishable between mice of either Ets2 genotype. These data suggest that Ets2 may regulate tumor development at an early initiation phase. The Ets2 dependent differences in the morphology of PyMT^{Y315/322F} hyperplastic growth support this view. The remarkable aspect of this difference is that it is likely due to stromal cell effects on hyperplastic epithelial cell organization because inactivation of Ets2 within the mammary epithelium by MMTV-Cre7 did not result in such differences in hyperplastic outgrowth (data not shown). The loss of Ets2 within the mammary epithelium does not effect time of tumor onset, and there was no detectable genetic selection for tumors with an active Ets2^{flox} allele. Thus, in spite of the known activation of Ets2 downstream of growth factor stimulated MAP kinases, Ets2 does not play a key role in PyMT^{Y315/322F} tumor cells. The strictly stromal role complements previous studies showing that stromal derived Ets2 function can regulate short-term transplanted tumor growth (Man et al., 2003).

What is the key Ets2 dependent stromal cell type(s) that regulates mammary tumor progression? Endothelial cells and inflammatory cells are two distinct cell types that have a direct influence on tumor progression. Ets2 is integral for the inflammatory response of macrophages by regulating both matrix metalloproteases (Man et al., 2003; Yamamoto et al., 1998) and cytokines (Wei et al., 2004), suggesting the possibility of an Ets2 regulation of mammary tumor formation via an inflammatory cell function. While Ets2 does not appear limiting for the production of VEGF in mammary tumors (Oshima et al., 2004), many studies have implicated Ets1 function in endothelial cells (Lelievre et al., 2001; Sato, 2001; Wernert et al., 1992). While Ets2 deficiency alone does not result in differences in CD31 positive tumor

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vasculature (Man et al., 2003) (Oshima et al., 2004), Ets2 deficiency may impact the function or maturation of tumor vasculature as suggested by Factor VIII staining of transplanted tumors (Man et al., 2003).

Both Cre transgenes used in this study showed evidence of mosaic expression or even silent alleles. However, the frequency of recombination was still high enough to reveal the Ets2 dependent support of PyMT^{Y315/322F} tumors. The mosaic nature of Cre expression in MORE mice has been noted previously (Hayashi et al., 2002). The apparent mosaic Cre expression from MMTV-Cre7 was reflected in two ways. First, both recombined and non-recombined tumors were identified in the same host. Secondly, multiple animals had no recombined Ets2^{fllox} allele in any tumor. However, we were able to take advantage of the mosaic MMTV-Cre7 expression and found there was no genetic selection for a functional Ets2 allele in mammary tumors.

The results of this study show that Ets2 deficiency limits the development of PyMT^{Y315/322F} initiated tumors exclusively through the stroma. Decreasing Ets2 activity slows the appearance of mammary tumors from a hyperplastic phase. It is possible that variation in the expression or activity of Ets2 within the human population may contribute to the variable progression of human breast cancers.

Materials and Methods

Mice

Generation of Ets2^{fllox} mice will be described elsewhere⁶. Ets2^{fllox} was backcrossed into the FVB/N genetic background 4 generations prior to this study. MORE mice on the C57BL/6J genetic background were purchased from the Jackson Laboratory (stock No. 003755). PyMT^{Y315/322F} (Webster et al., 1998) and MMTV-Cre7 (Andrechek et al., 2000) lines were

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maintained in FVB/N genetic background. Trigenic mice were derived from breeding Ets2^{flox/flox};PyMT^{Y315/322F} males with Ets2^{flox/+};MORE females or from breeding Ets2^{flox/+};PyMT^{Y315/322F} males with Ets2^{flox/flox};MMTV-Cre7 females. Tumor study mice possessed a majority FVB/N genetic background with the remaining from C57BL/6J and 129.

Genotyping

PCR was performed using tail biopsy or organ DNA. Mice were genotyped with the following primers: MORE and MMTV-Cre7, CTGGCATTCTGGGGATTGC and ACGGAAATCCATCGCTCGAC; PyMT^{Y315/322F}, GCTGACAAAGAAAGGCTGC and ATCCAGGTCCAGCCAGTCTA; Ets2^{flox}, GACCCACTTGCTCCAAAGAC (primer B) and GCTTCCCAGAGACTCTTCCC (primer C). Inclusion of primer A (GCCACAGCAAACCTCTTTCT) with the Ets2^{flox} genotyping primers allowed semi-quantitative analysis of the conversion of Ets2^{flox} to Ets2^{db2}. The percent Ets2^{db2} allele was estimated from standard curves containing DNA mixtures of Ets2^{flox} or Ets2^{db2} alleles.

Tumor studies

Animals were inspected for palpable tumors weekly in blinded manner. Once detected, the length and width of tumors were measured with calipers every 2-3 days. Tumor volume was estimated (length x width²/2). Small tumor biopsies were dissected and used for genotype analysis by PCR. Unaffected or mildly affected mammary glands were mounted on glass slides, fixed in Carnoy's fixative, and stained with carmine alum. Photographic documentation was performed with a Nikon Coolpix 990 camera and Adobe Photoshop software.

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RNA analysis

RNA was prepared with Trizol (Invitrogen). cDNA was prepared from 4 ug of total RNA using oligo(dT) priming and Superscript II reverse transcriptase (Invitrogen), and cDNA levels were measured by real-time PCR in a LightCycler instrument using the SYBR Green I PCR kit and LightCycler software (Roche). Gene expression was normalized to cyclophilin A (Cph) levels. The two primers for each target were as follows: Cph, AGACCAGCAAGAAGATCACC and GGAAAATATGGAACCCAAAG; Ets2, CCTGTCAATCTTTCATCAGC and AGTTCTGCAGGTCACATACG (flox region); PyMT^{Y315/322F}, CAGCAGGCATATAAGCAGCA and CACCTGGCATCACATTTGTC.

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References

- Andrechek ER, Hardy WR, Siegel PM, Rudnicki MA, Cardiff RD and Muller WJ. (2000). *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 3444-3449.
- Guy CT, Cardiff RD and Muller WJ. (1992). *Mol. Cell. Biol.*, **12**, 954-961.
- Hayashi S, Lewis P, Pevny L and McMahon AP. (2002). *Mech. Dev.*, **119 Suppl 1**, S97-S101.
- Hever A, Oshima RG and Hauser CA. (2003). *Exp. Cell Res.*, **290**, 132-144.
- Lelievre E, Lionneton F, Soncin F and Vandenbunder B. (2001). *Int. J. Biochem. Cell Biol.*, **33**, 391-407.
- Man AK, Young LJT, Tynan J, Lesperance J, Egeblad M, Hauser CA, Muller WJ, Cardiff RD and Oshima RG. (2003). *Mol. Cell Biol.*, **23**, 8614-8625.
- Neznanov N, Man AK, Yamamoto H, Hauser CA, Cardiff RD and Oshima RG. (1999). *Cancer Res.*, **59**, 4242-4246.
- Oshima RG, Lesperance J, Munoz V, Hebbard L, Ranscht B, Sharan N, Muller WJ, Hauser CA and Cardiff RD. (2004). *Cancer Res.*, **64**, 169-179.
- Sapi E, Flick MB, Rodov S and Kacinski BM. (1998). *Cancer Res.*, **58**, 1027-1033.
- Sato Y. (2001). *Cell Struct. Funct.*, **26**, 19-24.
- Tallquist MD and Soriano P. (2000). *Genesis*, **26**, 113-115.
- Webster MA, Hutchinson JN, Rauh MJ, Muthuswamy SK, Anton M, Tortorice CG, Cardiff RD, Graham FL, Hassell JA and Muller WJ. (1998). *Mol. Cell. Biol.*, **18**, 2344-2359.
- Wei G, Guo J, Doseff AI, Kusewitt DF, Man AK, Oshima RG and Ostrowski MC. (2004). *J. Immunol.*, **173**, 137-139.
- Wernert N, Raes M-B, Lassalle P, Dehouck MP, Gosselin B, Vandenbunder B and Stehelin D. (1992). *Am. J. Pathol.*, **140**, 119-127.

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Yamamoto H, Flannery ML, Kupriyanov S, Pearce J, McKercher SR, Henkel GW, Maki RA,
Werb Z and Oshima RG. (1998). *Genes Dev.*, **12**, 1315-1326.

Figure Legends

Figure 1. Ets2^{db2} delays PyMT^{Y315/322F} induced tumor appearance. A, Schematic of the Ets2^{fllox} allele, and its conversion to Ets2^{db2} in the presence of Cre. Exons are indicated by solid blocks with exon number noted above. Exons 9 and 10, encoding the C-terminal portions of the Ets2 DNA-binding domain, are flanked by LoxP sequences (►). Small arrows indicate PCR primer sites used to distinguish wild-type Ets2 lacking loxP sequences (176 bp product from primers B and C), Ets2^{fllox} (217 bp product from primers B and C) and Ets2^{db2} (280 bp product from primers A and C). B, PCR genotyping of Ets2 in the indicated tissues from Ets2^{fllox/db2};MORE mice (upper panel) shows recombination is highly efficient. Recombination of Ets2^{fllox} in Ets2^{fllox/fllox};MMTV-Cre7 mice (bottom panel) is primarily restricted to the mammary gland (mam. gl.). C, Tumor appearance was determined by palpation in Ets2^{fllox/+};MORE and Ets2^{fllox/db2};MORE mice expressing the PyMT^{Y315/322F} transgene. Median tumor onset was significantly delayed, from 137 to 174 days, in Ets2^{fllox/db2};MORE mice (Log-rank, $p = 0.007$). D, Volume of MORE;PyMT^{Y315/322F} tumors is shown as a function of elapsed time after tumor detection. Values represent the average and standard error of the largest tumors of 12 and 22 mice of either Ets2^{fllox/+} or Ets2^{fllox/db2} genotypes, respectively.

Figure 2. Characterization of mammary tumors in MORE mice. A, PCR genotyping of PyMT^{Y315/322F} tumors from MORE;Ets2^{fllox/+} and MORE;Ets2^{fllox/db2} mice shows the degree of variation seen in Ets2 recombination among different tumors. +, wild-type Ets2; f, Ets2^{fllox}; d, Ets2^{db2}. B, Real time RT-PCR of Ets2 and PyMT^{Y315/322F} mRNA expression in tumors of the indicated Ets2 genotype from MORE;PyMT^{Y315/322F} mice of Ets2^{fllox/+} or Ets2^{fllox/db2} genotype. C and D, Scatter plots show the percentage of Ets2^{db2} allele in individual MORE;PyMT^{Y315/322F}

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tumors from either $Ets2^{flox/+}$ or $Ets2^{flox/db2}$ mice. Mouse number is shown at the bottom. Each point represents an individual tumor.

Figure 3. $Ets2^{db2}$ decreases extent of epithelial filling of fat pads in $PyMT^{Y315/322F}$ mice. Wholemounts of the number four inguinal mammary glands from mice of the indicated age and genotype are shown. A, Wild-type FVB/N mouse at 114 days with typical arboreal epithelium. B, MMTV-PyMT at 49 days. Note the appearance of tumor foci and morphologically normal epithelium. C and E, $Ets2^{flox/+};MORE;PyMT^{Y315/322F}$ mammary glands at 98 days. D and F, $Ets2^{flox/db2};MORE;PyMT^{Y315/322F}$ mammary glands at 99 days. G, As measured from the nipple area, the average length of the epithelium as a function of fat pad length is shown for $MORE;PyMT^{Y315/322F}$ mice of either $Ets2^{flox/+}$ or $Ets2^{flox/db2}$ genotype. Error bars represent standard deviation. N=8 for both groups.

Figure 4. Targeting of $Ets2^{flox}$ by MMTV-Cre7 does not delay spontaneous tumor appearance. A, Tumor appearance was determined by palpation in MMTV-Cre; $PyMT^{Y315/322F}$ mice of either $Ets2^{flox/+}$ or $Ets2^{flox/flox}$ genotype. Median tumor appearance in both groups was 132 days (Log-rank, $p = 0.67$). B, PCR genotyping of MMTV-Cre7; $PyMT^{Y315/322F}$ tumors from $Ets2^{flox/+}$ and $Ets2^{flox/flox}$ mice shows the degree of variation seen in $Ets2$ recombination between different tumors. +, wild-type $Ets2$; f, $Ets2^{flox}$; d, $Ets2^{db2}$. C and E, Scatter plots of percent $Ets2^{db2}$ present in individual tumors as determined by PCR from MMTV-Cre7; $PyMT^{Y315/322F}$ mice of $Ets2^{flox/+}$ (panel C) or $Ets2^{flox/flox}$ (panel E) genotype. D, Fraction of recombined tumors as a function of all tumors genotyped (left side of panel) or after exclusion of tumors from mice with a silent MMTV-Cre7 allele (right side of panel) in $Ets2^{flox/+}$ and $Ets2^{flox/flox}$ animals.

Figure 1

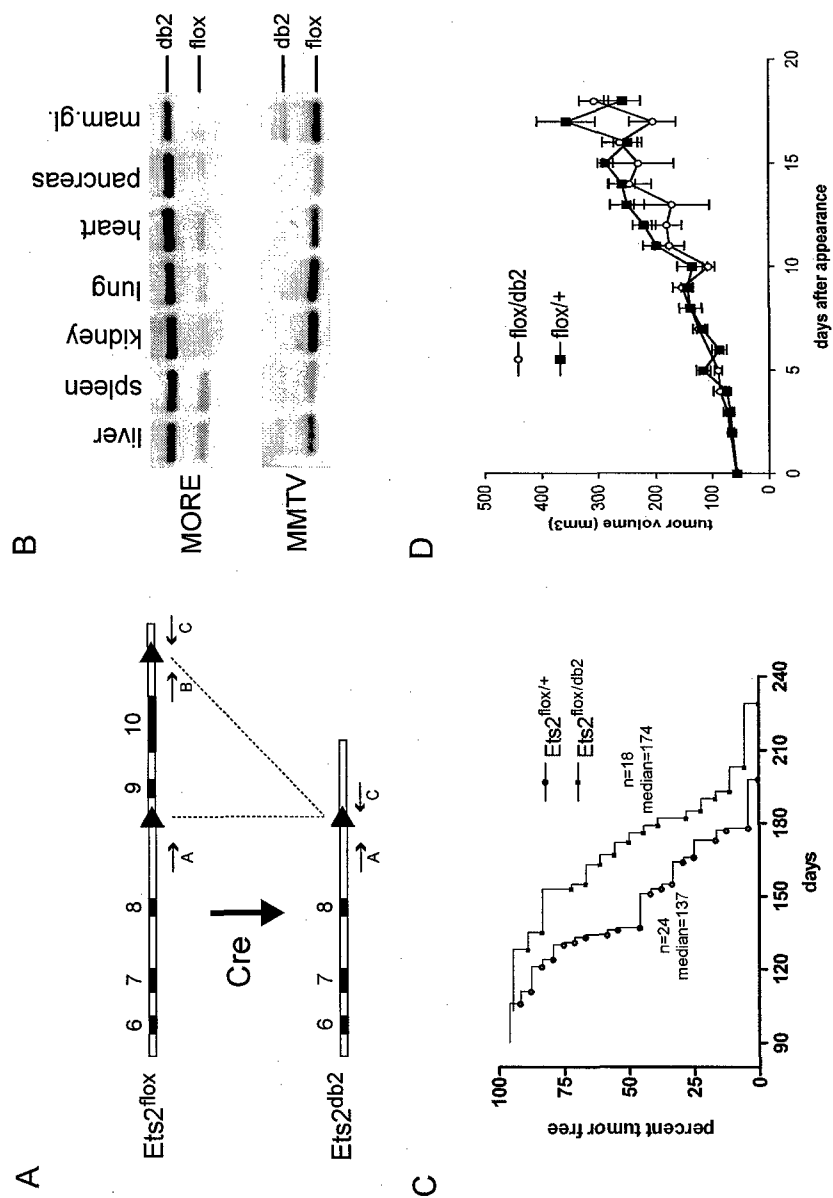


Figure 2

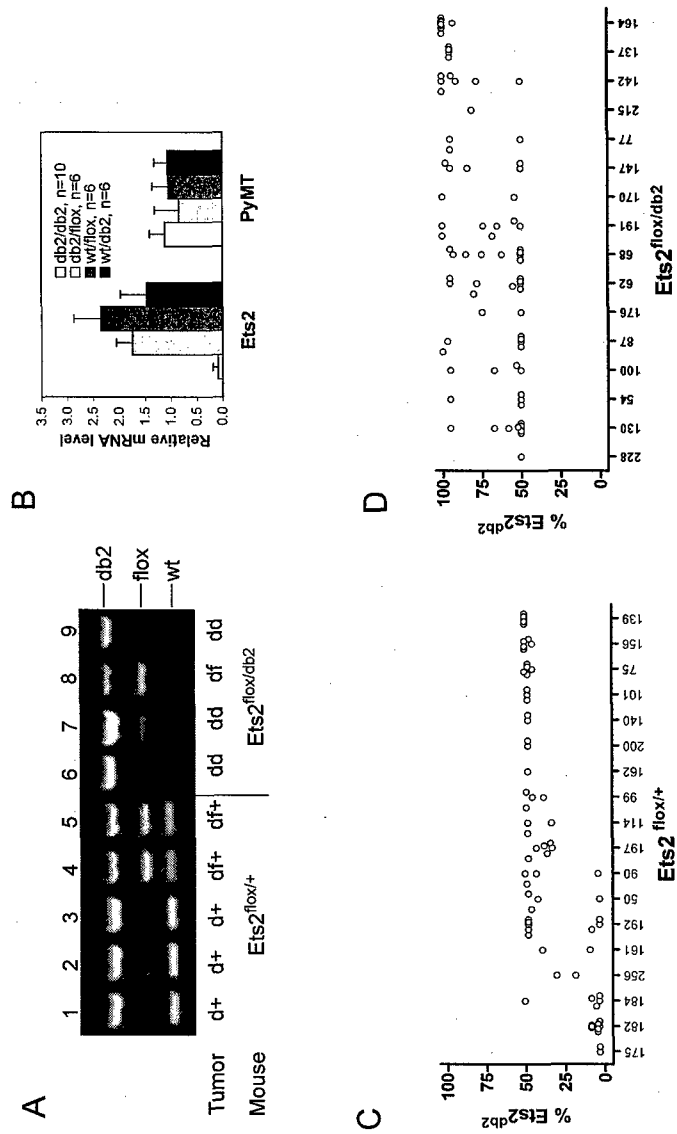


Figure 3

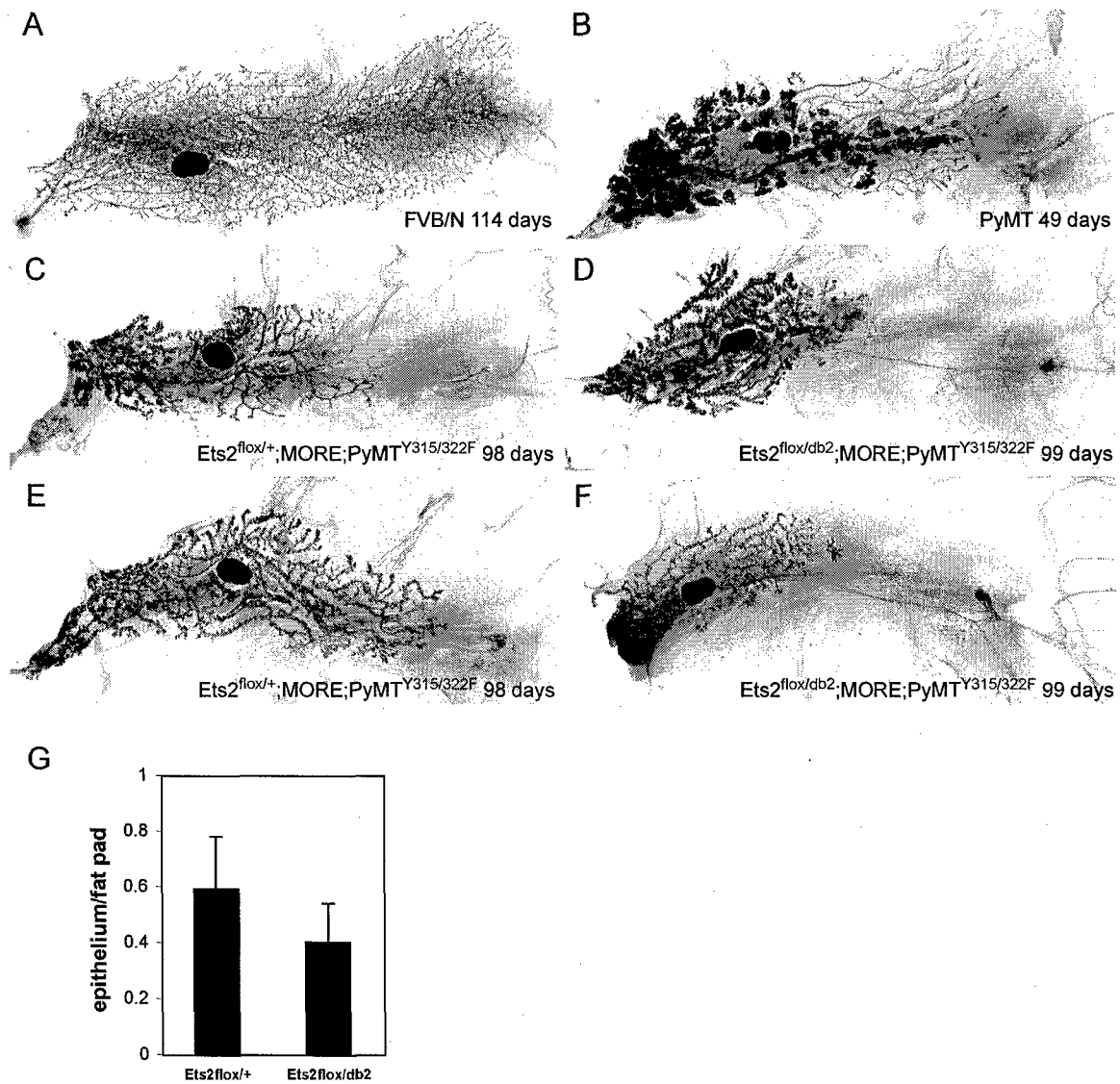


Figure 4

