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14. ABSTRACT Recent scientific advances have revealed that a malignant tumor can be viewed as an organ consisting of different types of interacting cells. Different tumor cell types play different roles in the growth and development of the tumor and thus in the end, all cells within the tumor may help pave the way for further tumor growth in a patient with cancer. Current work in the field indicates that fibroblasts surrounding breast cancers are particularly important for cancer progression, but no one knows why. Our experimental approach to this problem is a direct one; we target the mutation of genes in stromal fibroblasts surrounding the tumor in order to learn whether these genes are important for cancer progression. Using this approach we have shown that the Pten gene in fibroblasts is a major gene involved in suppressing epithelial breast cancers. In order to understand how <i>Pten</i> works in fibroblasts we will measure the immediate and long-term consequences of <i>Pten</i> mutation on the biology of all the cells surrounding the tumor, including the tumor cells themselves, as well as the matrix that holds these cells together. Because the entire system is so complex, we plan to study how the <i>Pten</i> gene behaves as a tumor suppressor by developing extremely detailed three-dimensional images of tumors where each image is annotated with detailed cancer related molecular information. This will allow us to use bioinformatics (computer assisted methods) to understand the molecular basis for how fibroblasts promote breast cancer. This information will lead to the design of novel therapeutic strategies that target fibroblasts and that could be used in combination with current therapies that target epithelial cells, to stop tumor growth and prevent recurrence or metastasis.					
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INTRODUCTION:

Emerging evidence points to a particularly important role of **fibroblasts** within the tumor stroma in promoting phenotypic changes that allow the extracellular matrix to evolve and contribute to the *invasiveness of epithelial tumor cells*. However, how stromal fibroblasts that promote cancer progression are poorly understood and are only beginning to emerge. The Leone laboratory has developed a system to conditionally manipulate the mouse genome in stromal fibroblasts of the mammary gland (*FSP-cre*). Using this genetic system we present preliminary data suggesting that *Pten* function in mammary stromal fibroblasts plays a critical role in the progression of *ErbB2*-initiated mammary epithelial tumors. The task at hand is to now genetically and biochemically identify the key '*stroma-specific*' tumor suppressor activities of *Pten*, and to then relate these stroma-specific functions to the structural and molecular events taking place during tumor progression.

The immense genetic and cellular diversity of the tumor microenvironment, however, has made the problem of cancer progression difficult to address by traditional experimental approaches. The Leone and Saltz groups have therefore joined expertise to develop novel *genetic* and *high-resolution 3D bioinformatics models* that integrate *spatial* and *molecular* information relating to the genetics and biochemical properties of the tumor microenvironment. These models will provide an effective platform for identifying the relationships between the different cell compartments of the tumor microenvironment that are critical for cancer progression. The proposed studies will fall into two major efforts, each divided into two phases. The first major effort involves the genetic analysis of *Pten* in mouse tumor models. The progress report outlined below describes the progress made in this phase of the study. The work is now being prepared for publication.

BODY:

Coordinated signaling between different cell types of the '*normal stroma*' is required during embryonic and adult development (Wiseman BS, Werb Z, 2002.). While cellular and ECM activities of the stroma are maintained in balance throughout development, they can be appropriately activated in response to extreme but normal physiological cues, such as wounding, inflammation or pregnancy (Schedin P, 2006). The stroma can also be inappropriately activated, such as in cancer (Nelson CM, Bissell MJ, 2006). Fibroblasts are a principal constituent of the stroma responsible for the synthesis of growth and survival factors, chemokines, structural components of the ECM and enzymes that control its turnover. In breast tumors, stromal fibroblasts are believed to adapt and continuously co-evolve along with tumor epithelial cells (Littlepage et al., 2005). Fibroblasts are implicated in fostering transformation and tumor growth by providing factors that induce epithelial cell proliferation, ECM remodeling and blood vessel recruitment (Bhowmick et al., 2004b; Mueller and Fusenig, 2004; Kalluri and Zeisberg, 2006). Despite extensive evidence for a role of the tumor stroma in carcinogenesis, relatively little is known about the signaling pathways involved in the communication between the different cellular compartments of the tumor microenvironment that contribute to the cancer phenotype.

Phosphatase and tensin homolog (*PTEN*) is a tumor suppressor with lipid and protein phosphatase activity (Myers et al., 1998) that impacts several signaling pathways, including phosphoinositide 3-kinase (PI3K), and Ras-MAPK-Erk1/2 signaling pathways. *Pten* inactivation in mice and humans leads to a disruption in cell polarity, cell architecture, and chromosomal integrity as well as in the promotion of cell cycle progression, cell growth and stem cell self-renewal (Di Cristofano and Pandolfi, 2000). Not surprisingly, its somatic or germ-line inactivation contributes to the genesis of many tumor types primarily epithelial in origin. While tremendous progress in understanding *PTEN* function in tumor cells has been made since its discovery over a decade ago, relatively little is known about its potential role in the tumor stroma. Patients with Cowden syndrome have germline mutations in *PTEN*, suggesting that the higher risk for developing breast cancer in these patients could be due to *PTEN* activity in either epithelial or stromal compartments. Here, we have generated a mesenchymal-specific *Fsp-cre* transgene and conditional alleles of *Pten* (*Pten^{loxP}*) in mice to ablate its function in mammary stromal fibroblasts *in vivo* and rigorously evaluate its role in the tumor microenvironment. We show that *Pten* ablation in mammary stromal fibroblasts results in the induction and activation of Ets2-P (T72), massive remodeling of the ECM, recruitment of innate immune cells, and enhanced progression and malignancy of mammary tumors of epithelial origin. These findings expand *Pten*'s repertoire as a tumor suppressor by identifying the fibroblast as a key site from which it exerts its powerful tumor suppressive influence on adjacent epithelium.

For well over a century, the pathology of cancer suggested that malignant tumors consist of a complex cellular system dependent on reciprocal signaling between tumor cells and the adjacent stroma. The signaling pathways involved in the communication between the various cell types in the tumor remain virtually unknown. We recently developed a mesenchymal-specific *cre* mouse and used it here to target the inactivation of *Pten* in mammary stromal fibroblasts of the mammary gland and examine, for the first time, its role in the tumor microenvironment *in vivo*. This work identified the *Pten*-Ets2 pathway as a key regulatory axis in stromal fibroblasts that profoundly attenuates malignant characteristics of the tumor microenvironment and suppresses mammary epithelial tumors.

The tumor suppressor functions of *Pten* have been extensively studied in the tumor cell, but whether it also plays a role in other cell compartments of the tumor was unknown. We show direct *in vivo* evidence for a critical tumor suppressor role of *Pten* in the tumor microenvironment. Genetic ablation of *Pten* in stromal fibroblasts of the mammary gland resulted in a dramatic perturbation of the tumor microenvironment by increasing ECM deposition and a host of activities imbedded in it that together favor the initiation and progression of mammary epithelial tumors. This novel function of *Pten* in maintaining homeostasis within the mammary microenvironment may also be relevant in the suppression of epithelial tumors of other organs. Such a role for *Pten* may extend beyond cancer, to conditions where the microenvironment is speculated to profoundly impact disease manifestation including in autoimmune syndromes, lung fibrosis and neurodegeneration. Interestingly, the stromal *Pten* expression signature identified here includes genes that have been causally linked with ECM deposition and inflammation in rheumatoid arthritis, lung fibrosis and neurodegeneration.

KEY RESEARCH ACCOMPLISHMENTS:

TASK 1 (month 3- 18): Paper in re-submission to *Nature* (Trimboli et al.) attached.

TASK 2 (months 3-18): Work dealing with *Pten* in progress. Paper in progress (Hui Wang et al.). Work behind approximately 8 months.

TASK 3 (month 12-18): See *Nature* (Trimboli et al.) article attached. This task is addressed in this article.

TASK 4 (month 18-24):Partly completed. Also addressed in *Nature* (Trimboli et al.) article attached. The component dealing with regional areas of stroma (macrophages) is ongoing.

TASKS 5-7 (month 1- 24): Work on these tasks is ongoing. The no cost extension is requested to complete this important component of this project. The “segmentation” component (see figure 1 below) has encountered significant technical hurdles dealing with image segmentation, but even so, we feel that these can be overcome in the next 6 months.

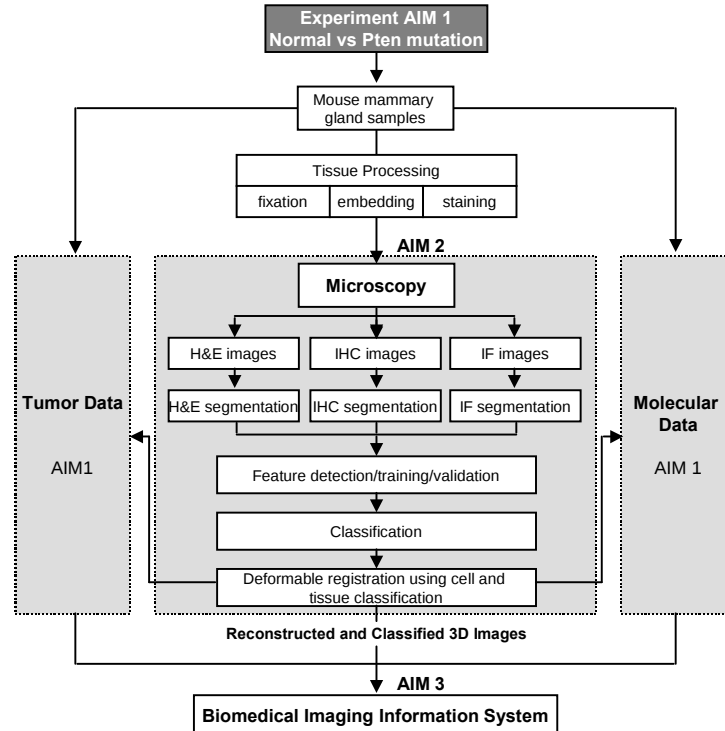


Figure 1: Scientific Processes Involved in Creation of BIIS

REPORTABLE OUTCOMES:

1. Manuscripts in preparation
 - A. Trimboli et al. *Pten* in Stromal Fibroblasts Suppresses Mammary Epithelial Tumors.
 - B. Cantemir et al. Tumor Fibroblasts and Ets2 Regulate Angiogenesis in Breast Cancer.
2. Submitted an NCI grant application (U01) that is related to this work.

CONCLUSIONS:

The mechanism by which Pten in the stroma regulates the expression of such an extensive set of genes likely involves complex transcriptional networks. The importance of the Pten-Ets2 axis in stromal fibroblasts is consistent with previous work from Oshima and colleagues that showed a critical cell non-autonomous role for Ets2 in mammary tumor growth (Tynan et al. 2005). Moreover, analysis of tumor-stroma gene expression signatures identified Ets2 activation as a key event associated with breast cancer patients having the worst prognosis (Park et al., 2007). In summary, this study offers a molecular basis for how altered signaling from the tumor stroma contributes to the most malignant characteristics of the breast cancer phenotype.

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APPENDIX:

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
Leone, Gustavo W.		Associate Professor	
eRA COMMONS USER NAME			
gleone			
EDUCATION/TRAINING (<i>Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.</i>)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Calgary, Calgary, Canada	B.Sc.	1988	Biochemistry
University of Calgary, Calgary, Canada	Ph.D	1994	Molecular Virology
Duke University Medical Center, Durham, N.C	Postdoc	1994-1998	Genetics
Ohio State University, Columbus, OH	Assoc Prof	1999-present	Cancer Genetics

A. Positions and Honors.

Academic Appointments

- 1987 – 1988 Undergraduate Research Assistant, Department of Microbiology and Infectious Diseases, University of Calgary (Supervisor: Dr. Patrick W.K. Lee)
- 1988 – 1994 Graduate Student, Department of Microbiology and Infectious Diseases, University of Calgary (Supervisor: Dr. Patrick W.K. Lee)
- 1990 – 1994 Graduate student assistant, University of Calgary, Project Lab (intensive laboratory training for undergraduates in virology and molecular biology)
- 1994 – 1998 Postdoctoral Fellow, Department of Genetics, Duke University Medical Center (HHMI), Durham, North Carolina. Role of E2F in Cell Cycle Control (Mentor: Dr. Joseph R. Nevins)
- 1999–present Associate Professor, Human Cancer Genetics Program, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio.

Honors and Awards

- 1989 – 1994 Alberta Heritage Foundation for Medical Research Studentship
- 1994 – 1997 Medical Research Council of Canada Fellowship and Alberta Heritage Foundation for Medical Research Fellowship
- 1997 – 1999 Medical Research Council of Canada Centennial Fellowship
- 1999 The Robert M. and Barbara R. Bell Basic Science of Cancer Award
- 1999 – 2001 Scholar Award, The 1999 V - Foundation
- 2001 – 2005 Scholar Award, PEW Charitable Trust
- 2004 – 2009 Scholar Award, Leukemia and Lymphoma Society

B. Selected peer-reviewed publications (Selected from a total of 70)

1. **Leone, G.**, Maybaum, L. And Lee, P.W.K. (1992). The Reovirus cell attachment protein possesses two independently active trimerization domains: Basis of dominant negative effects. ***Cell*** 71:479-488.
2. DeGregori, J.*, **Leone, G.***, Ohtani, K., Miron, A. and Nevins, J.R. (1995) E2F1 accumulation bypasses a G₁ arrest resulting from the inhibition of G₁ cyclin-dependent kinase activity. ***Genes & Development***, 9:2873-2887. *These authors contributed equally to this work.
3. Gilmore, R., Coffey, M., **Leone, G.**, McLure, K., Lee, P.W.K. (1996). Co-translational trimerization of the Reovirus cell attachment protein. ***The EMBO Journal*** 15(11):2651-2658.
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*corresponding author
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PI: Gustavo Leone, PhD
Co-PI: Joel Saltz, PhD
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Title: Mechanisms of MYC-induced Lymphomagenesis
PI: Gustavo Leone, Ph.D.

***Pten* in Stromal Fibroblasts Suppresses Mammary Epithelial Tumors**

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SUMMARY

The tumor stroma is believed to contribute to some of the most malignant characteristics of epithelial tumors. However, signaling between stromal and tumor cells is complex and remains poorly understood. Here we show that the genetic inactivation of *Pten* in stromal fibroblasts of mouse mammary glands accelerated the initiation, progression and malignant transformation of mammary epithelial tumors. This was associated with the massive remodeling of the extra-cellular matrix (ECM), innate immune cell infiltration and increased angiogenesis. Loss of *Pten* in stromal fibroblasts led to increased expression, phosphorylation (T⁷²) and recruitment of Ets2 to target promoters known to be involved in these processes. Remarkably, *Ets2* inactivation in *Pten* stroma-deleted tumors ameliorated disruption of the tumor microenvironment and was sufficient to decrease tumor growth and progression. Global gene expression profiling of mammary stromal cells identified a *Pten*-specific signature that was highly represented in the tumor stroma of breast cancer patients. These findings identify the *Pten*-Ets2 axis as a critical stroma-specific signaling pathway that suppresses mammary epithelial tumors.

Coordinated signaling between different cell types of the ‘*normal stroma*’ is required during embryonic and adult development¹. The stroma can be appropriately activated in response to extreme but normal physiological cues, such as wound, inflammation or pregnancy². The stroma can also be inappropriately activated in cancer^{3, 4}. In breast tumors, stromal fibroblasts are believed to adapt and continuously co-evolve along with tumor epithelial cells in order to foster transformation and tumor growth⁵. Fibroblasts are a principal constituent of the stroma responsible for the synthesis of growth and survival factors, angiogenic and immunological chemokines, and structural components of the ECM as well as enzymes that control its turnover^{6, 7}. Despite extensive evidence for a role of the tumor stroma in carcinogenesis, relatively little is known about the signaling pathways involved in the communication between the different cellular compartments of the microenvironment that contribute to the cancer phenotype.

Alterations in the phosphoinositide 3-kinase (PI3K) pathway are associated with the activation of tumor-associated stroma^{8, 9}. One of the main regulators of PI3K signaling is the phosphatase and tensin homolog (*PTEN*), a tumor suppressor with lipid and protein phosphatase activity^{10, 11}. *PTEN* inactivation disrupts multiple cellular processes associated with cell polarity, cell architecture, chromosomal integrity, cell cycle progression, cell growth and stem cell self-renewal^{12, 13}. Germ-line inactivation of a single allele of *PTEN* in both human and mice contributes to the genesis of a variety of tumor types of epithelial origin¹⁴. While tremendous progress in understanding *PTEN* function in tumor cells has been made since its discovery over a decade ago, relatively little is known about its potential role in the tumor stroma. Here, we show that *Pten* ablation in mammary stromal fibroblasts of mice results in massive remodeling of the

ECM and tumor vasculature, recruitment of innate immune cells, and increased malignancy of mammary epithelial tumors. Gene expression profiling of *Pten*-deleted stromal fibroblasts identified an *Ets2*-specific transcription program associated with many of these aggressive tumor phenotypes. Remarkably, the concomitant inactivation of *Ets2* in the mammary stroma reversed the increased malignancy caused by *Pten* deficiency. These findings expand *Pten*'s repertoire as a tumor suppressor by identifying the fibroblast as a key site from which it exerts its powerful tumor suppressive influence on the adjacent tumor epithelium.

RESULTS

***Pten* in stromal fibroblasts suppresses mammary tumors of epithelial origin**

To rigorously evaluate the role of *Pten* in the tumor microenvironment of breast cancer we generated mice containing a mesenchymal-specific *Fsp-cre* transgene¹⁵ and conditional alleles of *Pten* (*Pten*^{loxP}; Supplementary Fig. 1). Cell type-marker analysis using a β -galactosidase *Rosa26*^{LoxP} reporter allele showed specific *Fsp-cre* expression in stromal fibroblasts surrounding the mammary epithelial ducts, with no expression in cytokerin-positive epithelial cells, F4/80-positive macrophages and CD31-positive endothelial cells (Fig. 1a, Supplementary Fig. 2a, 2b). Southern, PCR and Western blot assays demonstrated efficient cre-mediated deletion of *Pten*^{loxP} in stromal fibroblasts isolated from *Fsp-cre;Pten*^{loxP/loxP} mammary glands (data not shown and Fig. 1b, 1c). Examination of mammary sections by immunohistochemistry (IHC) showed deletion of *Pten*^{loxP} that was confined to stromal fibroblasts, with no collateral deletion in epithelial ducts or the adjacent myoepithelium (Fig. 1d, Supplementary Fig. 3a, 3b). Interestingly,

this resulted in the expansion of the ECM, but did not lead to the transformation of the mammary epithelium (Fig 1d, 2b).

We then examined the role of stromal *Pten* on mammary tumorigenesis using two established mouse models of breast cancer, *MMTV-ErbB2/neu (ErbB2)*¹⁶ and *MMTV-rtTA;teto-MYC (MYC)*^{17, 18}. While not representative of all known genetic abnormalities in breast cancer, these two mouse models have unique molecular, cellular and clinical features that can be used to evaluate different aspects of this heterogeneous disease. To avoid possible confounding effects caused by *Pten* deletion in mesenchymal cells of other organs, mammary glands from *Fsp-cre;Pten^{loxP/loxP}* (n=5), *ErbB2;Pten^{loxP/loxP}* (n=12), *MYC;Pten^{loxP/loxP}* (n=20), *ErbB2;Fsp-cre;Pten^{loxP/loxP}* (n=16) and *MYC;Fsp-cre;Pten^{loxP/loxP}* (n=26) donors were transplanted into syngeneic wild-type recipients and tumor development was monitored over the course of several months¹⁹. By genetically marking the stroma with the *Rosa26^{LoxP}* reporter allele, we could show that both the epithelium and its associated stroma were effectively transplanted into host female mice (Supplementary Fig. 4). By 16 weeks post-transplantation, loss of *Pten* in stromal fibroblasts dramatically increased the incidence of *ErbB2*-driven mammary tumors (Fig. 2b). By 26 weeks most of these females met the criteria for early removal due to excessive tumor burden, whereas few control *ErbB2* females had palpable tumors (Fig. 2a-d). Stromal deletion of *Pten* also dramatically accelerated development of *MYC* mammary tumors (Fig. 2e-g). By 16 weeks post-transplantation, most of these lesions progressed to adenoma, carcinoma *in situ* and invasive carcinoma (Fig. 2h). Histological examination showed that *ErbB2*- and *MYC*-tumor cells in *Pten* stromal-deleted tumors retained their typical oncogene-specific morphology. *ErbB2*-cells had small nuclei, fine

chromatin and abundant eosinophilic cytoplasm¹⁹, whereas *MYC*-tumor cells had pleomorphic nuclei with coarse chromatin and amphophilic cytoplasm. In contrast to non-deleted tumors, which typically had relatively little stromal contribution^{19, 20}, *Pten* stromal-deleted tumors had a significant amount of stroma surrounding and infiltrating the epithelial masses (Fig. 2d, 2h). PCR-based and immunohistochemical assays confirmed that all tumors had an intact *Pten*^{loxP} allele in the epithelial compartment (Supplementary Fig. 5a, 5b and data not shown). Thus, the analysis of two distinct breast cancer tumor models identified a potent tumor suppressor role for *Pten* in stromal fibroblasts of the mammary gland.

***Pten* in stromal fibroblasts controls ECM and innate immune functions.**

To investigate the tumor suppressive mechanism of *Pten* action in stromal fibroblasts, we profiled the transcriptome of mammary stromal fibroblasts isolated from *Pten*^{loxP/loxP} (n=3) and *Fsp-cre;Pten*^{loxP/loxP} (n=3) females. Details of sample collection, processing of Affymetrix oligo-arrays and expression data are available in the Methods section. Briefly, we implemented class comparison analyses of all probe sets on the Affymetrix mouse genome 430 2.0 array to identify genes differentially expressed between the two genetic groups. We also used an unbiased approach similar to Gene Set Enrichment Analysis⁹ to identify *a priori* defined groups of genes that were significantly differentially expressed. The analysis of over 14,000 mouse genes identified 129 upregulated and 21 downregulated genes in response to *Pten* deletion (Supplementary Fig. 6a, 6b; >4-fold at p <0.001; Supplementary Tables 1 and 2). Quantitative RT-PCR assays of a subset of genes confirmed >85% of these expression changes using

independent fibroblast samples (Supplementary Fig. 6c, 6d, and Supplementary Table 3) and the lack of macrophage-, endothelial- and epithelial-specific expression confirmed the purity of the fibroblast preparations used for these microarrays (Supplementary Figure 6d). Functional annotation^{21, 22} (GO) of *Pten*-responsive targets revealed a remarkable bias toward genes encoding proteins involved in ECM remodeling, wound healing and inflammation (Fig. 3a). Given this unexpected convergence of function in *Pten* stromal-deleted mammary glands, we performed a more thorough cellular and molecular analysis of *Pten*-deleted stroma. Staining of consecutive mammary gland sections with H&E and Mason's trichrome stains indicated enhanced deposition of collagen in *Pten*-deleted stroma that was independent of *ErbB2*-oncogene expression (Fig. 3b, and Supplementary Fig. 7a). IHC and Western blot assays using collagen type-specific antibodies showed that the non-cellular material consisted mostly of type-I collagen and not the basement membrane type-IV collagen (Fig. 3b, and Supplementary Fig. 7b, 7c). GO analysis also suggested that *Pten* in stromal fibroblasts influenced the expression of a complex network of chemokines, cytokines and receptors known to support chronic inflammation^{21, 22}. Quantification of innate and adaptive immune cells showed significant infiltration of F4/80-positive macrophages into stromal *Pten*-deleted mammary glands (Fig. 3d, 3e), which was also independent of *ErbB2*-oncogene expression (Supplementary Fig. 8). The abundance of B- and T- cells did not change in response to stromal deletion of *Pten* (data not shown). From these experiments we conclude that ablation of *Pten* in stromal fibroblasts recapitulates two key events associated with tumor malignancy: increased ECM deposition and innate immune cell infiltration.

Loss of stromal *Pten* activates an Ets2-dependent transcriptional program

Along with the remarkable remodeling of the tumor microenvironment, loss of *Pten* in stromal fibroblasts resulted in the activation of the Ras, JNK and Akt pathways. Western blot analysis using protein lysates derived from *Pten*-deleted stromal fibroblasts demonstrated an increase in the phospho-specific forms of Akt (T³⁰⁸ and S⁴⁷³) and JNK (T¹⁸³ and Y¹⁸⁵) (Fig 3a). Immunohistochemical assays confirmed the activation of Akt and JNK in stromal fibroblasts, and interestingly, also revealed a profound activation of these two pathways in ductal epithelial cells adjacent to the *Pten*-deleted stroma (Fig. 3g and data not shown). This analysis also showed increased levels of phospho-Erk1/2 in *Pten*-deleted stromal fibroblasts (Fig. 3g), however, this increased could not be detected in primary cultured fibroblasts, presumably due to the constitutive *Pten*-independent activation of Erk1/2 by serum-stimulation²³.

Among the many expression changes observed in *Pten*-deleted stromal fibroblasts we noted that there was a significant increase in *Ets2* mRNA levels (2.8 fold, $p < 0.001$). This induction is notable because the Ets2 transcription factor is known to be transcriptionally induced by MAPK²⁴⁻²⁶ activation and its function to be post-translationally enhanced by the Akt- and JNK-mediated phosphorylation of its pointed domain at threonine 72 (Ets2^{T72})^{23, 27}. Quantitative RT-PCR and Western blot assays confirmed the higher levels of *Ets2* mRNA and protein in *Pten*-deleted relative to control fibroblasts (~2.5-fold, $p < 0.001$; Fig. 4a, 4b). Immunofluorescence (IF) assays using vimentin and phospho-specific Ets2^{T72} antibodies revealed a marked increase of P-Ets2^{T72} in stromal fibroblasts and interestingly, in the adjacent epithelial ductwork as well (Fig.

3c, 3d). Loss of *Pten* in stromal fibroblasts resulted in the induction of a number of genes involved in ECM remodeling and macrophage recruitment (Supplementary Fig. 6c), two of which, *Mmp9* and *Ccl3*, are known to be direct transcriptional targets of Ets2^{28, 29} (Fig. 4e). The increase of *Mmp9* expression in *Pten* stromal-deleted *ErbB2*-tumors appears to be of pathological relevance since *in situ* zymography, a functional assay enabling real-time monitoring of a fluorescent protease cleavage substrate³⁰, showed robust activation of Mmp9 in these tumor samples (Fig. 4f). Chromatin immunoprecipitation (ChIP) assays showed an increase in the loading of Ets2 onto the *Mmp9* and *Ccl3* promoters in *Pten*-deleted mammary fibroblasts (Fig. 4g), suggesting a direct role for Ets2 in the transcriptional regulation of these two target genes. Together, these data illustrate the extensive molecular reprogramming that takes place in the tumor microenvironment and in tumor cells, as a consequence of ablating *Pten* in stromal fibroblasts.

Stromal *Ets2* promotes mammary tumorigenesis

To determine whether Ets2 promotes a microenvironment conducive to tumor growth we analyzed the consequences of ablating a conditional allele of *Ets2* (*Ets2*^{loxP})³¹ in mammary stromal fibroblasts of a well-characterized mouse model of breast cancer, *MMTV-PyMT* (*PyMT*)³². The *PyMT* oncogene initiates the rapid onset and progression of mammary tumors and thus represents an ideal model for evaluating any potential delay that loss of *Ets2* might have on tumorigenesis. The complete or near-complete ablation of *Ets2* in stromal fibroblasts was facilitated by using *Fsp-cre* mice carrying conventional and conditional knockout alleles of *Ets2* (DNA-binding domain-*Ets2*^{db/LoxP})³³. PCR-based analysis of genomic DNA showed efficient deletion of *Ets2*^{loxP} in mammary

fibroblasts of *Fsp-cre;Ets2^{db/loxP}* mice (Fig. 5a) and Western blot and IF assays confirmed the loss of its protein product (Fig. 5b and 5c, respectively). Importantly, ablation of *Ets2* in these cells had no detectable physiological consequence on the development of mammary glands, either during puberty or pregnancy (MCO, unpublished observations). The evaluation of *PyMT;Fsp-cre;Ets2^{db/loxP}* and control *PyMT;Ets2^{db/loxP}* mice over a period of three months showed that ablation of *Ets2* in mammary fibroblasts significantly reduced tumorigenesis (Fig. 5d). *PyMT;Fsp-cre;Ets2^{db/loxP}* mice exhibited decreased tumor load (Fig. 5e) and slower progression to adenoma and early carcinoma than *PyMT;Ets2^{db/loxP}* control mice (Fig. 5f). Analysis of *Mmp9* expression by quantitative RT-PCR showed low levels of its mRNA in normal fibroblasts (with or without *Ets2*) and high levels in tumor-associated fibroblasts containing *Ets2*, which were reduced back to the normal low levels upon in *Ets2*-deleted tumor fibroblasts (Fig. 6a). *In situ* zymography assays measured a 4-5 fold decrease in Mmp9 activity in tumor sections from *PyMT;Fsp-cre;Ets2^{db/loxP}* mice relative to *PyMT;Ets2^{db/loxP}* controls (Fig. 6d and data not shown). Because Mmp9 activity is known to mediate the release of matrix-bound VEGF-A to its active isoforms, including VEGF₁₆₄³⁴, we visualized the spatial distribution of VEGF₁₆₄ and Mmp9 by immunofluorescent staining in consecutive frozen tumor sections. These assays showed that the accumulation of VEGF₁₆₄, which was particularly acute within collagen1A-rich stromal locations overlapping Mmp9 activity, was significantly decreased in stromal-deleted *Ets2* tumors (Fig. 6b, 6d). Given that VEGF₁₆₄ is a specific ligand for VEGF Receptor 2 (VEGFR2; FLK-1; KDR), one of the most potent mediators of VEGF-induced endothelial signaling and angiogenesis³⁵, we also evaluated VEGFR2 status by immuno-staining tumor sections with antibodies

specific for CD31 (endothelial-specific marker) and the phospho-activated form of the murine VEGF receptor (VEGFR2^{Y1173})³⁶. This analysis revealed a four-fold decrease in the number of CD31/ VEGFR2^{Y1173} double-positive cells in *Ets2*-deleted versus non-deleted tumor samples (Fig. 6c, 6e). Together, we conclude that loss of *Ets2* in stromal fibroblasts resulted in decreased *Mmp9* expression and activity in the tumor ECM and reduced VEGFR2^{Y1173}-activation in the tumor vasculature.

Loss of *Ets2* diminishes tumor formation in *Pten* stromal-deleted mammary glands

Given the apparent role of stromal *Ets2* in promoting *PyMT*-driven mammary tumors, we entertained the hypothesis that *Ets2* may be contributing to the remodeling of the tumor microenvironment caused by stromal *Pten* deletion that led to enhanced tumorigenesis in *ErbB2;Fsp-cre;Pten^{loxP/loxP}* mice. To directly test this possibility, we compared tumor incidence and burden in *Pten^{loxP/loxP}*, *Fsp-cre;Pten^{loxP/loxP}* and *Fsp-cre;Pten^{loxP/loxP}Ets2^{db/loxP}* mammary glands that were orthotopically injected with an established *ErbB2*-initiated mammary tumor cell line (NT 2.5)³⁷. This orthotopic model recapitulated the consequences of deleting *Pten* in the mammary stroma that were observed in the genetically engineered *ErbB2*-mouse model described earlier in this study. Indeed, the incidence of tumors at injected sites and the tumor load in *Fsp-cre;Pten^{loxP/loxP}* females was markedly higher than in control *Pten^{loxP/loxP}* females (Fig. 7a, 7b). Importantly, both the tumor incidence and tumor loads were significantly reduced in mammary glands doubly deleted for stromal *Pten* and *Ets2* (*Fsp-cre;Pten^{loxP/loxP}Ets2^{db/loxP}*). Evaluation of tumor sections revealed decreased number of macrophages and recruitment of new vasculature in these doubly-deleted mammary

glands (Fig. 7d, 7e). Loss of *Pten* and *Ets2*, however, failed to fully reduce the tumor load and the excessive collagen deposition to control levels (compare *Pten*^{loxP/loxP} and *Fsp-cre;Pten*^{loxP/loxP};*Ets2*^{db/loxP} in Fig. 7b, 7d and 7e), suggesting that additional effectors must also contribute towards Pten's tumor suppressor functions. From these data, we conclude that *Ets2* is a major component of the Pten tumor suppressive axis that acts in the stromal fibroblast compartment of mammary glands.

Mouse fibroblast Pten expression signature distinguishes normal from tumor stroma in breast cancer patients.

To determine the relevance of these findings in mice to human breast cancer, we compared the stromal fibroblast Pten-expression signature identified in *Fsp-cre;Pten*^{loxP/loxP} mice to the expression signatures derived from laser-captured tumor stroma (49 samples) and adjacent normal stroma (52 samples) in breast cancer patients³⁸. Details of sample processing and data analysis are available in the Supplementary Methods section. First, we identified 137 human orthologs from the 150 differentially expressed mouse genes detected by the Affymetrix oligo-arrays (Supplementary Fig. 6). Of these 137 orthologs, 129 genes were represented in the expression platform used³⁸ (Agilent) for the analysis of human patient stroma samples (McGill Cancer Center's Breast Stroma Microarray data GSE9014 and GSE4823). Only 71 of these 129 genes had highly variable gene expression across all human stromal samples (a variance cutoff of >0.5). The heat map generated for the human stroma dataset showed that this 71 gene-subset derived from the mouse Pten-signature was sufficient to distinguish normal from tumor stroma in all patients (Fig. 8a; $p=3.9e^{-15}$ as determined by Wilcoxon's test).

Interestingly, 12 of the 137 human orthologs identified by the *Pten*-signature (Fig. 8a, highlighted in red) were previously shown to be differentially expressed in the tumor stroma of breast cancer patients and to be associated with recurrence³⁸. This overlap between differentially expressed genes in mouse (71 genes) and human stroma (163 genes) is highly significant ($p = 2.5e-8$, Fisher's Exact Analysis). These analyses suggest that the fibroblast *Pten*-expression signature identified by our stroma mouse model represents a significant subset of the total gene signature expressed in the stroma of human breast cancer. We interpret these results to mean that a portion of the transcriptome regulated by *Pten* in mammary stromal fibroblasts is dysregulated in the tumor stroma of breast cancer patients.

DISCUSSION

Histopathology and molecular studies suggest that malignant tumors consist of a complex cellular system that is dependent on reciprocal signaling between tumor cells and the adjacent stroma. However, the signaling pathways that mediate the communication between the various cell types in the tumor remain virtually unknown. We recently developed a mesenchymal-specific *cre* mouse¹⁵ and used it here to examine the consequences of inactivating *Pten* in mammary stromal fibroblasts. Using this system we show, for the first time, that *Pten* in stromal fibroblasts has a critical role in the suppression of epithelial mammary tumors that is, in part, mediated through an *Ets2*-regulated transcriptional program.

The tumor suppressor functions of *PTEN* have been extensively studied in the tumor cell³⁹⁻⁴¹. We show here that genetic ablation of *Pten* in mammary stromal

fibroblasts of mice alters the expression profile of these cells to increase ECM, chemokine and cytokine production in the tumor microenvironment. As a result, *Pten* stromal-deleted tumors exhibit high levels of collagen, macrophage recruitment and vascular networks, which together favor the initiation and progression of mammary epithelial tumors. Remarkably, side-by-side evaluation of histopathology by independent pathologists could not distinguish tumors between *Pten* stromal-deleted mice and human breast cancer patients, highlighting the importance of modeling stromal cell compartments of the tumor microenvironment. The mechanism by which *Pten* in the stroma exerts its tumor suppressor role likely involves the control of multiple signaling pathways, including components of the Ras, Akt and JNK networks, which together culminate in the regulation of Ets2 transcriptional activity. The fact that loss of *Ets2* in mammary stromal fibroblasts diminished the oncogenic consequences of deleting *Pten* in these cells underscores the importance of the stromal Pten-Ets2 axis in stromal fibroblasts during tumor suppression. These observations are consistent with previous work from Oshima and colleagues that showed a critical cell non-autonomous role for *Ets2* in the growth of mammary tumors in mice⁴² and with the identification of Ets2 activation as a key event associated with breast cancer in human patients having poor prognosis⁴³⁻⁴⁵. The relevance of the mouse Pten-Ets2 tumor suppression axis identified here to human breast cancer is highlighted by the high correspondence between the mouse and human stromal expression signatures. The observation that the dire consequences of targeting this *Ets2*-driven stromal program are tumor-specific, sparing normal mammary development, emphasizes the potential utility of stromal-specific strategies for therapeutic intervention in human breast cancer.

In summary, this work identifies *Pten-Ets2* as a key regulatory axis in stromal fibroblasts that suppresses mammary epithelial tumors by profoundly attenuating some of the most malignant characteristics of the tumor microenvironment. This novel function of *Pten* may be relevant in the suppression of epithelial tumors of other organs, but may also extend beyond cancer, to conditions where the microenvironment may impact disease manifestation, such as in autoimmune syndromes⁴⁶, lung fibrosis⁴⁷ and neurodegeneration⁴⁸. Interestingly, the stromal *Pten* expression signature identified here includes genes that have been causally linked to ECM deposition and inflammation in rheumatoid arthritis, lung fibrosis and neurodegeneration (Listed in Supplementary Table1 and Table 2). These data offers a molecular basis for how altered *Pten* signaling in the tumor stroma may elicit broad responses in a variety of cells in the tumor microenvironment that contribute to disease manifestation.

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METHODS SUMMARY

Transgenic mice. Generation of *Fsp-cre* mice has been described¹⁵. *Pten*^{loxP} mice were created following the strategy described in Supplementary Fig. S1. *Ets2*^{loxP} mice were generated by standard techniques³¹. Animals were maintained and euthanized following institutional guidelines. Tenth generation congenic (N10) FVB/N animals were used for transplantation and orthotopic injection studies.

Tissue processing, histology. All tissues were either fixed with 4% PFA and embedded in OCT, or fixed with formalin and embedded in paraffin. Frozen sections were used for X-gal staining as described¹⁵. To perform gelatinase *in situ* zymography 10µm frozen sections were incubated with 40 ug/ml DQ-gelatin (Molecular Probes) for 10hours at room temperature as described by Mook *et al*⁴⁹.

Isolation of primary mammary fibroblasts. Primary mammary fibroblasts were purified following the protocol published previously with minor modifications⁵⁰. Mammary glands were dissected from 8 week-old female mice, minced and digested with collagenase (0.15% Collagenase I, 160 U/ml Hyaluronidase, 1 ug/ml hydrocortisone and 10 ug/ml insulin with penicillin and streptomycin) in a 5% CO2 incubator overnight at 37°C. Collagenase was neutralized with 10% FBS-DMEM medium. Digested tissue was resuspended in medium and subjected to gravity for 12-15 min. Pellets were washed three times to collect epithelial organoids, and supernatants were subjected four more times to gravity and then cultured.

RNA and microarray analysis. RNA was harvested with Trizol according to manufacturer's instructions (Invitrogen). RNA quality and concentration were assessed by Bioanalyzer and Nanodrop RNA 6000 nano-assay. RNA samples were hybridized to Affymetrix GeneChip Mouse genome 430 2.0 platform at the Microarray Shared Resource Facility, Ohio State University Comprehensive Cancer Center.

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FIGURE LEGENDS

Figure 1 Stromal fibroblast-specific deletion of *Pten*.

(a) *Fsp-cre* expression in the mammary gland. Whole mount X-gal stained mammary glands from *Fsp-cre;Rosa^{+/-loxP}* and *Rosa^{+/-loxP}* (top inset) mice. Higher magnification micrograph of whole mount (bottom left) and cross section (bottom right) of *Fsp-cre;Rosa^{+/-loxP}* X-gal stained mammary gland; lu, lumen.

(b) *Pten* deletion in the *Fsp-cre;Pten^{loxP/loxP}* mice by PCR-based assays. DNA extracted from tail biopsies (lanes 1-4) or purified primary mammary stromal fibroblasts (lanes 5-7) with the indicated genotypes were used as template for PCR-based measurement of *Pten* deletion using primers described in Methods.

(c) Ablation of *Pten* protein in mammary stromal fibroblasts. Representative Western blot analysis of mammary fibroblast lysates derived from four different 8 week-old female mice with the indicated genotypes.

(d) *Pten* inactivation is restricted to mammary stromal fibroblasts. Paraffin sections from *Pten^{loxP/loxP}* and *Fsp-cre;Pten^{loxP/loxP}* mammary glands were processed for IHC using a *Pten*-specific antibody. Lower panels represent higher magnification of boxed areas in upper panels. lu, lumen; epi, epithelial compartment; str, stromal compartment; red dotted line indicates the border between the epithelial and stromal compartments.

Figure 2 Loss of *Pten* in mammary stromal fibroblasts accelerates tumorigenesis.

- (a) Tumors derived from *ErbB2;Pten^{loxP/loxP}* and *ErbB2;Fsp-cre;Pten^{loxP/loxP}* mammary glands, 26 weeks post-transplantation.
- (b) Percentage of mammary glands with the indicated genotypes that developed tumors by 16 weeks post-transplantation. Tumorigenicity was determined based on palpation or histological presentation of adenoma/carcinoma at each implantation site and statistically analyzed using Fisher's Exact test.
- (c) Total tumor burden in mammary glands with the indicated genotypes at 26 weeks post-transplantation. A mixed ANOVA model was used to compare the groups. A random effect for the mouse within donor effect was used to account for the correlations.
- (d) H&E stained sections of mammary glands with the indicated genotypes that were harvested just before transplantation (0 weeks) and at the indicated times post-transplantation.
- (e) Tumors derived from *MYC;Pten^{loxP/loxP}* and *MYC;Fsp-cre;Pten^{loxP/loxP}* mammary glands, 10 weeks post-transplantation.
- (f) Percentage of mammary glands with the indicated genotypes that developed tumors by 10 weeks post-transplantation. Tumorigenicity was determined based on histological presentation of adenoma/carcinoma at each implantation site. Note that no tumors developed in *MYC;Pten^{loxP/loxP}* mammary glands and hence no statistical analysis was performed.
- (g) Total tumor burden in mammary glands with the indicated genotypes at 26 weeks post-transplantation. A mixed ANOVA model was used to compare the groups. A random effect for the mouse within donor effect was used to account for the correlations.

(h) Paraffin sections of mammary glands with the indicated genotypes were harvested at 10 weeks post-transplantation and stained with H&E. Note the range of tumor stage observed in *MYC;Fsp-cre;Pten^{loxP/loxP}* glands.

Figure 3 Characterization of ECM deposition and immune cell infiltration.

- (a) Schematic representation of the biological processes affected by differentially expressed genes (<4 fold).
- (b) Tissue sections from 8 week-old *Pten*^{loxP/loxP} and *Fsp-cre;Pten*^{loxP/loxP} mammary glands were stained with H&E, Masson's Trichrome and Collagen I-specific antibodies, respectively.
- (c) Quantification of collagen deposition (trichrome-positive area) in mammary glands with the indicated genotypes. Trichrome-stained sections were imaged using an Aperio Scanscope CS whole-slide scanner. Areas of skin and muscle, which also stain positive, were manually encircled and excluded from the analysis. Values shown represent the mean with standard deviation; Wilcoxon Rank Sum test was used for the comparison between groups.
- (d) Sections from mammary glands with the indicated genotypes were stained with the macrophage-specific marker F4/80.
- (e) Quantification of stromal cells stained positive for the macrophage-specific marker F4/80 in mammary glands with the indicated genotypes. Values shown represent the mean with standard deviation; Wilcoxon Rank Sum test was used for the comparison between groups.
- (f) Western blot analysis of whole-cell lysates derived from stromal fibroblasts with the indicated genotypes and antibodies; blots were probed with anti-tubulin as a loading control.
- (g) Sections from mammary glands with the indicated genotypes stained with the phospho-Akt^{473/308}, phospho-JNK^{183/185}, and phospho-Erk1/2 specific antibodies.

Figure 4 Loss of *Pten* in stromal fibroblasts leads to activation of Ets2.

(a) qRT-PCR analysis of *Ets2* expression in *Pten*^{loxP/loxP} and *Fsp-cre;Pten*^{loxP/loxP} stromal fibroblasts. Total RNA was isolated from fibroblasts with the indicated genotypes and analyzed by real-time RT-PCR assays. Values shown represent the average with standard deviation; statistical analysis used the Student t-test.

(b) Western blot analysis of whole-cell lysates derived from fibroblasts with the indicated genotypes.

(c) Quantification of (Fig 4d) mammary epithelial and stromal cells that stain positive for nuclear phospho-Ets2^{T72} in *Pten*^{loxP/loxP} and *Fsp-cre;Pten*^{loxP/loxP} animals. Values shown represent the mean with standard deviation; Wilcoxon Rank Sum test was used for the statistical comparison between groups.

(d) Mammary tissue sections from *Pten*^{loxP/loxP} and *Fsp-cre;Pten*^{loxP/loxP} animals were processed for IF using a phospho-Ets2^{T72}-specific antibody. Note that loss of *Pten* in the mammary stroma increased Ets2 phosphorylation in both the stromal and epithelial compartments. Dotted-white line indicates the stromal-epithelial boundary.

(e) qRT-PCR analysis of *Mmp9* expression in *Pten*^{loxP/loxP} and *Fsp-cre;Pten*^{loxP/loxP} stromal fibroblasts. Total RNA was isolated from fibroblasts with the indicated genotypes and analyzed by real-time RT-PCR assays. Values shown represent the average with standard deviation; statistical analysis used the Student t-test. Note that *Mmp9* expression is increased in *Pten*-deleted fibroblasts and further increased in *Pten*-deleted fibroblasts derived from mammary glands expressing *ErbB2* in the epithelial compartment.

(f) *In situ* zymogen IF assays in 26 week post-transplanted *ErbB2;Pten^{loxP/loxP}* and *ErbB2;Fsp-cre;Pten^{loxP/loxP}* mammary glands (bottom panels). Top panels are H&E staining of consecutive sections from the same mammary gland in bottom panels.

(g) ChIP assays using Ets2-specific or IgG control antibodies in stromal fibroblasts with the indicated genotypes. Values shown represent the average with standard deviation; statistical analysis used the Student t-test.

Figure 5 Ets2 ablation in stroma fibroblasts restricts mammary tumorigenesis.

- (a) Ets2 genotyping and (b) Western blot of fibroblasts purified from mammary glands of *PyMT;Ets2^{db/loxP}* (lane 1) or *PyMT;Fsp-Cre;Ets2^{db/loxP}* mice (lane2).
- (c) Immunofluorescence staining of cultured mammary fibroblasts from 9 week-old mice with Vimentin (green) and p-Ets2(T⁷²) (red) antibodies, and counterstained with DAPI. Scale bar is 50μm.
- (d) Representative images of gross tumors dissected four weeks post tumor initiation from *PyMT;Ets2^{db/loxP}* (left) or *PyMT;Fsp-cre;Ets2^{db/loxP}* (right) mice.
- (e) Comparison of total mammary tumor volume of *PyMT;Ets2^{db/loxP}* (n = 20), *PyMT;Fsp-cre;Ets2^{db/loxP}* (n = 21) mice; significance based on Wilcoxon Rank Sum test.
- (f) H&E staining of tumors harvested from *PyMT;Ets2^{db/loxP}* or *PyMT;Fsp-cre;Ets2^{db/loxP}* mice; Scale bar is 500μm for top panel and 50μm for bottom panel.

Figure 6 Ets2 inactivation in tumor fibroblasts impairs vascular endothelial signaling and reduces tumor angiogenesis

(a) Quantitative RT-PCR analysis of *Mmp9* mRNA expression in primary mammary fibroblasts of indicated genotypes (* $P < 0.01$, $n = 3$, Students t-test).

(b) Quantification of VEGF₁₆₄ of the IF staining (Fig 6d) in tumor stroma area in tumors collected one week post tumor initiation(* $P < 0.01$, $n = 3$, Students T-test). Error bars represent s.d.

(c) Quantification of tumor endothelial cells (Fig 6e) co-expressing CD31 and phospho-VEGFR2 (* $P < 0.01$, $n = 3$, Student T-test).

(d) Consecutive sections stained for (from left to right): Trichrome, Mmp9 gelatinase activity and VEGF₁₆₄, and counterstained with DAPI from *PyMT;Ets2^{db/loxP}* and *PyMT;Fsp-cre;Ets2^{db/loxP}* mammary tumors harvested one week post tumor initiation. Scale bars are 50 μ m.

(e) Tumor vascular endothelial cells visualized by IF double staining with CD31 (green) and p-VEGFR2(Tyr1173) (red), and counterstained with DAPI in mammary tumors collected one week post tumor initiation *PyMT;Ets2^{db/loxP}* and *PyMT;Fsp-cre;Ets2^{db/loxP}* mice. Scale bars are 50 μ m.

Figure 7 Loss of *Ets2* in stromal fibroblasts diminishes tumor growth in stromal *Pten*-deleted mammary glands.

(a) Orthotopic injection of the *ErbB2*-expressing tumor cell line NT 2.5 into *Pten*^{loxP/loxP};*Ets2*^{db/loxP};*Pten*^{loxP/loxP} and *Fsp-cre*;*Pten*^{loxP/loxP} and *Fsp-cre*;*Ets2*^{db/loxP};*Pten*^{loxP/loxP} mammary glands. Mammary glands from at one month post-injection were sectioned and stained with H&E.

(b) The *Pten*^{loxP/loxP};*Ets2*^{db/loxP} (n=10) and *Pten*^{loxP/loxP} (n=10) control groups were combined (+(*), n=20) after it was determined there was no statistical difference in the tumor incidence/load between these two control groups. Values shown represent the mean with standard deviation; Wilcoxon Rank Sum test was used for the comparison between groups.

(c) Sections from mammary glands with the indicated genotypes stained with the macrophage-specific marker F4/80. Frozen sections from 8 week-old mammary tissue with the indicated genotypes stained with the endothelial-specific antibody, CD31, and H&E (consecutive section). Note that loss of *Pten* in the mammary stroma resulted in a disruption of the normal vascular architecture.

(d) Quantification of stromal cells stained positive for the macrophage-specific marker F4/80 in mammary glands with the indicated genotypes. Values shown represent the mean with standard deviation; Wilcoxon Rank Sum test was used for the comparison between groups.

(e) Quantification of collagen deposition (trichrome-positive area) in mammary glands with the indicated genotypes. Trichrome-stained sections were imaged using an Aperio Scanscope CS whole-slide scanner. Areas of skin and muscle, which also stain positive,

were manually encircled and excluded from the analysis. Values shown represent the mean with standard deviation; Wilcoxon Rank Sum test was used for the comparison between groups.

Figure 8 Analysis of the PTEN null mouse fibroblast microarray data.

(a) Heat map comparing 71 human orthologs from the PTEN null fibroblast list, both up- and down-regulated (y-axis), with the genes that are differentially expressed in human tumor stroma from the McGill study. Red and green regions indicate up-regulate and down-regulated genes, respectively, between the normal and tumor stroma sets from human patients.

(b) Gene expression changes assessed by qRT-PCR to confirm microarray results for representative genes that are differentially expressed between fibroblasts with and without *Pten*. Gene expression was analyzed in independent experiments and the averages between duplicates are shown with standard deviation.

(c) Venn diagram depicting the overlap between the mouse *Pten*-deleted fibroblast and human stroma microarray data. This overlap is highly significant (p-value=2.5e-8; Fisher's Exact Analysis).

SUPPLEMENTARY METHODS

Transgenic mice. Animals were housed according to federal and OSU ULAR regulations.

***Fsp-cre*.** The generation of the *Fsp-cre* mouse line has been previously described¹.

***Ets2^{loxP}*.** The *Ets2* conditional transgenic mouse line was generated such that the *Ets2* pointed domain is “floxed”². The pointed domain is encoded by exons 3-5 and important for the protein-protein interaction and signal transduction.

***Ets2^{db}*.** *Ets2^{db}* mouse was a gift from Dr. Oshima R.G. (Burnham Institute for Medical Research, La Jolla, California).

***Pten^{loxP}*.** *LoxP* sites were introduced into two *HpaI* sites within introns 3 and 5 of the *Pten* gene, respectively, to flank exons 4 and 5. Exon 5 encodes the lipid phosphatase domain. Tissue-specific expression of *cre* will excise exons 4 and 5, generating a loss-of-function *Pten^d* allele (Supplementary Figure 1; G.W. and M.C.O. unpublished data.). A list of PCR primers can be found on Supplementary Table 3.

Mammary tissue transplantation. Transplant procedure was based on a previously method³. The day prior to surgery, recipient mice were anesthetized with Isoflurane (Abbott Laboratories) and a 25mm x 25mm square area along the scapular region was shaved. At the time of surgery, inguinal and groin mammary tissue (5x5mm in size; minus lymph node) was removed from eight to nine-week-old donor females and placed subcutaneously into the scapular region of wild-type hosts through two 5-10mm incisions on the left and right side under aseptic conditions. The small incisions were closed using

a 9mm wound clip. Animals were monitored twice a week until tumor onset. Mice were sacrificed either at specific time points, when the tumor was about 2 cm in size or it presented a health problem to the animal such as exterior ulceration at the site of the tumor. The *myc* tumor model is an inducible system⁴, but donor mice were never given doxycycline. Instead, recipient mice were started on chow containing doxycycline (1g/kg) prior to the transplant procedure, and then continued until the end of the 10 week study.

Orthotopic mammary gland injection. The neu-expressing mouse mammary carcinoma cell line NT2.5 was provided by Dr. Kaumaya (Ohio State University, Columbus, OH) and was maintained as described⁵. Eight week old female mice of each genotype were anesthetized and injected with 5×10^5 NT2.5 cells at both inguinal mammary glands. Tumor initiation was monitored by palpating twice a week. All the mice were euthanized three weeks after injection. Tumor volume was calculated by formula $V = 1/2 \times \text{length} \times (\text{width})^2$.

Tissue processing and X-gal staining. Large individual tumors (typically ~1 to 2cm) or the remainder of transplanted tissues were removed, divided and either fixed in 4% PFA for 24-48hrs or embedded directly in OCT (Sakura, Torrance, CA). Fixed tissue samples were embedded in paraffin and cut into 5 μm sections for H&E, IHC or IF staining. For each sample collected, two sets of sections were obtained at 25 μm intervals for analysis. Corresponding OCT embedded tissue was sectioned (7 μm) in a similar manner for X-gal or IF staining. For X-gal staining, frozen tissue sections were dried 15 min at RT before fixing in a glutaraldehyde solution (0.2% glutaraldehyde, 1.25mM EGTA, pH 7.3 and

2mM magnesium chloride in 1x PBS) for 30 min. The sections were washed with LacZ wash buffer (2mM magnesium chloride, 0.01% sodium deoxycholate, 0.02% IGEPAL CA-630 (Sigma) in PBS) for 5 min three times and then stained in LacZ staining solution (4 mM potassium ferricyanide, 4mM potassium ferrocyanide, 1mg/mL X-gal in LacZ wash buffer) protected from light at 37°C overnight (~18 hrs). Stained sections were washed in PBS for 5 min three times and then rinsed with water for 2 min before counter-staining with nuclear fast red (NFR). .

Immunohistochemistry and immunofluorescence. IHC or IF was performed using paraffin sections with the following antibodies: Pten (1:100, Cell signaling), Collagen I (1:100, Abcam- 30 min), F4/80 (1:50, Caltag), P-Akt(S473, 1:50, Cell Signaling), P-JNK(1:50, Cell Signaling), P-Erk1/2(1:100, Cell Signaling), P-Ets2(T72, 1:125, M.C.O lab), Cytokeratin 8/18 (1:300; Research Diagnostics- 30 min), E-cadherin (1:700, BD-Pharmingen- 30 min), mouse α -SMA (1:200, Sigma-30 min) and Collagen IV(1:100, Chemicon- 30 min). In general, paraffin sections were deparaffinized and the antigen retrieval accomplished by incubation in antigen retrieval solution (DAKO) at >95°C (30 min). **IHC.** Staining was developed using the biotin/avidin/horseradish peroxidase system from Vector Laboratories according to manufacturer's instructions. All IHC slides were counterstained with hematoxylin and images obtained using an Eclipse 50i microscope (Nikon) and an Axiocam HRc camera (Zeiss). **IF.** Staining was developed using secondary antibodies conjugated to AlexaFluor dyes following standard protocol (Invitrogen; Molecular Probes). For Pten IF the signal was amplified using a biotinylated secondary antibody and Texas Red conjugated to streptavidin. All IF sections were

counterstained with DAPI and images obtained using an Axioscope 40 microscope (Zeiss) equipped with an Axiocam HRc camera (Zeiss). Frozen sections of mammary glands for IF were fixed at 4°C in either 4% paraformaldehyde in PBS or acetone (CD31 and Vimentin). Samples were treated with phospho-Ets2 (T72) (1:125; M.C.O. lab), Vimentin (1:50; Santa Cruz Biotech), VEGF₁₆₄ (1:100, R&D Systems), phospho-VEGFR2Y1175 (1:100, Cell Signaling) and CD31 (1:50, BD Biosciences) antibodies. Fluorescent images were obtained as above. CD31 and phospho-VEGFR2Y1175 images were quantified with software written by Dr. Huang K. VEGF₁₆₄ images were quantified with MetaImaging Series 6.1 software. IHC cells were counted manually and reported as a percentage of positive cells from the total cell population.

Gelatinase *in situ* zymography. This was performed as described, with minor modification⁸. Briefly, frozen sections (10µm) were quickly fixed with cold acetone, rehydrated with PBS and then incubated with 40 µg/ml DQ-gelatin (Molecular Probes) in reaction buffer (50mM Tris-HCl, 150mM NaCl, 5mM CaCl₂ and 0.2mM NaN₃, pH7.6) for 10hrs. The reaction is quenched with 10mM EDTA-PBS wash. Nuclei are counterstained with DAPI. A consecutive slide is stained with H&E to localize the MMP9 activity.

Western blot. One to two million cells were lysed with RIPA buffer (50mM pH7.4 Tris-HCl, 150mM NaCl, 1mM EDTA, 1% NP-40, 1% Sodium Deoxycholate and 0.1% SDS) containing protease and phosphatase inhibitors (Roche). Primary antibodies for PTEN, Akt, P-Akt, P-JNK, P-Erk, Erk and Ets2 were all used at 1:1000 dilution, while tubulin

was used at 1:5000 dilution. Washed membranes were blotted with either HRP-conjugated anti mouse IgG or anti rabbit IgG antibodies and developed with ECL.

RNA isolation and microarray analysis. Cells were harvested with Trizol and RNA was extracted according to manufacturer's instructions (Invitrogen). Total RNA quality and concentration were assessed by Bioanalyzer and Nanodrop RNA 6000 nano assay. RNA was hybridized to Affymetrix GeneChip, Mouse genome 430 2.0 platform at the Microarray Shared Resource Facility, Ohio State University Comprehensive Cancer Center. The data were analyzed using WEDGE++ expression analysis⁷. Heat map representation (Supplementary Figure 6) was performed using the TIGR Multiexperiment Viewer program [MeV v4.1](#).

Quantitative realtime PCR. Quantitative gene expression was performed using 50 ng cDNA per reaction. Taqman Roche Universal Probe Library system probe and primers (Roche) following manufacturer's instructions. Reactions were carried out on the Icyler iQ Real-Time machine (Bio-Rad).

Chromatin Immunoprecipitation and qPCR. Chromatin immunoprecipitation (ChIP) assays were performed as described by Hu *et al.*⁸ Primary fibroblasts were cross-linked with 1% formaldehyde and soluble chromatin was prepared with sonication to an average DNA length of 200–1000bp. Sheared soluble chromatin was pre-cleared with tRNA-blocked Protein G-agarose, and 10% of the pre-cleared chromatin was set aside as input control. Immunoprecipitation was carried out with 5µg of Ets2 antibody or rabbit IgG

overnight at 4 °C. Immune complexes were pulled down with Protein G-agarose, washed, and eluted with elution buffer (0.1 M NaHCO₃, 1% SDS), and de-crosslinked with 200mM NaCl at 65 °C overnight with 20µg of RNase A (Sigma). DNA was purified with the Qiagen PCR purification kit following proteinase K treatment according to the manufacturer's instructions. Samples were analyzed by real-time PCR as indicated above. The threshold for the promoter being studied was adjusted by that of input values and represented as relative abundance. All qRT-PCR reactions were analyzed by melt curve analysis and agarose gels to confirm the specificity of the reaction.

Generating the Human Stroma Heat map with the fibroblasts - PTEN null genes.

Analysis of the PTEN null mouse microarray data using WEDGE++⁶ led to the identification of 195 differentially expressed probe sets matching to 150 unique mouse genes. A search for human orthologs using Ensembl and MGI databases yielded in a list of 137 genes. These genes were queried against the McGill Cancer Center's Breast Stroma Microarray data (GSE9014 and GSE4823). 129 of the 137 genes were represented on the Agilent Custom Array used in the McGill study. A heat map was generated for the human stroma dataset (52 normal stroma and 49 tumor stroma samples). To achieve better resolution on the heat map, and with the aim of identifying only those genes that had highly variable gene expression across all samples, a variance cutoff of >0.5 was used to generate a subset of 71 genes. The heat map (Figure 8a) shows the ability of these 71 genes to separate the normal and tumor stroma samples based solely on their gene expression profiles. This partitioning is highly significant (p-value=3.9e-

15), as determined by Wilcoxon's test on the average expression of PTEN null-signature in all samples.

Interestingly, comparison of 137 human orthologs of the PTEN null list with the 163 genes associated with recurrence in tumor stroma from the McGill study showed that there are 12 genes (highlighted in red; Fig 8a) present on both gene lists. This overlap is again highly significant (p-value=2.5e-8; Fisher's Exact Analysis).

Statistical Analysis

Animal numbers and experiments are as indicated in the figures. Wilcoxon -rank test was used for most statistical analyses unless mentioned otherwise in the figure legend.

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Supplementary Figures:

Supplementary Figure 1: Scheme for the generation of *Pten* conditional mice.

Three *loxP* sites were introduced into two Hpa1 restriction sites (H) within introns 3 and 5 on the *Pten* construct, resulting in the loss of these restriction sites (HΔ). Two of the *loxP* sites were associated with the insertion of a neo cassette, used as the selectable marker. An EcoR V (RV) restriction digest was performed to generate the final construct for delivery into ES cells and integration into the genome via homologous recombination. Limited *-cre* expression resulted in the removal of the neo cassette with the remaining two *loxP* sites flanking *Pten* exon 4 and exon 5. Tissue specific expression of *cre* removes *Pten* exon 4 and exon 5 from the genome and results in loss of functional *Pten* expression.

Supplementary Figure 2: Cellular location of *Fsp-cre* expression.

(a) IHC staining for the epithelial markers Cytokeratin 8/18 (Ck8/18) was performed on paraffin sections from X-gal stained mammary tissue (8 week old mice). The Ck 8/18 expression did not overlap with the X-gal marked *cre* expression generated by *Fsp-cre* and the *Rosa26^{loxP}* reporter.

(b) Macrophage (F4/80) and endothelial cells (CD31) were isolate by flow cytometry, using the indicated cell marker, and epithelial and fibroblasts cells were isolated by cell culture from *Fsp-cre;Pten^{f/f};Rosa26^{loxP}* mice. RNA was purified from each cell type and the expression of the conditional *lacZ* reporter gene was quantified by qRT-PCR.

Supplementary: Figure 3: Further characterization of *Fsp-cre* expression.

(a) IHC analysis on non-X-gal stained slides was used to confirm the loss of conditional Pten protein expression due to *Fsp-cre* in mammary gland from 8-9week old mice. Lower panels are higher magnification images of the area enclosed by the rectangle in the upper panels. The dotted red line indicates the epithelial-stroma boundary, lu- Lumen, epi-epithelium and str-stroma.

(b) Double IF was performed to confirm that loss of Pten expression had not occurred in the myoepithelium. Paraffin sections of mammary tissue from 8-9week old mice were used and myoepithelial cells identified by α -smooth muscle actin (Sma) staining; lu- Lumen, epi-epithelium and str-stroma.

Supplementary Figure 4: Fibroblast viability after transplant

Mammary tissue from *Fsp-cre;Rosa26^{loxP}* and *Rosa26^{loxP}* donors were transplanted into wild-type hosts, removed at 16 weeks post-transplantation and assayed for β -galactosidase activity using X-gal staining. This confirmed that fibroblasts would be viable long enough to allow tumors to be formed using the *MMTV-ErbB2* model, which has a 6-7 month latency period.

Supplementary Figure 5: Verification of *Fsp-cre* expression in the 26 week post transplant tumors for the *MMTV-ErbB2* model.

(a) Hematoxylin and eosin (H&E) and X-gal staining of frozen tissue sections shows increased tumor stroma in the *MMTV-ErbB2;Fsp-cre;Pten^{loxP/loxP}* transplants. Tumors from the *MMTV-ErbB2;Pten^{loxP/loxP}* transplants have little to no significant stroma. Unit size is 2mm. Lower β -gal panels are higher magnifications of the corresponding areas outline above by the black squares.

(b) IHC staining for PTEN revealed that the PTEN protein was absent in the stroma (Str) and present in the tumor epithelium (T). The boundary is marked with the dotted red line.

Supplementary Figure 6: Global gene expression analysis in stromal *Pten*-deleted mammary fibroblasts. Total RNA was isolated from primary mammary fibroblasts derived from 8 week-old mice and processed for microarray analysis using Affimetrix chips (see Methods).

(a) Heat-map of upregulated genes in *Pten*-deleted stromal fibroblasts (<4-fold and $p < 10^{-6}$). Genes are listed on the right in descending order magnitude-change.

(b) Heat-map of downregulated genes in *Pten*-deleted stromal fibroblasts (<4-fold and $p < 10^{-6}$). Genes are listed on the right in descending order magnitude-change.

(c) Quantitative RT-PCR analysis of gene expression of representative genes involved in inflammation and wound healing. Gene expression was analyzed in independent experiments and the average between duplicates of a representative sample are shown with standard deviation (d)

Supplementary Figure 7: Collagen expression in the *Pten* deleted mammary gland.

(a) Masson's Trichrome staining of mammary gland sections with the indicated genotypes at 16 weeks post-transplantation.

(b) Epithelial cells produce collagen IV as part of the basement membrane, which surround the mammary ducts. To determine if the observed collagen deposits are a result of the expansion of the basement membrane, IF staining was performed using a collagen IV antibody. There was no expansion of the basement membrane as the collagen IV staining (red) was only observed adjacent to the epithelia cells (green; E-cadherin).

(c). To determine if these ECM deposits were due to an increase in pro-collagen I, protein lysates from primary fibroblasts isolated from 8 week old non-transplanted mammary glands were used for western analysis. An increase in both the pro-collagen and its processed form was observed.

Supplementary Figure 8: F4/80 expression in the *MMTV-ErbB2* model in the presence or absence of *Pten*. Sections from mammary glands transplants with the indicated genotypes were stained with the macrophage-specific marker F4/80.

Supplementary Table 1: *Pten*-responsive genes upregulated more than fourfold in the *Fsp-cre;Pten^{loxP/loxP}* vs. *Pten^{loxP/loxP}* primary mammary stromal cells

Gene Symbol	Gene name	Difference	Reference	Role
<i>Ccl3</i>	chemokine (C-C motif) ligand 3	77.5	1.	inflammation
<i>Irg1</i>	immunoresponsive gene 1	51.6	2.	inflammation
<i>Il1b</i>	interleukin 1 beta	45	3.	inflammation
<i>Ccl12</i>	chemokine (C-C motif) ligand 12	35.5	4.	wound fibrosis
<i>Clec4e</i>	C-type lectin domain family 4, member e	33.2	5.	inflammation
<i>Arg1</i>	arginase 1, liver	20.9	6.	inflammation
<i>Fpr-rs2</i>	formyl peptide receptor, related sequence 2	19.2	7.	inflammation
<i>Fcgr1g</i>	Fc receptor, IgE, high affinity I, gamma polypeptide	18.3	8.	inflammation
<i>Marco</i>	macrophage receptor with collagenous structure	18	9.	inflammation
<i>Ccl6</i>	chemokine (C-C motif) ligand 6	17.2	10.	inflammation
<i>Fpr1</i>	formyl peptide receptor 1	15.9	11.	wound
<i>F13a1</i>	coagulation factor XIII, A1 subunit	15.8	12.	wound
<i>Mmp12</i>	matrix metalloproteinase 12	15.7	13.	wound fibrosis
<i>Lcp1</i>	lymphocyte cytosolic protein 1	14.2	14.	inflammation
<i>Lyzs</i>	lysozyme	14.1	15.	inflammation
<i>S100a3</i>	S100 calcium binding protein A3	13.6	16.	wound ECM
<i>Mpeg1</i> /// <i>LOC671359</i>	macrophage expressed gene 1 /// similar to macrophage expressed gene 1	13.4	17.	inflammation
<i>Msr1</i>	macrophage scavenger receptor 1	13.3	18.	inflammation
<i>Tyrbp</i>	TYRO protein tyrosine kinase binding protein	13.3	19.	Wound ECM
<i>Igfbp5</i>	insulin-like growth factor binding protein 5	13.2	20.	wound fibrosis
<i>Clec4n</i>	C-type lectin domain family 4, member n	13	21.	inflammation
<i>Rac2</i>	RAS-related C3 botulinum substrate 2	12.6	22.	inflammation
<i>Ccl4</i>	chemokine (C-C motif) ligand 4	12	23.	inflammation
<i>Gp49a</i> /// <i>Lilrb4</i>	glycoprotein 49 A /// leukocyte immunoglobulin-like receptor, subfamily B, member 4	11.8	24.	inflammation
<i>Cybb</i>	cytochrome b-245, beta polypeptide	11.6	25.	inflammation
<i>Laptn5</i>	lysosomal-associated protein transmembrane 5	11.4	26.	inflammation
---	CDNA clone MGC:107680 IMAGE:6766535	11.4	N/A	N/A
<i>Plek</i>	pleckstrin	11.3	27.	inflammation
<i>Fcgr2b</i>	Fc receptor, IgG, low affinity IIb	11.1	28.	wound
<i>Clec4d</i>	C-type lectin domain family 4, member d	10.7	29.	inflammation
<i>Cxcl4</i>	chemokine (C-X-C motif) ligand 4	10.6	30.	inflammation
<i>Prq1</i>	proteoglycan 1, secretory granule (Srgn)	10.4	31.	inflammation
<i>Sema4f</i>	sema domain, immunoglobulin domain (Ig), TM domain, and short cytoplasmic domain	10.3	32.	other (neuronal)
<i>Pik3ap1</i>	phosphoinositide-3-kinase adaptor protein 1	10	33.	inflammation
<i>Cd14</i>	CD14 antigen	9.8	34.	inflammation
<i>Cd300lf</i>	CD300 antigen like family member F	9.7	35.	inflammation
<i>Lzp-s</i>	P lysozyme structural	9.3	36.	inflammation
<i>Itgb2</i>	integrin beta 2 (CD18)	9.1	37.	wound healing
<i>Hal</i> /// <i>LOC638196</i>	histidine ammonia lyase /// similar to Histidine ammonia-lyase (Histidase)	8.9	38.	inflammation
<i>Cd48</i>	CD48 antigen	8.6	39.	inflammation
<i>Ptpcr</i>	protein tyrosine phosphatase, receptor type, C (CD45)	8.6	40.	inflammation
---	---	8.5	N/A	N/A

<i>Ctss</i>	cathepsin S	8.5	41.	inflammation
<i>Cd36</i>	CD36 antigen	8.3	42.	inflammation
<i>Ctsc</i>	cathepsin C	8.1	43.	inflammation
<i>Adam8</i>	a disintegrin and metallopeptidase domain 8	8	44.	inflammation
<i>ErbB3</i>	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	8	45.	inflammation
<i>Ms4a6d</i>	membrane-spanning 4-domains, subfamily A, member 6D	7.9	46.	inflammation
<i>Phb</i>	Prohibitin	7.9	47.	inflammation
<i>Hcls1</i>	hematopoietic cell specific Lyn substrate 1	7.8	48.	inflammation
<i>Bcl2a1a</i> /// <i>Bcl2a1b</i> /// <i>Bcl2a1d</i>	B-cell leukemia/lymphoma 2 related protein A1a /// B-cell leukemia/lymphoma 2 related protein A1b /// B-cell leukemia/lymphoma 2 related protein A1d	7.8	49.	inflammation
<i>Ncf1</i>	neutrophil cytosolic factor 1	7.7	50.	inflammation
<i>Rapgef5</i>	Rap guanine nucleotide exchange factor (GEF) 5	7.6	51.	inflammation
<i>Lcp2</i>	lymphocyte cytosolic protein 2	7.6	52.	inflammation
<i>Afp</i>	alpha fetoprotein	7.6	53.	inflammation
<i>Emr1</i>	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	7.4	54.	inflammation
<i>Krt23</i>	keratin 23	7.3	55.	inflammation
<i>Rassf4</i>	Ras association (RalGDS/AF-6) domain family 4	7.3	56.	other
<i>Il2rg</i>	interleukin 2 receptor, gamma chain	7.2	57.	inflammation
<i>C3ar1</i>	complement component 3a receptor 1	7.2	58.	inflammation
<i>2310026E23Rik</i>	RIKEN cDNA 2310026E23 gene	7.1	N/A	N/A
<i>BC032204</i>	cDNA sequence BC032204	7	N/A	N/A
<i>Moxd1</i>	monooxygenase, DBH-like 1	7	59.	inflammation
<i>Pld4</i>	phospholipase D family, member 4	6.9	60.	inflammation
<i>Cd72</i>	CD72 antigen	6.8	61.	inflammation
<i>Gatm</i>	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	6.8	62.	inflammation
<i>Cd68</i>	CD68 antigen	6.7	63.	inflammation
<i>Fgf5</i>	fibroblast growth factor 5	6.6	64.	wound ECM
<i>Itgb8</i>	integrin beta 8	6.6	65.	wound ECM
<i>Ms4a6b</i>	membrane-spanning 4-domains, subfamily A, member 6B	6.5	66.	inflammation
<i>Tnf</i>	tumor necrosis factor	6.4	67.	inflammation
<i>Cfp</i>	complement factor properdin	6.4	68.	inflammation
<i>Slc28a2</i> /// <i>LOC381417</i>	solute carrier family 28 (sodium-coupled nucleoside transporter), member 2 /// similar to solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	6.3	69.	inflammation
<i>AI851790</i>	expressed sequence AI851790	6.2	N/A	N/A
<i>Igsf4a</i>	immunoglobulin superfamily, member 4A (Cadm1)	6.2	70.	other (tumor suppressor)
<i>Cxcl2</i>	chemokine (C-X-C motif) ligand 2	6.2	71.	inflammation
<i>Igsf6</i>	immunoglobulin superfamily, member 6	6	72.	inflammation
<i>Osm</i>	oncostatin M	6	73.	Wound
---	Transcribed locus	6	N/A	N/A
<i>1100001H23Rik</i>	RIKEN cDNA 1100001H23 gene	5.8	N/A	N/A
<i>Hdgfrp3</i> /// <i>Tm6sf1</i>	hepatoma-derived growth factor, related protein 3 /// transmembrane 6 superfamily member 1	5.8	74.	inflammation
<i>Hemt1</i>	hematopoietic cell transcript 1	5.7	75.	other
<i>Csf2rb2</i>	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	5.7	76.	inflammation
<i>Hk3</i>	hexokinase 3	5.6	77.	other

				metabolism
<i>Lpxn</i>	leupaxin	5.6	78.	inflammation
<i>Fcgr3</i>	Fc receptor, IgG, low affinity III	5.6	79.	inflammation
<i>Was</i>	Wiskott-Aldrich syndrome homolog (human)	5.5	80.	inflammation
<i>Selpl</i>	selectin, platelet (p-selectin) ligand	5.5	81.	inflammation
<i>C5ar1</i>	complement component 5a receptor 1	5.3	82.	inflammation
<i>L1cam</i>	L1 cell adhesion molecule	5.3	83.	wound ECM
<i>Igsf4c</i>	immunoglobulin superfamily, member 4C	5.3	84.	other adhesion
<i>Ncf4</i>	neutrophil cytosolic factor 4	5.1	85.	inflammation
<i>Pilrb1</i>	paired immunoglobulin-like type 2 receptor beta 1	5.1	86.	other
<i>Ly9</i>	lymphocyte antigen 9	5.1	87.	inflammation
<i>Krt19</i>	keratin 19	5	88.	inflammation
<i>Ikzf1</i>	IKAROS family zinc finger 1	5	89.	inflammation
<i>Nckap1l</i>	NCK associated protein 1 like (Hem 1)	5	90.	inflammation
<i>Clec4a2</i>	C-type lectin domain family 4, member a2	5	91.	inflammation
<i>LOC668101</i>	similar to SIRP beta 1 isoform 2	4.9	N/A	N/A
<i>Ptpn6</i>	protein tyrosine phosphatase, non-receptor type 6	4.8	92.	inflammation
<i>BC013712</i>	cDNA sequence BC013712	4.8	93.	inflammation
<i>Ccr1</i>	chemokine (C-C motif) receptor 1	4.8	94.	inflammation
<i>AI662270</i>	expressed sequence AI662270	4.8	N/A	N/A
<i>Npy</i>	neuropeptide Y	4.7	95.	inflammation
<i>Irf8</i>	interferon regulatory factor 8	4.7	96.	inflammation
<i>Slc13a3</i>	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	4.7	97.	other Krebs cycle
<i>Ms4a6c</i>	membrane-spanning 4-domains, subfamily A, member 6C	4.7	N/A	N/A
<i>Tlr13</i>	toll-like receptor 13	4.6	98.	inflammation
<i>Dock10</i> /// <i>LOC630691</i>	dedicator of cytokinesis 10 /// similar to Dedicator of cytokinesis protein 10 (Protein zizimin 3)	4.6	99.	wound ECM
<i>Apold1</i>	apolipoprotein L domain containing 1	4.5	100.	other (neuro)
<i>Mrc1</i>	mannose receptor, C type 1	4.5	101.	inflammation
<i>Ms4a7</i>	membrane-spanning 4-domains, subfamily A, member 7	4.5	102.	inflammation
<i>Edil3</i>	EGF-like repeats and discoidin I-like domains 3	4.4	103.	wound ECM
<i>Pla2g7</i>	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	4.4	104.	inflammation
<i>Kctd12</i>	potassium channel tetramerisation domain containing 12	4.4	105.	other markers
<i>Tlr7</i>	toll-like receptor 7	4.3	106.	inflammation
<i>Gpr109a</i>	G protein-coupled receptor 109A	4.3	107.	inflammation
<i>Vav3</i>	vav 3 oncogene	4.3	108.	Wound ECM
<i>Cd53</i>	CD53 antigen	4.3	109.	inflammation
<i>Plp1</i>	proteolipid protein (myelin) 1	4.3	110.	inflammation
<i>Lonrf3</i>	LON peptidase N-terminal domain and ring finger 3	4.2	N/A	N/A
<i>Lgi1</i>	leucine-rich repeat LGI family, member 1	4.2	111.	other neuro
<i>Gm1960</i> /// <i>Cxcl3</i>	gene model 1960, (NCBI)	4.2	112.	inflammation
<i>Cd52</i>	CD52 antigen	4.2	113.	inflammation
<i>Sh3bgrl2</i>	SH3 domain binding glutamic acid-rich protein like 2	4.1	114.	other heart devel
<i>Rnf128</i>	ring finger protein 128 (Grail)	4.1	115.	inflammation
<i>Ncf2</i>	neutrophil cytosolic factor 2	4.1	116.	inflammation
<i>Cd93</i>	CD93 antigen	4.1	117.	inflammation
<i>Cd84</i>	CD84 antigen	4.1	118.	inflammation

<i>Rasgef1b</i>	RasGEF domain family, member 1B	4	119.	other develop
<i>Ebi3</i>	Epstein-Barr virus induced gene 3	4	120.	inflammation
<i>Pdgfb</i>	platelet derived growth factor, B polypeptide	4	121.	wound fibrosis

Biological roles affected by the genes upregulated in *Fsp-Cre;Pten^{loxP/loxP}*

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Supplementary Table 2: *Pten*-responsive genes downregulated more than fourfold in the *Fsp-cre;Pten^{loxP/loxP}* vs. *Pten^{loxP/loxP}* primary mammary stromal cells

Gene Symbol	Gene name	Difference	Reference	Role
<i>Igfbp2</i>	insulin-like growth factor binding protein 2	69.3	122.	other
<i>Fmod</i>	fibromodulin	22.3	123.	ECM
<i>Prelp</i>	proline arginine-rich end leucine-rich repeat	19.1	124.	ECM
<i>Rerg</i>	RAS-like, estrogen-regulated, growth-inhibitor	9.7	125.	ECM
<i>Sned1</i>	sushi, nidogen and EGF-like domains 1	8.4	126.	ECM
<i>Atoh8</i>	atonal homolog 8 (<i>Drosophila</i>)	7.9	127.	other
<i>Efs</i>	Embryonal Fyn-associated substrate	6.7	128.	inflammation
<i>Fndc1</i>	fibronectin type III domain containing 1	6.2	129.	ECM
<i>Sfrp1</i>	secreted frizzled-related sequence protein 1	5.8	130.	ECM
<i>E430002G05Rik</i> (<i>Pamr1</i>)	peptidase domain containing associated with muscle regeneration 1		131.	ECM
<i>Wisp2</i>	WNT1 inducible signaling pathway protein 2	5.3	132.	other
<i>Ptprz1</i>	protein tyrosine phosphatase, receptor type Z, polypeptide 1	5.0	133.	ECM
<i>Gas6</i>	growth arrest specific 6	4.9	134.	inflammation
<i>Igf1</i>	insulin-like growth factor 1	4.7	135.	ECM
<i>Id4</i>	inhibitor of DNA binding 4	4.7	136.	other
<i>Smoc1</i>	SPARC related modular calcium binding 1	4.6	137.	ECM
<i>Pde8b</i>	phosphodiesterase 8B	4.6	138.	other
<i>Pcsk6</i>	proprotein convertase subtilisin/kexin type 6	4.4	139.	ECM
<i>Mfap4</i>	microfibrillar-associated protein 4	4.4	140.	ECM
<i>Syt13</i>	synaptotagmin XIII	4.3	141.	other
<i>Mgp</i>	matrix Gla protein	4.3	142.	ECM/calcification

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Supplementary Table 3: Primers list

qRT-PCR (Roche; Universal Probe Library Assay Design Center)

GENE	Link UPL	L / R	Left primer	Right primer	
CD48	NM_007649.4	L387/R441	cgagttgaagataaccctgga	tcgacgctcagcttattgatt	
C1orf38 (BC013712)	NM_001033308.2	L1698/ R1743	aagaaacagcagaacatacagagc	ctgcggttctacaacttgagg	
Ccl12	NM_011331.2	L100/R175	ccatcagtcctcaggtattgg	cttccggacgtgaatcttct	
Ccl3	NM_011337.2	L119/R212	tgcccttgctgttcttctct	gtggaatcttccggctgtag	
Ccl4	NM_013652.2	L98/ R153	gccctctctctcctcttgct	gaggggtcagagcccattg	
Cdh1	NM_009864.2	L2282/R2325	atcctcgccctgctgatt	accaccgttctctccgta	
Csf1r	NM_001037859.1	L2355/R2410	ccgagggagactccagcta	gactggagaagccactgtcc	
Cybb	NM_007807.2	L1428/ R1481	tgccaacttctcagctaca	gtgcacagcaaatgattgg	
Ets-2	NM_011809.2	L210/ R264	cagtttctgtgggacactca	aaggggagcacagcaaca	
Fmod	NM_021355.2	L1038/ R1095	cagggcaacaggatcaatg	ctgcagcttggagaagtcat	
Gas6	NM_019521.2	L1608/R1661	ggatttgcactacaggtcga	ttacttcccagggtgttcc	
Igfbp2	NM_008342.2	L664/R728	ggaggagcccaagaagtgtg	ggagatccgctccaggac	
Igfbp5	NM_010518.2	L1042/ R1098	ggcgagcaaaccaagataga	agggtcttctcagcatctcg	
IL1b	NM_008361.3	L632/ R679	tgtaatgaaagacggcacacc	tcttcttgggtattgcttg	
Itgb8	NM_177290.3	L2124/ R2170	acttctctgtccctatctcca	atctgccaccttcacactcc	
Krt23	NM_033373.1	L660/R700	tcatgaagaaacgcatgag	cctgaagtcactcggaag	
L4 (Rpl4)	NM_024212.2	L15/ R89	agcagccgggtagagagg	atgactctcccttttcggagt	
LacZ		L180/R226	ggcgattaagtgggtaacg	attcactggccgctgtttta	
Lcp1	NM_008879.2	L197 / R248	tcaacagacgggctgattc	agagcagcctgtcaagca	
Mmp12	NM_008605.3	L1247/R1299	ttgtggataaacactactggaggt	aatcagcttgggtaagca	
Mmp9	NM_013599.2	L1307 /R1374	acgacatagacggcatcca	gctgtggtcagttgtgtgtg	
Ncf2	NM_010877.4	L1037/ R1083	cttcggattcaccctcagtc	gaggtggtggaatatcggatt	
Pla2g7	NM_013737.5	L310/ R379	gtatccgggagtcagtgacg	agcgcctgcagtttgagt	
Plek	NM_019549.1	L1114/ R1156	agtggatcaagccatccag	tcagtgtattctcgggttcctc	
Prelp	NM_054077.2	L82/ R133	cagaagagtgtcccagagtc	atgccctcatgatccaggt	
VE-Cadherin	ENSMUST00000034339	L1589/R1645	gttcaagtttgcctgaagaa	gtgatgtggcgggtgtgt	
Wisp2	NM_016873.1	L297/R362	tcctctgattcttcaatgg	gtgtccaaggacaggcaca	

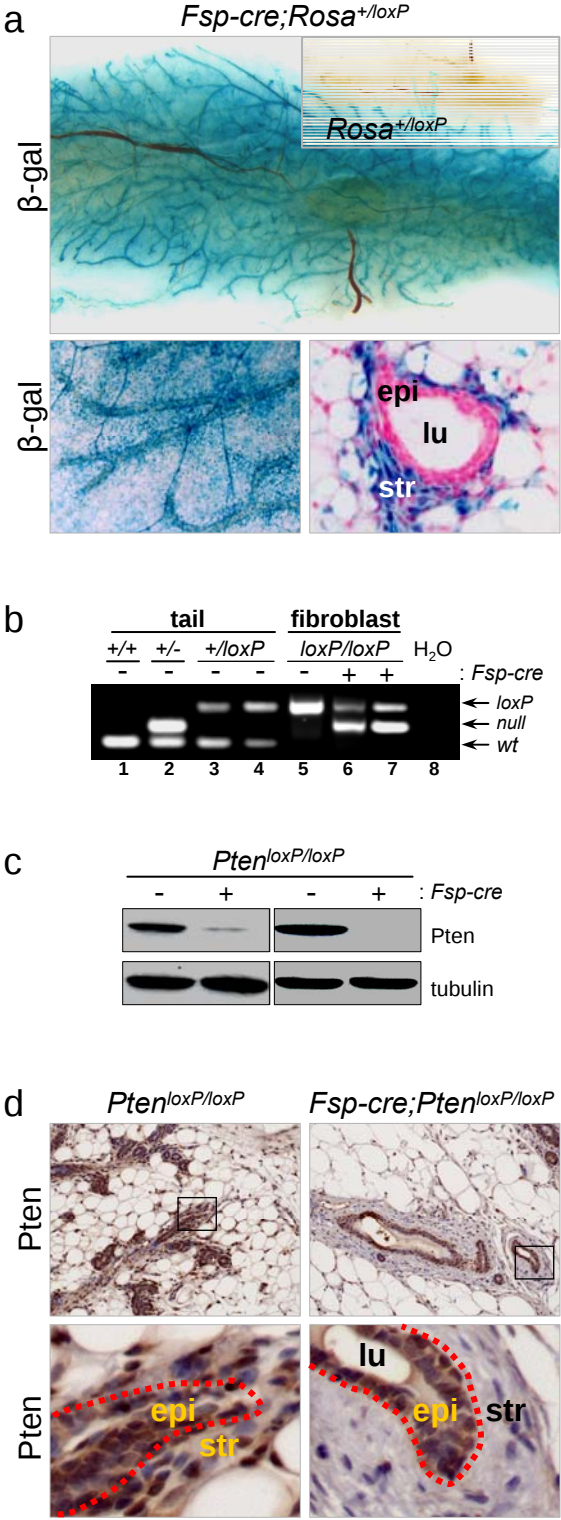
ChIP primers

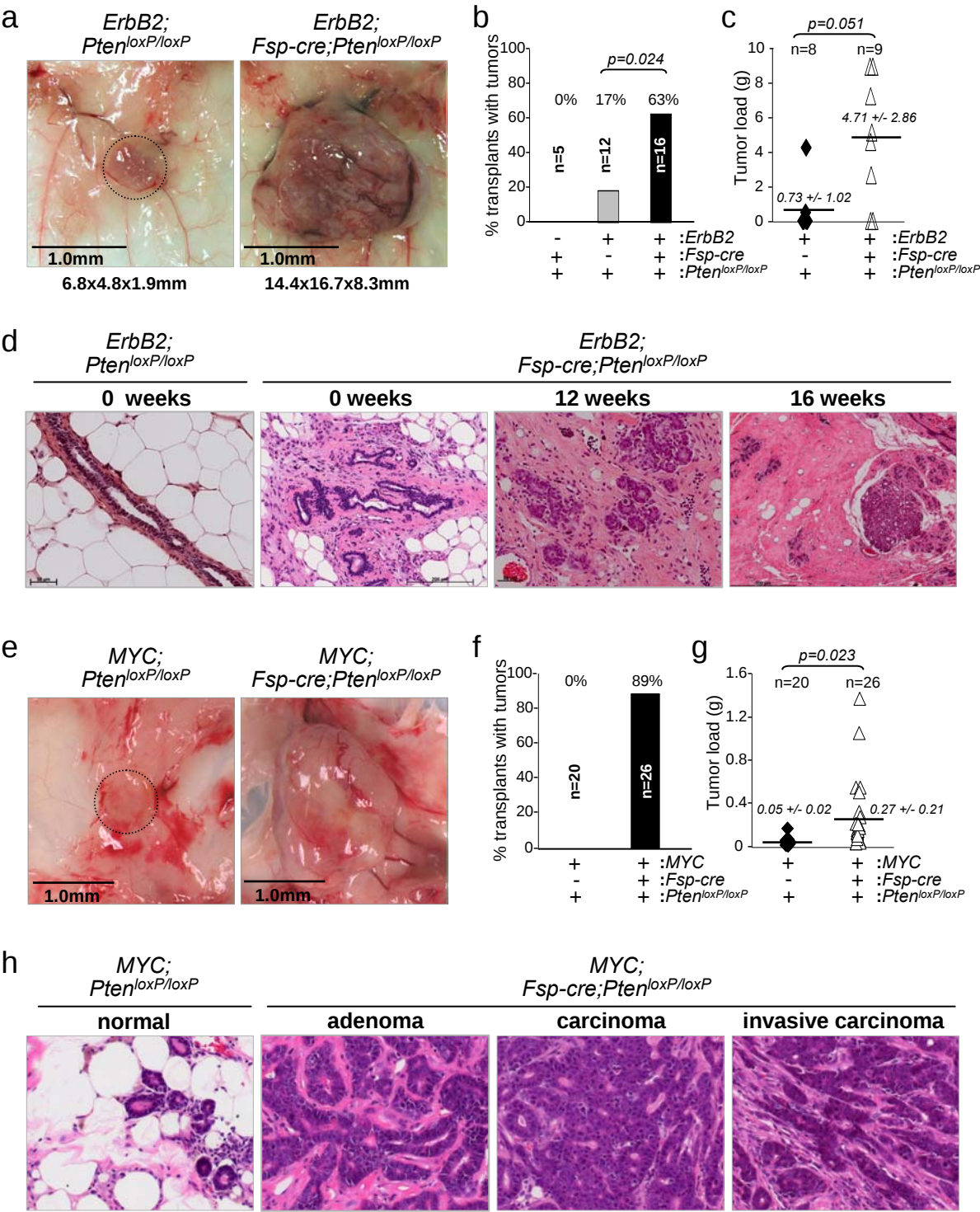
GENE	NCBI	L / R	Left primer	Right pri	
Mmp9	NT_039207	L381 / R325	gatggggcattcacctagc	gcctcaggtctccca	
Ccl3	NT_096135.5	L260 / R176	gtcctacctctctgctca	tcagctctcaactcgt	

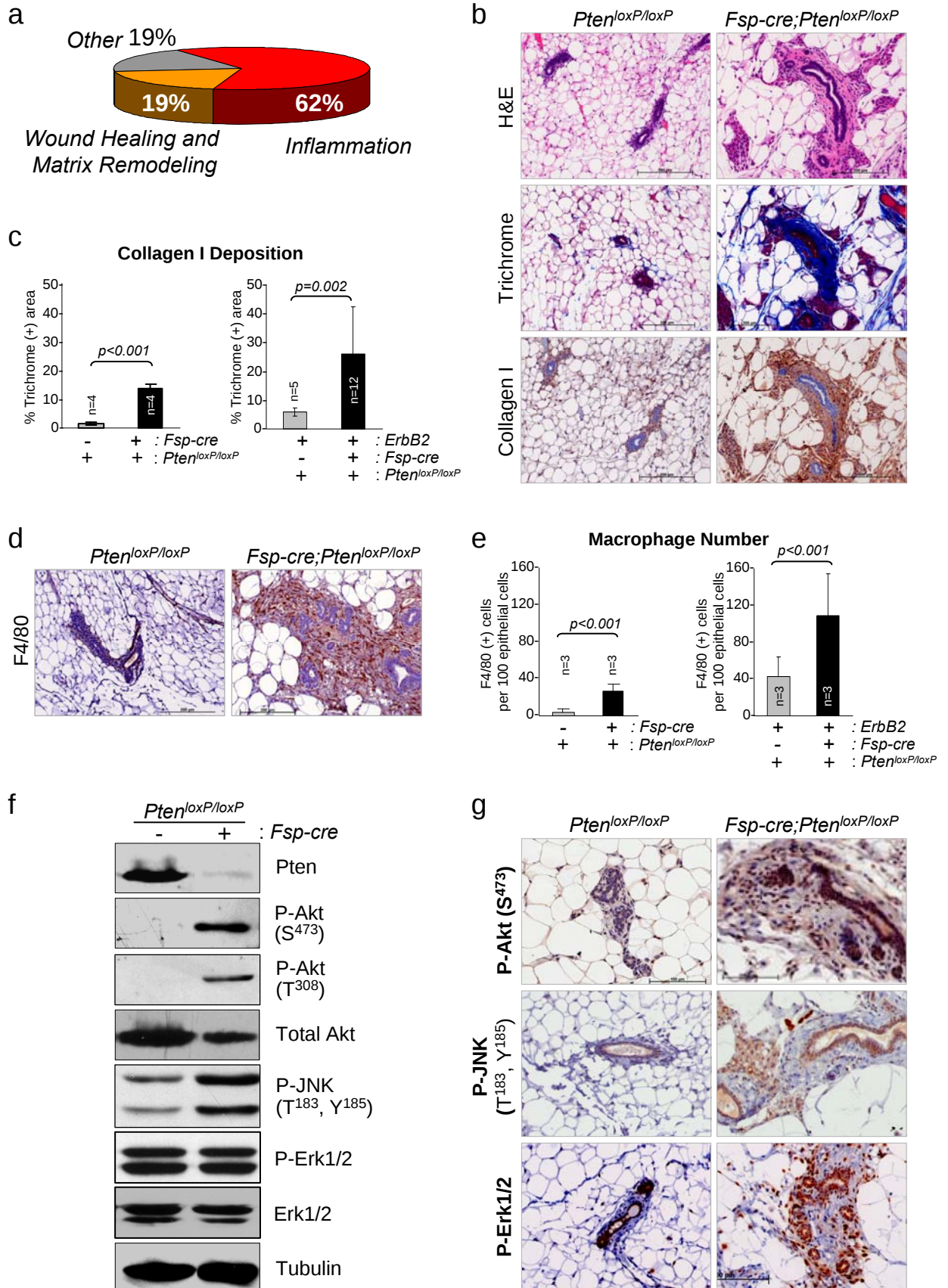
PCR genotyping

GENE	sens	Primers	Product size (bp)
MMTV-Neu	forward	GGAACCTTACTTCTGTGGTGTGAC	

<i>MMTV-Neu</i>	reverse	TAGCAGACACTCTATGCCTGTGTG	500bp
<i>MMTV-PyMT</i>	forward	CAGGGCGGGTCTGAGTCCATGG	
<i>MMTV-PyMT</i>	reverse	GGAAAGTCACTAGGAGCAGGG	200bp
<i>Fsp-cre</i>	forward	ATGCTTCTGTCCGTTTGCCG	
<i>Fsp-cre</i>	reverse	CAATGCGATGCAATTCCTC	1082bp
<i>Ets2^{db}</i>	forward	AATGACAAGACGCTGGGCGG	
<i>Ets2^{db}</i>	reverse	CGTCCCTACTGGATGACAGCGG	200bp
<i>Pten^{loxP/WT}</i>	common	GGGTTACACTAACTAAACGAGTCC	
<i>Pten^{loxP}</i>	loxP	GAATGCCATTACCTAGTAAAGCAAGG	300bp/220bp
<i>Pten^{-/-}</i>	deleted	GAATGATAATAGTACCTACTTCAG	280bp
<i>Ets2^{loxP/WT}</i>	common	CGCTTGCTAGGCAAGTGCTCTACC	
<i>Ets2^{loxP}</i>	loxP	GCTGACACAGGGTTTTGGTGTCTATGC	400/320bp
<i>Ets2^{-/-}</i>	deleted	CTAAGCCAGCCTGGCTACAGAACC	450bp
<i>Rosa26^{loxP}</i>	common	AAAGTCGCTCTGAGTTGTTAT	
<i>Rosa26^{WT}</i>	WT	GCGGGAGAAATGGATAT	550bp
<i>Rosa26^{-/-}</i>	transgene	GCGAAG-AGTTTGTCTCAACC	260bp
<i>teto-myc</i>	forward	GGAATGGCAGAAGGCAGG	
<i>teto-myc</i>	reverse	GCAGTAGCCTCATCATCACTAGATGG	580 bp
<i>MMTV-rtTA</i>	forward	AGTATGCCGCCATTATTACGAC	
<i>MMTV-rtTA</i>	reverse	CGATGGTAGACCCGTAATTGTT	170 bp







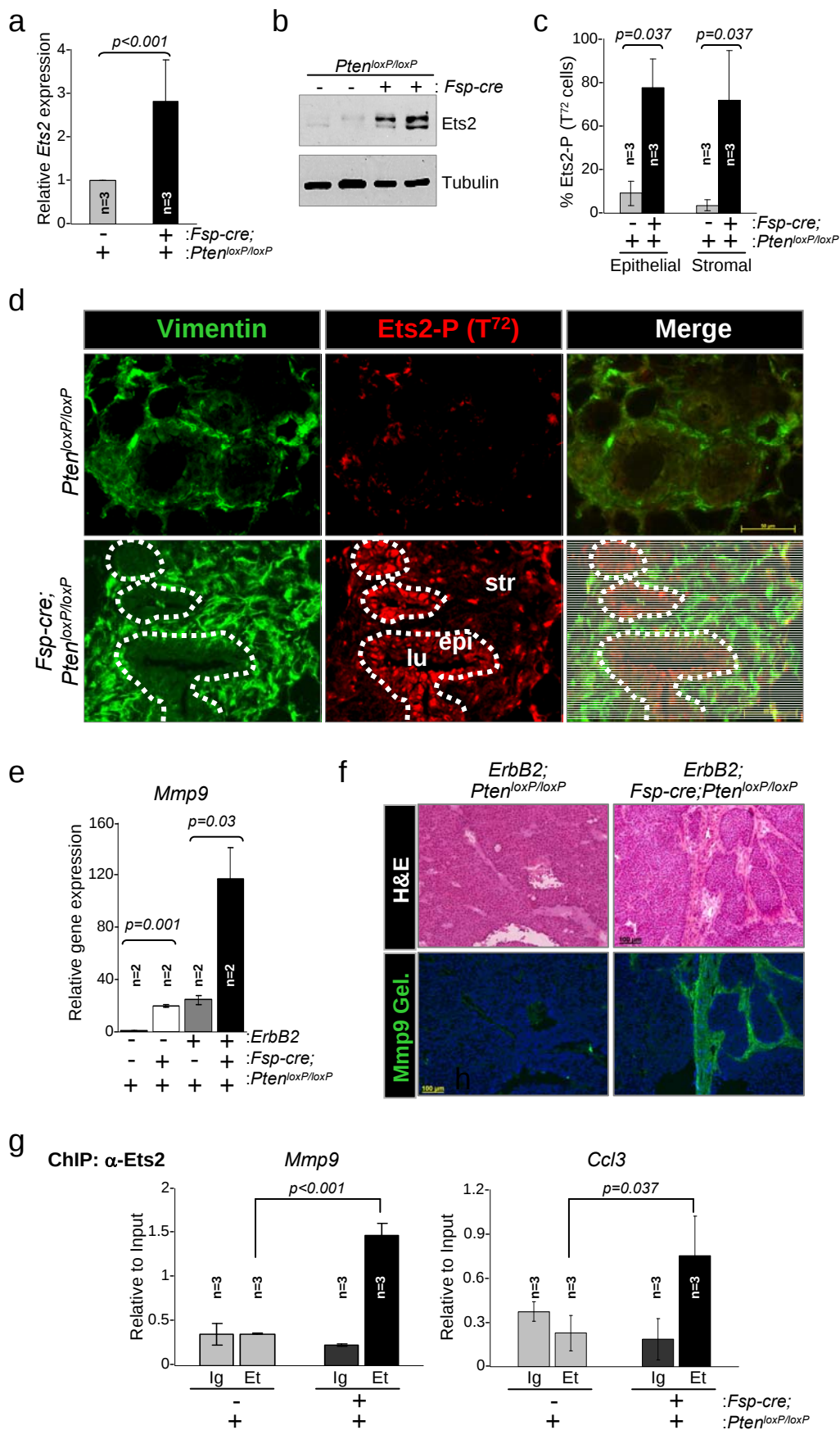


Figure 5

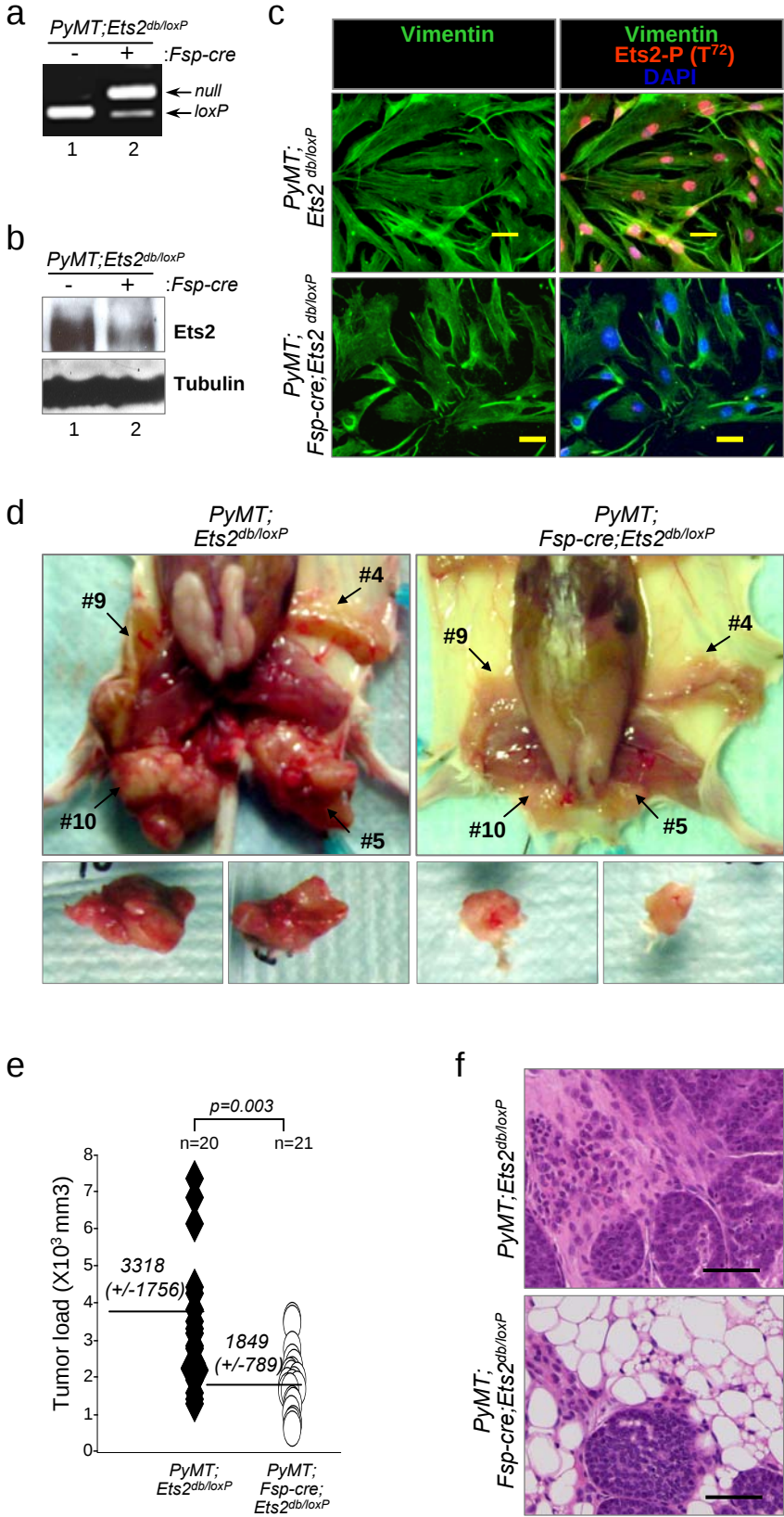
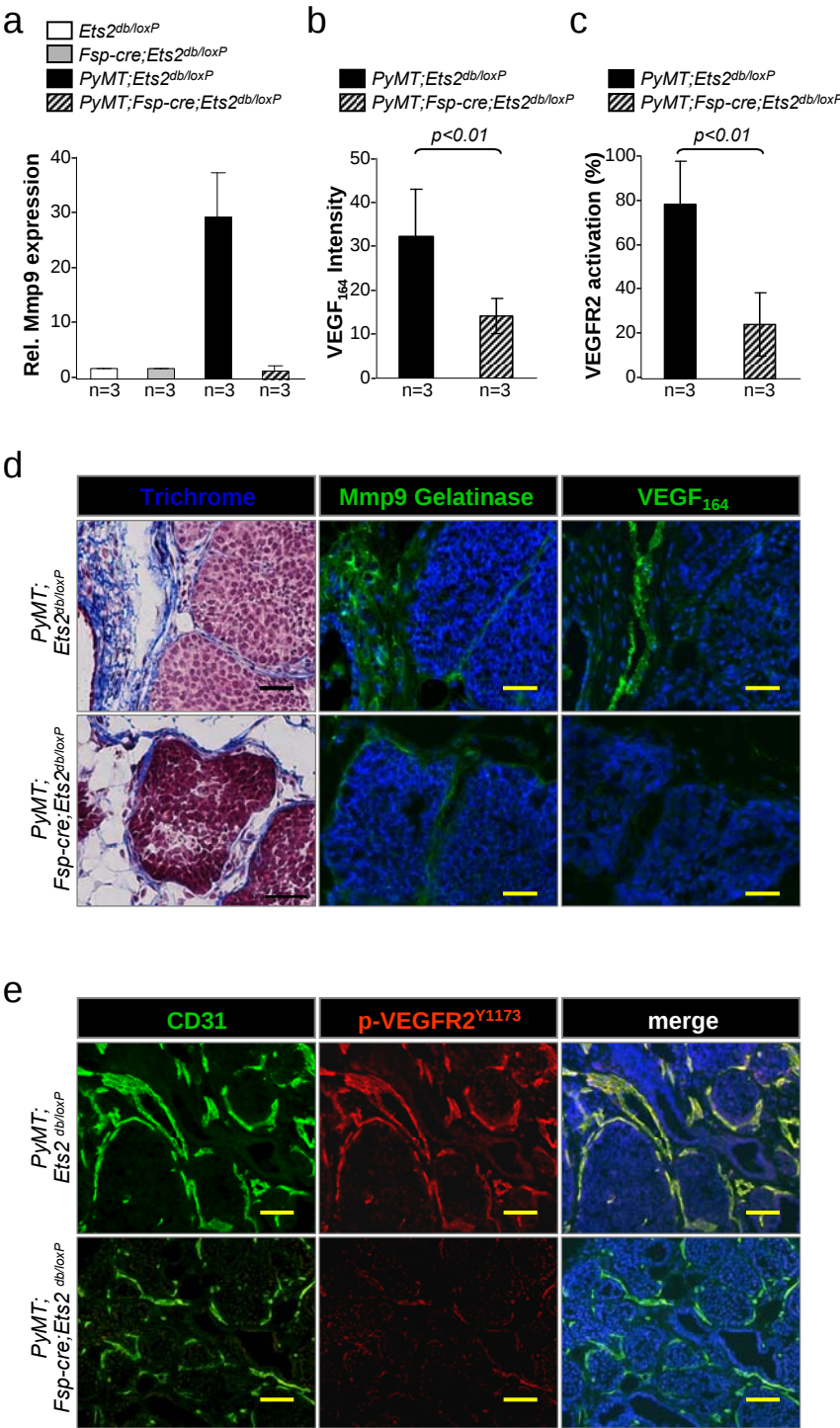


Figure 6



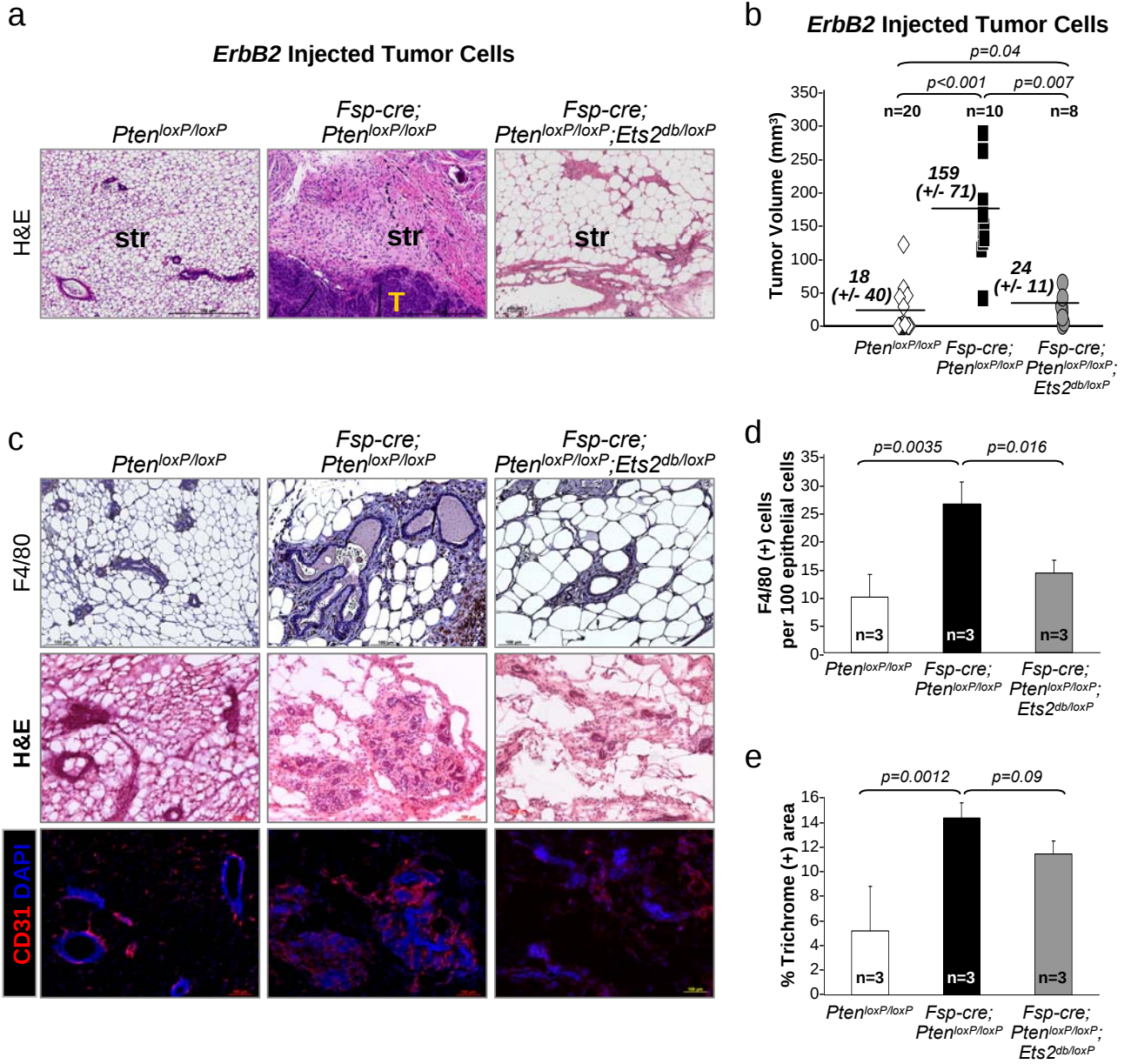
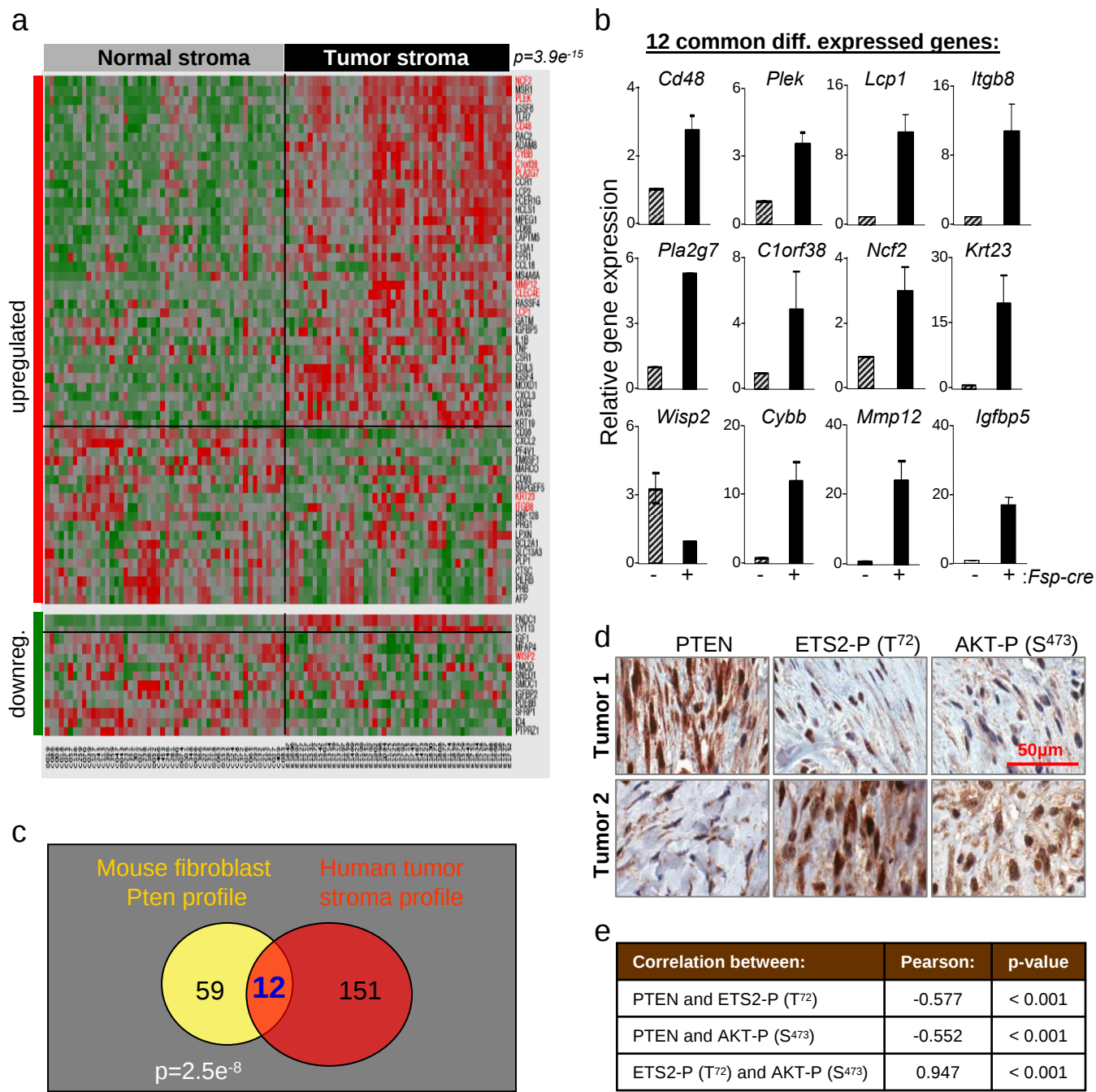


Figure 8



Generation of mice with Pten conditional knockout allele

