ANIMAL MODELS

Dual Pten/Tp53 Suppression Promotes Sarcoma Progression by Activating Notch Signaling

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Accepted for publication February 11, 2013.
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Soft tissue sarcomas are a heterogeneous group of tumors associated with poor clinical outcome. Although a subset of soft tissue sarcomas is characterized by simple karyotypes and recurrent chromosomal translocations, the mechanisms driving cytogenetically complex sarcomas are largely unknown. Clinical evidence led us to partially inactivate Pten and Tp53 in the smooth muscle lineage of mice, which developed high-grade undifferentiated pleomorphic sarcomas, leiomyosarcomas, and carcinosarcomas that widely recapitulate the human disease, including the aberrant karyotype and metastatic behavior. Pten was found haploinsufficient, whereas the wild-type allele of Tp53 invariably gained point mutations. Gene expression profiles showed up-regulated Notch signaling in Pten<sup>−/−</sup> Tp53<sup>−/−</sup> tumors compared with Pten<sup>−/−</sup> Tp53<sup>+/−</sup> tumors. Consistently, Pten silencing exacerbated the clonogenic and invasive potential of Tp53-deficient bone marrow—derived mouse mesenchymal stem cells and tumor cells and activated the Notch pathway. Moreover, the increased oncogenic behavior of Pten<sup>−/−</sup> Tp53<sup>−/−</sup> and shPten-transduced Pten<sup>−/−</sup> Tp53<sup>−/−</sup> tumor cells was counteracted by treatment with a γ-secretase inhibitor, suggesting that the aggressiveness of those tumors can be attributed, at least in part, to enhanced Notch signaling. This study demonstrates a cooperative role for Pten and Tp53 suppression in complex karyotype sarcomas while establishing Notch as an important functional player in the cross talk of these pathways during tumor progression. Our results highlight the importance of molecularly subclassifying patients with high-grade sarcoma for targeted treatments. (Am J Pathol 2013, 182: 2015–2027; http://dx.doi.org/10.1016/j.ajpath.2013.02.035)

Supported by American Cancer Society grant RSG-08-161-01-DDC, Edna’s Foundation of Hope, Spanish Ministerio de Educacion y Ciencia postdoctoral fellowship (M.V.G.), a National Cancer Center fellowship (M.V.G.), members of the NYU Cancer Institute Histopathology and Immunohistochemistry cores for NYUCI Center Support grant NIH/National Cancer Institute 5 P30CA16087-31, and the Molecular Cytogenetics core facility at Memorial Sloan-Kettering.

sarcomas (HGUPSs: the most common sarcomas diagnosed in the extremities), leiomyosarcomas (LMSs; which arise in smooth muscle), and carcinosarcomas (CSSs; a mixed epithelial/connec
tive tissue malignancy).\textsuperscript{1,3}

Despite the heterogeneity of STSs, there are hints that some alterations are shared among multiple tumor types. Individuals with Li-Fraumeni syndrome, caused by a germ-line TP53 mutation, have an increased tendency to develop STSs, and several different sarcomas have shown disruptions in the müllerian hormone type II receptor (\textit{Amhr2}).\textsuperscript{4} Individuals with neurofibromatosis type 1, who carry \textit{NF1} mutations leading to up-regulation of Ras signaling, are also predisposed to develop STS. Mouse models of these diseases replicate the broad spectrum of neoplasia seen in human patients, but it has been difficult to engineer \textit{in vivo} models that develop non-syndromic forms of sarcoma, particularly HGUPS and LMS.

There may be a requirement for multiple genetic hits (eg, intramuscular delivery of an adenovirus expressing the Cre recombinase in mice with conditional mutations in both \textit{Kras} and \textit{Tp53} initiated high-grade sarcomas with myofibroblastic differentiation).\textsuperscript{5} Similarly, uterine LMSs that arose after \textit{Tp53} and \textit{BRCA1} were conditionally deleted under the anti-müllerian hormone type II receptor (\textit{Amhr2}), and simultaneous inactivation of both genes significantly accelerated tumor progression. Despite their usefulness, these models likely represent only a portion of all HGUPSs, LMSs, and/or CSSs, given the molecular heterogeneity of these tumors. In fact, to our knowledge, there is no mouse model of CS.

We have, therefore, sought to generate mouse models based on genetic defects widely found in tumor tissue from patients with STS in the hope that these models would allow us to dissect molecular pathways frequently involved in sarcoma formation and metastasis and to test the efficacy of new therapies. Herein, we describe novel conditional mice heterozygous for \textit{Tp53} and/or \textit{Pten} in the smooth muscle (SM) lineage. These mice develop HGUPS, LMS, and CS, which recapitulate the histological and biological features of the corresponding human neoplasia, including their metastatic behavior, and provide insight into the molecular mechanisms involved in these tumors.

Materials and Methods

Generation of Knockout Mice, Animal Husbandry, and Genotyping

\textit{Pten}^{+/−}\textsuperscript{7,8} and \textit{Tp53}^{+/-}\textsuperscript{8,9} (National Cancer Institute mouse repository, Bethesda, MD) were intercrossed, and the progeny were crossed with \textit{Tagln-cre23} [\textit{Tg(Tagln-cre23)}\textit{H1Her/J}; Jackson Labs, Bar Harbor, ME] mice for smooth muscle–specific deletion of one or two allelic of \textit{Pten} and \textit{Tp53}. The mouse cohort followed up for overall survival up to 27 months included 90 animals: 13 \textit{Tagln-cre}^{−/−}\textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+}, 28 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+}, 16 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+}, 5 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/Δ}\textit{Tp53}^{Δ/+}, and 28 control mice (including 2 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+}, 7 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+}, 1 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+}, 3 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/Δ}\textit{Tp53}^{Δ/+}, and 15 \textit{Tagln-cre}^{−/+}\textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+}). Another mouse cohort was maintained to increase the amount of tumors isolated and explanted. B6;129-G\textit{t(Rosa)}26Sortm2ShoI \textit{mice (Rosa26-LSL-EGFP}, number 004077; Jackson Labs\textsuperscript{10}) and B6.129S4-G\textit{t(Rosa)}26Sortm1SorI \textit{mice (Rosa26-LSL-LaCZ, number 003474; Jackson Labs\textsuperscript{11}) were obtained. All mice were handled according to New York University (NYU) Institutional Animal Care and Use Committee approved protocols (numbers 061108-03 and 100108-01). For genotyping, tail DNA was subjected to polymerase chain reaction analysis using the primers and protocols described.\textsuperscript{7,8,10,11}

Mouse Histopathological and IHC Characteristics

Mouse tissue samples collected were fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin, according to standard protocols. Sections (5 μm thick) were prepared for antibody detection and H&E staining. Immunohistochemistry (IHC) was conducted following the standard avidin-biotin immunoperoxidase staining procedure. Diaminobenzidine was used as the chromogen, and hematoxylin was used to counterstain nuclei. Antibodies used were desmin (DER11, number 2530; Ventana Medical Systems, Tucson, AZ) and proliferating cell nuclear antigen (PCNA; PC10, number MS106; Neomarkers, Fremont, CA).

Generation of Cell Lines and Culture Conditions

Tumor fragments were minced in HBSS with antibiotics/antimycotics (Hyclone Thermo, South Logan, UT) and dissociated by incubation on 100 U/mL collagenase V (Sigma, St. Louis, MO) for 30 minutes at 37°C. Twenty-four hours later, media were changed to mouse mesenchymal stem cell medium that consists of Iscove’s modified Dulbecco’s medium (Gibco, Grand Island, NY) with 2 mmol/L l-glutamine (Hyclone Thermo), 9% fetal bovine serum (Cell Gro, Manassas, VA), and 9% donor horse serum (Cell Gro).

RNA Extraction, RT-PCR, and Real-Time PCR Analyses

Total RNA was extracted using Qiazol (Qiagen, Valencia, CA), followed by RNasea Mini Kit (Qiagen). Semi-quantitative RT-PCR was performed by reverse transcribing 500 ng of total RNA with a Taqman Assay kit (Applied Biosystems, Austin, TX), followed by standard PCR using RNA-specific primers. Quantitative real-time RT-PCR was performed using FastStart SYBR Green MasterMix (Roche, Basel, Switzerland) and BioRad iCycler equipment (Hercules, CA).

Expression Analysis

Expression profiling of \textit{Pten}^{Δ/+}\textit{Tp53}^{Δ/+} (n = 4) and \textit{Pten}^{−/+}\textit{Tp53}^{Δ/+} (n = 5) murine HGUPS was performed using the
Affymetrix Genechip system (Affymetrix, Santa Clara, CA). Total RNA was extracted with Qiazol (Qiagen), and quality and quantity were determined using a Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Nanodrop ND-1000 (Thermo Scientific, Waltham, MA). Total RNA (100 ng) was used to prepare cDNA following the Affymetrix 3’IVT Express Kit labeling protocol (Affymetrix). Standardized array processing procedures recommended by Affymetrix were performed, including hybridization, fluids processing, and scanning of the Affymetrix Mouse Genome-430 2.0 arrays. The raw data (.cel) files were normalized for probe-level summarization by the robust multichip average and analyzed by using Pavlidis template matching (P < 0.05). For these steps, GeneSpring software version GX11 (Agilent Technologies) was used. Gene set enrichment analysis (GSEA)\textsuperscript{12,13} was used to identify significantly enriched gene expression patterns underlying Pten\textsuperscript{-}/-TP53\textsuperscript{-}/- HGUPS, by querying the C2 (curated pathways) and C5 (Gene Ontologies) categories of the GSEA MSigDB, version 3.0 (Broad Institute, Boston, MA). The raw array data were deposited at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE42103).

DNA Extraction, Loss of Heterozygosity, and Sequencing Analyses

For genomic DNA extraction, formalin-fixed, paraffin-embedded (FFPE) tissues (10 μm thick) were cut and placed on uncharged glass slides. Tissues were deparaffinized with xylene, washed in ethanol, and digested with proteinase K. DNA was extracted using the Qiagen FFPE genomic DNA isolation kit. Total DNA quantity and quality were quantitated using NanoDrop ND-1000 (Thermo Scientific). Primer sequences used for DNA amplification are listed in Table 1. For sequencing, PCR fragments were cloned into pCRII-TOPO vector, and colonies were sequenced using T7 and M13R primers.

Western Blot Analysis

Cell pellets were lysed using radioimmunoprecipitation (RIPA) assay buffer (Thermo Scientific), supplemented with protease and phosphatase inhibitors (Roche). Tumors were lysed with the same buffer, adding 0.1 mg/mL aprotinin, 0.1 mg/mL leupeptin, and 0.5 μg/mL pepstatin. Protein (30 μg) was resolved on 4% to 20% Tris-glycine SDS-PAGE gels (Invitrogen, Carlsbad, CA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS)-Tween 0.01% for 1 hour, and incubated overnight at 4°C with primary antibody [PTEN, number 9552 (Cell Signaling, Danvers, MA) and tubulin, number T0206 (Sigma)]. Membranes were incubated with horseradish peroxidase—conjugated secondary antibodies for 1 hour before development using Enhanced Chemiluminescence Plus (ECL Plus), a Western blot detection kit (GE Healthcare, Piscataway, NJ).

Bone Marrow Extraction

Bone marrow (BM) was collected by flushing the long bones of murine tibias and femurs with an insulin syringe. BM was washed with HBSS twice. Cells were plated in a 75-mm\textsuperscript{2} culture dish in complete isolation media, which consisted of RPMI 1640 (Gibco, Carlsbad, CA), 9% fetal bovine serum (Cell Gro), 9% horse serum (HyClone Thermo), 100 U/mL penicillin/streptomycin (Thermo Scientific), and 2 mmol/L L-glutamine (Invitrogen). After 48 hours, adherent cells were washed with PBS and fresh complete isolation medium was added every 3 to 4 days. After 2 weeks in culture, cells were lifted with 0.25% trypsin (Gibco) and plated for different experiments in complete expansion medium (mouse mesenchymal stem cell medium).

Adenovirus Infection

BM-derived cells were infected in low serum with Ad5—CMV—Cre—green fluorescent protein (GFP). Adenoviruses were purchased from Baylor College of Medicine (Houston, TX). At 7 hours after infection, mouse mesenchymal stem cell medium was added and changed every 2 days. After 5 days, cells were plated.

### Table 1 Primers Used for Sequencing, Genotyping, and Expression Array Validation

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<tr>
<th>Gene</th>
<th>No.</th>
<th>Sequence</th>
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Lentiviral Infection

Lentiviruses were propagated using previously described methods. GIPZ short-hairpin (shPten) and empty vector were purchased from Open Bioso systems (Huntsville, AL). Cell lines were infected 3 × and selected with 1 µg/mL puromycin 48 hours after transfection to generate stable cell lines.

Clonability

A total of 0.5, 1, or 2 × 10^3 cells were seeded in 6-well plates. Medium was replaced every 3 days, and after 5 to 10 days, cells were fixed and stained with 1% crystal violet. After extensive washing, colonies were counted. When indicated, cells were treated with 500 nmol/L Compound E (ALX-270-415-C250; Enzo Life Sciences, Farmingdale, NY).

Growth Curves

A total of 1 × 10^3 cells were plated in 96-well plates. Every 2 days, cells were fixed and stained with 1% crystal violet. After extensive washing, 15% acetic acid was used to dissolve the stain, and color intensity was quantified at 595 nm. Relative growth is represented as the percentage of growth compared with the control. When indicated, cells were treated with 500 nmol/L Compound E.

Fibronectin Transwell Invasion Assay

A suspension of 8 × 10^3 cells was added to cell culture inserts (Falcon, Austin TX) containing a polycarbonate filter with 8-µm-diameter pores coated with 100 µg/mL fibronectin and 2.5% bovine serum albumin. Cells were incubated for 19 hours under standard culture conditions. Tumor cells remaining on the top side of the membrane were removed, and cells that had migrated to the underside were fixed and stained with 1% crystal violet. Five preset fields per insert were imaged and scored. When indicated, cells were treated 1 day before seeding and during the experiment with 500 nmol/L Compound E.

β-Galactosidase Staining

Tissues were fixed in 1% paraformaldehyde/0.2% glutaraldehyde in PBS containing 2 mmol/L MgCl2, 5 mmol/L EGTA, and 0.02% NP-40 at 4°C for 2 hours. After rinsing in PBS and staining with X-Gal, 4 hours, tissues were dehydrated with isopropanol and embedded in paraffin. Tissue was divided into sections (5 µm thick) and counterstained with eosin.

Fluorescence-Activated Cell Sorter Analysis

Fluorescence-activated cell sorting of primary BM cells was performed on a BD LSR II flow cytometer. Antibodies were purchased from eBioscience (San Diego, CA): c-kit APC number 17-1171-82, Stem cell antigen-1 (Sca) PE-Cy7 number 25-5981-82, Myeloid differentiation antigen (Gr1) APC-Cy7 number 47-5931-82, Macrophage-1 antigen (Mac1) APC-Cy7 number 47-0112-82, TER-119 APC-Cy7 number 47-5921-82, B220 APC-Cy7 number 47-0452-82, CD4 APC-Cy7 number 47-0042-82, CD8 APC-Cy7 number 47-0081-82, and IL7R APC-Cy7 number 47-1271-82. Data were analyzed using FlowJo software version X (10.0.0) (Tree Star, Ashland, OR).

Karyotyping

Subconfluent cultures were treated with 0.05 µg/mL Kar- 

yoMAX Colcemid (Invitrogen) for 40 to 60 minutes before harvesting. Cells were trypsinized to a single-cell suspen-

sion, pelleted at 180 × g for 8 minutes, and resuspended in warm 0.075 mol/L KCl. After an 8-minute incubation at 37°C, the hypotonic solution was diluted with three parts of Carnoy’s fixative (3:1 methanol/glacial acetic acid) and gently mixed, and the cells were pelleted as before. The supernatant was removed, and the cell pellet was loosened by gently flicking the base of the tube. The cells were then fixed in three changes of fixative. Fixed cell suspensions 

were stored at −20°C. Fixed metaphase preparations were dropped onto dry slides, and the quality of spreading was assessed by phase microscopy. Spreading was adjusted by altering the drying time, increasing local humidity (water bath), or applying heat (hotplate). Slides were then air dried and aged (at 37°C for several days or at 60°C for several hours). Aged slides were immersed in 0.08 µg/mL DAPI in 2 × standard saline citrate for 3 minutes, rinsed, air dried, mounted in antifade solution (Vectashield; Vector Labs, Burlingame, CA), and stored at 4°C. DAPI-stained slides were scanned using a Nikon E800 epifluorescence micro-

scope (Melville, NY) equipped with a digital imaging system (Applied Spectral Imaging, Vista, CA). Metaphase images were inverted to resemble conventional G-banding and karyotyped using BandView software (Applied Spec-

tral Imaging, ASI; Carlsbad, CA). Where possible, a minimum of 20 metaphases was examined for each sample. To avoid confusion, abnormal karyotypes were described according to the International System for Human Cytogenetic Nomenclature, rather than the Standardized Genetic Nomenclature for Mice, because the experimental systems are intended to model human disease.

Patients and Tumor Collection

Paraffin blocks of the tumor specimens were obtained from the NYU Pathology Department at Bellevue Hospital Center (H10457-014; New York, NY) and Northwestern University (STU7147; Chicago, IL).

Extraction of Tumor Genomic DNA

For FFPE samples, tissue sections from blocks were placed into microcentrifuge tubes (five sections, 10 µm thick), and
consecutive H&E-stained slides were obtained from each block. Tumor-enriched areas were identified, and core punches were taken from the corresponding region of the block. DNA was extracted from FFPE cores using a Qiagen DNeasy kit, according to the manufacturer’s directions.

Mass Spectrometric Genotyping

A two-step process was used as previously described. DNA extracted from FFPE blocks was quantified using picogreen analysis. Whole genome amplified DNA was used as input for multiplex PCR using primers from OncoMap 3 and OncoMap 3 Extended (Dana-Farber Cancer Institute, Boston, MA), which together compose 1047 independent assays interrogating 983 unique mutations across 116 genes. Mass spectrometric genotyping using iPLEX technology (Sequenom Inc, San Diego, CA) was performed, and candidate mutations identified were subsequently subject to a second round of homogeneous Mass-Extend, using independent primers and probes. Candidate calls were classified as aggressive or conservative, depending on their apparent robustness. Sample quality was considered adequate for analysis if >80% of the attempted genotypes resulted in identifiable products.

Statistical Analysis

Statistical significance was determined using GraphPad Prism Software version 5 (La Jolla, CA). Overall survival was analyzed by Kaplan-Meier curves, and the log-rank (Mantel-Cox) test was used to determine differences in survival. Statistical significance was defined as P < 0.05. Two-tailed Student’s t-tests were used to compare time points.

Results

PTEN and TP53 Loci Are Concurrently Deleted in Many Human LMS and HGUPS Tumors

We performed mutational analysis of 40 human LMS samples and 7 normal myometria using OncoMap3 Core and Extended panels, assessing 1047 mutations from 116 genes (Supplemental Table S1 and Supplemental Table S2). Independent validation of 73 candidate mutations on 44 genes only confirmed six mutations in two genes, EGFR and TP53, none of which co-occurred in the same tumor sample (Supplemental Figure S1A). Specifically, three (7.5%) of the LMSs harbored a low-frequency germ-line single-nucleotide polymorphism in EGFR (S703F), and another three cases (7.5%) carried mutations in TP53 (one V157F and two R273C amino acid substitutions). Overall, the incidence of hotspot mutations in the most commonly altered oncogenes and tumor suppressors was extremely low in our LMS cohort, in accordance with a recent analysis covering fewer candidate mutations.

We then searched for chromosomal aberrations in several tumor suppressor loci using published single-nucleotide polymorphism array analysis of DNA from 27 LMSs and three MFHs/HGUPPs paired with their corresponding normal tissue (Supplemental Figure S1B). (HGUPS, alias undifferentiated sarcoma, has, at times, been coextensive with MFH or malignant fibrous histiocytoma; a history of the changes in subclassification is given by Matushansky et al.) The most frequent alteration observed was partial deletion of chromosome 10 at the PTEN locus, in approximately 50% to 70% of cases. Moreover, 85% of LMSs showed a reduced copy number of PTEN and/or TP53. Strikingly, although 11% of LMS tumors displayed only TP53 loss, 67% harbored concomitant reduction of both PTEN and TP53 (Supplemental Figure S1B).

In agreement with the genomic findings, data mining of several independent expression profiles revealed that human HGUPS (undifferentiated/MFH) and LMS had significantly lower expression of PTEN and TP53 than either liposarcomas (Supplemental Figure S1C) or other STSs (Supplemental Figure S1D), and 33% of HGUPS and LMS had concomitantly reduced mRNA levels of both PTEN and TP53.

In summary, concurrent reduction of PTEN and TP53 levels, resulting from genetic deletions or chromosomal losses, is characteristic of a large subgroup of human HGUPS and LMS.

Partial Inactivation of Pten and Tp53 in the SM Lineage Leads to Various Sarcoma Subtypes with Ability to Metastasize

These findings encouraged us to investigate the differential effect of reduced gene dosage of Pten, Tp53, or combinations of both in early progenitors of the SM lineage. We, therefore, crossed Pten<sup>−/−</sup>Tp53<sup>−/−</sup> (Pten<sup>D</sup>/<sup>D</sup> and Tp53<sup>D</sup>/<sup>D</sup>) and Tagln (transgelin)-<sup>cre</sup> mice to generate all of the possible genetic combinations of mice defective in one or both alleles of these two tumor suppressors. Immunostaining of Tagln-cre Rosa26-LSL-LacZ reporter mouse<sup>11</sup> tissues revealed diffuse LacZ signal in the myometrium and in the SM layers of the bladder (Supplemental Figure S2A), confirming cre-mediated recombination in the SM of Tagln-cre mice. The progeny of the Tagln-cre x Pten<sup>D</sup>/<sup>D</sup>Tp53<sup>D</sup>/<sup>D</sup> crosses followed an almost mendelian distribution, except for Tagln-cre<sup>+</sup>/Pten<sup>D</sup>/<sup>D</sup>Tp53<sup>D</sup>/<sup>D</sup> and Tagln-cre<sup>−/−</sup>/Pten<sup>D</sup>/<sup>D</sup>Tp53<sup>D</sup>/<sup>D</sup> mice, which were deemed less viable. Tagln-cre<sup>−/−</sup>/Pten<sup>D</sup>/<sup>D</sup>Tp53<sup>D</sup>/<sup>D</sup> mice were smaller at birth and presented complex pathological characteristics, which resulted in a dramatically reduced lifespan (average, 68.2 ± 9.5 days). This is consistent with our previous report<sup>24</sup> showing that these mice develop SM hyperplasia and incipient abdominal leiomyosarcomas.

Only 4 (10%) of 40 Tagln-cre<sup>−/−</sup>/Pten<sup>D</sup>/<sup>D</sup>Tp53<sup>D</sup>/<sup>D</sup> (hereafter, Pten<sup>D</sup>/<sup>D</sup>Tp53<sup>D</sup>/<sup>+</sup>) mice developed sarcomas (Table 2), suggesting that heterozygous Pten works as a strong tumor suppressor in this context. Sarcoma penetrance in Tagln-
cre+/Pten+/-/Tp53Δ+ (hereafter, Pten+/-/Tp53Δ+) mice was higher, affecting 6 (22%) of 27 mice. Concomitant inactivation of a Tp53 and a Pten allele led to the highest sarcoma incidence, affecting 14 (38%) of 37 Tagln-cre+/PtenΔ+ Tp53Δ+ mice (hereafter, PtenΔ+/Tp53Δ+), and the shortest overall survival (244.1 ± 29 days) compared with Pten heterozygous mice (376.9 ± 33 days; P = 0.0024) and Tp53 heterozygous mice (546.4 ± 36 days; P < 0.0001) (Figure 1A). Partial loss of Pten, thus, synergizes with Tp53 heterozygosity in sarcomagenesis by accelerating tumorigenesis, progression, or both.

Histopathological evaluation of PtenΔ+/Tp53Δ+ and Pten+/- Tp53Δ+ tumors led to the diagnosis of a variety of lesions (Table 2), including HGUPS, LMS, and CS, predominantly affecting the genitourinary tract and the retroperitoneum (Figure 1B). HGUPS displayed cellular atypia, hypercromatic nuclei, numerous aberrant mitoses, and local areas of necrosis (Figure 1C). LMSs were characterized by spindle cells with cigar-shaped nuclei, arranged in intersecting bundles and positive for SM markers, such as desmin (Figure 1C) or smooth muscle actin (data not shown). CSs were composed of their characteristic mixed areas of mesenchymal and epithelial origin, as shown by positivity for desmin (Figure 1C) and cytokeratins (data not shown), respectively. The proliferative index as per PCNA immunostaining was similar in HGUPS, LMS, and CS (approximately 60%). Pten+/-/Tp53Δ+ sarcomas included 50% LMS and 33.3% HGUPS, whereas PtenΔ+/Tp53Δ+ mice had a higher

<table>
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<td>6/27 (22.2)</td>
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*Data are given as number/total (percentage).

+, wild-type allele; Δ, floxed allele; CS, carcinosarcomas; HGUPS, high-grade undifferentiated pleomorphic sarcomas; LMS, leiomyosarcomas.

Figure 1  Conditional Pten and Tp53 heterozygous deletion in the SM lineage leads to HGUPS, LMS, and CS. A: Survival plot for mice with the indicated genotypes as a function of days. A statistically significant decrease in lifespan was found for Pten+/Tp53Δ+ mice compared with Pten+/Tp53Δ+ mice (***P = 0.0024) and Pten+/-/Tp53Δ+ mice (**P < 0.0001). B: Macroscopic images of a uterine LMS in a Pten+/Tp53Δ+ mouse (top panel) and an abdominal HGUPS in a Pten+/Tp53Δ+ mouse (bottom panel). C: H&E and IHC stainings of sections of the three sarcoma subtypes found on Pten+/Tp53Δ+ mice with antibodies against desmin and PCNA. The percentage of PCNA-positive cells is indicated. Scale bars, ×20 (250 μm); ×40 (125 μm). D: H&E and IHC staining for desmin in metastases found in the indicated organs. The top two rows correspond to a Pten+/Tp53Δ+ LMS, and the bottom panel depicts a Pten+/Tp53Δ+ CS. E: Top panel, scheme of primers used for Pten and Tp53 PCR-based assays to genotype or sequence for mutations and/or deletions. ORF, open reading frame. Bottom panel, electrophoresis of PCR products from genomic DNA isolated from tumor tissue using the indicated primer pairs. Floxed denotes the recombined allele after cre-mediated recombination. F: Gel electrophoresis of PCR amplification products for Tp53 (top two lanes) and Pten (bottom five lanes) in DNA from short-term cultures of the corresponding murine tumors. In parenthesis, primer pairs used for detection. GAPDH is used as a loading control.
incidence of undifferentiated sarcomas (64% HGUPS and 21% LMS). CS incidence was 22% and 14%, respectively (Table 2). Both mouse lines also developed nonmesenchymal tumors, as summarized in Supplemental Table S3.

Some PtenΔ/+T5p3Δ/+ mice and PtenΔ/+T5p3Δ/+ tumors (particularly the CSs) were able to colonize distant organs, including liver, pancreas, or lungs (Figure 1D), but metastases were detected much earlier in the life of PtenΔ/+T5p3Δ/+ mice. This observation underlines the ability of the PtenΔ/+T5p3Δ/+ model to recapitulate the highly aggressive behavior of human sarcomas, which normally display hematogenous spread through the portal system to reach distal organs.

Pten But Not Tp53 Is Haploinsufficient for Sarcoma Tumor Suppression in PtenΔ/+T5p3Δ/+ Mice

We asked whether sarcoma formation associates with loss of heterozygosity of the remaining Pten and/or Tp53 wild-type alleles in PtenΔ/+T5p3Δ/+ and PtenΔ/+T5p3Δ/+ mice. The wild-type allele of Pten was occasionally found partially deleted (3 of 10 PtenΔ/+T5p3Δ/+ and 0 of 4 PtenΔ/+T5p3Δ/+), and sequencing did not reveal point mutations (Figure 1, E and F, and Supplemental Table S4). The remaining Tp53 allele, however, was invariably found mutated in short-term cultures derived from resected murine tumors (six of six cases), recurrently in the DNA-binding domain (Supplemental Table S4). This suggests that Tp53 must be fully inactivated to allow sarcomagenesis initiated by Pten heterozygosity to proceed. We conclude that Pten is generally haploinsufficient for sarcoma tumor suppression, whereas there is strong selective pressure to completely abrogate Tp53 function to allow sarcoma development.

Interestingly, tumor cells from PtenΔ/+T5p3Δ/+ and PtenΔ/+T5p3Δ/+ mice showed numerous structural and numerical changes (Supplemental Figure S3). These tumors, thus, recapitulate the chromosomal instability characteristic of the corresponding human lesions.

Pten Down-Regulation in PtenΔ/+T5p3Δ/+ Bone Marrow–Derived Mesenchymal Stem Cells and in Tumor Cells Increases Their Clonogenic and Invasive Capacity

PtenΔ/+T5p3Δ/+ tumors progress much more rapidly than PtenΔ/+T5p3Δ/+ lesions. To determine which advantage Pten suppression confers on T5p3-deficient murine tumors, we compared the growth properties of BM-derived mesenchymal stem cells (MSCs) from these mice, as a surrogate of the plausible cell of origin of HGUPS and LMS.27,28 We first confirmed that a fraction of BM-MSC and their progeny are targeted in our mouse model on Cre recombination using fluorescence-activated cell sorter analysis of Tagln-cre/Rosa26-LSL-EGFP mice BM. This analysis showed 6.46% ± 0.52% (n = 4) of Lin−Sca1−c-kit− cells, which encompass the MSC pool, as positive for GFP (Supplemental Figure S2B). Then, BM-MSCs were isolated from PtenΔ/+T5p3Δ/+ and PtenΔ/+T5p3Δ/+ mice, and recombination was acutely triggered by Ad5-CMV-Cre-GFP infection in vitro. We observed that after infection, PtenΔ/+T5p3Δ/+ MSCs displayed significantly higher clonogenic potential (Figure 2A) and proliferative capacity (Figure 2B) than PtenΔ/+T5p3Δ/+ or wild-type MSCs. These results were confirmed in MSCs isolated from the corresponding PtenΔ/+T5p3Δ/+ and PtenΔ/+T5p3Δ/+ mice, in which recombination had occurred genetically, in vivo (data not shown).

To determine whether the enhanced oncogenic properties of PtenΔ/+T5p3Δ/+ tumor cells were caused by Pten down-regulation, we stably infected two cell lines derived from PtenΔ/+T5p3Δ/+ HGUPS with a lentivirus carrying a short-hairpin RNA for Pten (shPten). In both cell lines, reduced Pten expression (Figure 2C) was accompanied by a significant increase in colony formation (Figure 2D) and cell invasion (Figure 2E), without major effects in proliferation (Figure 2E). These in vitro properties are consistent with the more aggressive tumor behavior and reduced overall survival observed in PtenΔ/+T5p3Δ/+ mice; they further indicate that the differences between PtenΔ/+T5p3Δ/+ and PtenΔ/+T5p3Δ/+ tumor behavior can be directly attributed to lower Pten levels.

Notch Signaling Is Increased in PtenΔ/+T5p3Δ/+ Murine HGUPS Compared with PtenΔ/+T5p3Δ/+ Tumors

We compared the gene expression profiles of PtenΔ/+T5p3Δ/+ (n = 4) and PtenΔ/+T5p3Δ/+ (n = 5) murine HGUPS tissues to identify transcriptional changes that could account for their distinct phenotypic behavior. Functional annotation (DAVID)27,28 and GSEA12,13 revealed that the most significant up-regulated pathways in PtenΔ/+T5p3Δ/+ tumors were Notch (P = 0.012), Akt (P = 0.011), mitogen-activated protein kinase (MAPK; P < 0.0001), and NF-kB (P < 0.0001) (Figure 3, A and B). Further analyses of individual genes within those pathways showed Notch1, Pdk1, Jak1, Tradd, Map3k9, Pak2, and Ripk1 as significantly up-regulated (Figure 3C). Array data were validated by quantitative real-time PCR for Notch1, Jak1, and Ripk1 in PtenΔ/+T5p3Δ/+ and PtenΔ/+T5p3Δ/+ tumor-derived cell lines (Figure 3D). These data demonstrate that partial loss of Pten in a T5p3 heterozygous context results in a distinct molecular profile, which includes up-regulation of Notch signaling.

Increased Notch Signaling Partially Accounts for the Greater Invasive and Clonogenic Potential of PtenΔ/+T5p3Δ/+ Murine HGUPS

Because the Notch pathway exerts key roles in tumorigenesis,29 maintains MSCs in an undifferentiated state,30 and can be targeted pharmacologically,31 we selected it for further analyses. We sought to determine whether Notch signaling was responsible for the increased invasive and clonogenic potential of PtenΔ/+T5p3Δ/+ tumor cells compared with PtenΔ/+T5p3Δ/+ cells. Indeed, treatment with the γ-secretase
inhibitor (GSI) Compound E, which suppressed Hes1 levels (Figure 4A), reduced the invasive capacity of Pten\(^{+/+}\)Tp53\(^{+/+}\) cells (Figure 4B), suggesting that these tumors particularly rely on Notch signaling for their aggressive behavior. Surprisingly, treatment of Pten\(^{+/+}\)Tp53\(^{+/+}\) cells had the opposite effect (Figure 4B). Furthermore, treatment with this GSI counteracted the ability of shPten, which induced Hes-1 levels (Figure 4C), to enhance the clonogenic and invasive potential of Pten\(^{+/+}\)Tp53\(^{+/+}\) sarcoma cells (Figure 4, D and E). These results demonstrate that Notch activation subsequent to Pten silencing in a Tp53-deficient background contributes to the increased aggressiveness of double-heterozygous tumors. In sum, higher Notch signaling is characteristic of HGUPS that have dual Pten/Tp53 suppression, and it may contribute to a faster disease course in patients with this genetic makeup.

**Discussion**

The extremely low frequency of point mutations in conventional oncogenes, highlighted by our extensive analysis of tumor samples, has typically limited our ability to model LMS and HGUPS in mice. Herein, we demonstrate that concurrent chromosomal deletions encompassing the Pten and Tp53 loci, which are common hallmarks of human HGUPS and LMS,\(^{16}\) act synergistically to initiate these tumors in our genetically engineered mice. Similar genomic alterations in some LMS and HGUPS\(^{32}\) suggest that they may share common pathways of transformation. Our large-scale mutation profiling of human LMS using the Oncomap platform found nucleotide changes in TP53 and EGFR in 6 of 40 patients; other rare mutations previously reported for HGUPS and LMS were not found in our cohort, including those in PTEN and HRAS.\(^{33-36}\) These findings underline the low incidence of recurrent point mutations in classic oncogenes and tumor suppressors characteristic of these tumors. In contrast, published single-nucleotide polymorphism and gene expression arrays reveal frequent concurrent 10q22-23 genomic losses and down-regulation of TP53 and TP53\(^{37-42}\) providing a rationale for genetically engineering these lesions in mice to model HGUPS and LMS.

Genetic alterations of TP53 and Pten are among the most frequent causal events in many cancers, and their combined inactivation has shown strong oncogenic potential in other mouse models. Loss of Tp53 alone was sufficient to initiate sarcomas in our model, although tumor progression was dramatically accelerated in conjunction with Pten inactivation. Conditional inactivation of both Pten and Tp53 in other mouse models has been shown to cause metastatic bladder cancer,\(^{43}\) glioblastoma,\(^{44}\) and invasive, but not metastatic, prostate cancer.\(^{7}\) In contrast to our findings, those studies showed Tp53 depletion to be generally insufficient for transformation: both alleles of Tp53 and Pten needed to be inactivated to generate tumors that recapitulate the biological
behavior of the corresponding human lesions. These differences in results suggest that there are context-dependent tumor suppressor roles for \( \text{Tp53} \) and \( \text{Pten} \) in epithelial and mesenchymal transformation and tumorigenesis.

Complete Pten inactivation has been shown to trigger a prosenescence response (Pten-initiated cellular senescence), which acts as a potent antitumoral barrier \( \text{in vivo} \) that can be bypassed by \( \text{Tp53} \) genetic silencing.\(^7\) In our model, partial Pten inactivation was accompanied by \( \text{Tp53} \) point mutations in both \( \text{Pten}^{+/+} \text{Tp53}^{+/+} \) (data not shown) and \( \text{Pten}^{+/+} \text{Tp53}^{+/+} \) murine tumors. This could explain why human sarcomas with complex genetics do not select for complete loss of PTEN; partial loss has been reported to occur in 39% of 111 LMS and HGUPS\(^33\)—and emphasize the significance of PTEN haploinsufficiency for sarcoma initiation and progression.

Partial Pten/Tp53 inactivation in our mice led to multiple histological phenotypes (HGUPS, LMS, and CS), suggesting a common molecular \(^32\) and a common cellular origin for these tumors. The appearance of multiple histological phenotypes characterized by various degrees of differentiation might be explained by targeting of different cells of origin that represent various stages of differentiation along the SM lineage, each producing a specific histological phenotype. Alternatively, it could be that a specific cell of origin, perhaps a progenitor, subsequently differentiates into multiple tumor phenotypes through the accumulation of stochastic secondary events. In fact, the variety of tumors observed, ranging from HGUPS to well-differentiated LMS, may also reflect how transforming events affect the ability of a single precursor cell to differentiate.

In recent years, there has been much debate over the cell of origin for various sarcoma subtypes, including synovial, Ewing, and HGUPS. Xenografted and genetically engineered mouse models\(^\text{25,46,47} \) have revealed some of these tumors to be derived from mesenchymal stem cells or progenitors, rather than somatic, differentiated cell types. We propose that cre-mediated inactivation of Pten or Tp53 target MSC-like cells committed toward the SM lineage. The fact that there were no other tumors of mesenchymal origin (ie, liposarcomas, osteosarcomas, or chondrosarcomas) supports this hypothesis. It is also possible, however, that other mesenchymal lineages, such as the adipocytic or chondrocytic, are not as susceptible to transformation triggered by Tp53 and/or Pten defects. The appearance of carcinosarcomas is particularly puzzling: they might originate from a common epithelial/mesenchymal precursor within the uterus,\(^\text{48} \) or the carcinomatous component might result from transgene expression in some epithelial cells of the uterus, supporting a combined tumor model.\(^\text{49} \) A third possibility is that transformation of the epithelium could be non-cell autonomous, resulting from aberrant signaling from the surrounding sarcoma cells.

One of the salient characteristics of our genetically engineered mouse model is its spontaneous development of metastases. We found metastases in both \( \text{Pten}^{+/+} \text{Tp53}^{+/+} \)
and Pten\textsuperscript{\textasciitilde}+/+Tp53\textasciitilde+/+ mice, although the combined inactivation of Tp53 and Pten defines a subgroup of tumors with much faster dissemination. This suggests a particularly aggressive pattern of Pten/Tp53-inactivated tumors, as supported by our in vitro data.

Pten suppression in a Tp53 heterozygous background strongly promoted the clonogenic capacity of MSCs and the invasive potential of established murine tumor cell lines. Faster proliferation was also apparent in MSCs in response to partial Pten/Tp53 conditional inactivation, but not in already established Pten\textasciitilde+/+Tp53\textasciitilde+/+ tumors, perhaps because of additional hits acquired during tumor progression, which are partly redundant with the proliferative effects of Pten suppression. Nonetheless, the suppression of Pten in a Tp53-inactivated background induced more malignant behavior and more rapid disease course.

Expression profiling of Pten\textasciitilde+/+Tp53\textasciitilde+/+ and Pten\textasciitilde+/+Tp53\textasciitilde+/+ tumors revealed an up-regulation of the MAPK pathway on Pten inactivation. Aberrant MAPK signaling occurs in many human malignancies, where it promotes tumor proliferation and metastasis. In particular, Ras, a central signal transduction mediator, has been previously involved in carcinogenesis.\,\textsuperscript{5} Therefore, selective inhibition of the MAPK pathway, currently possible through highly specific compounds being tested in the clinic (ie, BRAF and MEK inhibitors), may serve as a viable therapeutic approach against double Pten/Tp53-defective HGUPSs.

In addition, the microarray profile showed a significant up-regulation of the Notch signaling pathway in Pten/Tp53 heterozygous tumors compared with Pten\textasciitilde+/+Tp53\textasciitilde+/+ heterozygous tumors. Furthermore, the increased invasive and clonogenic potentials conferred by Pten suppression (or shPten) were largely dependent on Notch signaling. The highly conserved Notch pathway plays critical roles in development and cancer in a cell-specific manner, leading to oncogenic or tumor-suppressive effects in different contexts.\,\textsuperscript{29} Tumor-promoting effects of Notch signaling have been described in human osteosarcoma,\,\textsuperscript{50} Ewing sarcoma,\,\textsuperscript{51} and rhabdomyosarcoma,\,\textsuperscript{52} and they have recently been implicated in the regulation of self-renewal in HGUPS.\,\textsuperscript{53} A link between NOTCH1 and PTEN deregulation has been reported in human melanoma, in which tumors lacking PTEN show hyperactivated phosphatidylinositol 3-kinase/AKT signaling and NOTCH1 up-regulation via NF-\kappaB.\,\textsuperscript{54} Interestingly, the NF-\kappaB pathway was found significantly up-regulated in Pten\textasciitilde+/+Tp53\textasciitilde+/+ mice compared with Pten\textasciitilde+/+Tp53\textasciitilde+/+ mice, according to our array data (Figure 3). Conversely, in other tumor models, such as T-ALL, phosphatidylinositol 3-kinase—AKT has been shown to contribute to NOTCH1-induced transformation, with the PTEN status determining the cellular response to GSI.\,\textsuperscript{55} In our system, treatment of Pten/Tp53 heterozygous HGUPSs with a GSI strongly inhibited their in vitro oncogenic properties, in contrast to Tp53 heterozygous HGUPSs that
paradoxically showed opposite effects. These data lend support to the notion that context is important for determining the effects of Notch signaling and inhibition.

At present, most HGUPSs and LMSs are treated with similar chemotherapy regimens, despite their histological and molecular heterogeneity.18,56 Previous observations in a Pten-null murine LMS model24 and data not shown revealed antiproliferative effects of mTor inhibition in both Pten-wt and Pten-het murine HGUPS. However, inhibition of mTOR has been deemed incapable of achieving durable therapeutic responses in patients with sarcoma,57 suggesting the possibility of feedback and compensatory effects, such as those shown in other tumor types.58 Our observation that Pten het/Tp53 het murine tumors are particularly sensitive to Notch inhibition raises the possibility that HGUPS or LMS patients bearing this genetic makeup might be successfully treated with NOTCH inhibitors either alone or in combination with mTOR inhibitors. In contrast, this treatment might be harmful for Pten wt/TP53 het cases, according to our preclinical studies.

These observations suggest that classifying HGUPS and LMS patients based on tumor genetic and expression criteria could improve patient outcomes by predicting disease progression and providing a decision-making tool for designing individualized treatments (as proposed in Supplemental Figure S4).

Acknowledgments

We thank Martha Vega for genotyping, Elizabeth Charytonowicz and Xavier Jirau-Serrano for supervising the mouse colony, members of the Affants laboratory (Camille Loubry, Jasper Mullenders, and Beatriz Aranda-Orgilles) for reagents and discussions, and Jordi Barretina and Emmanuelle Palescandolo (Broad Institute) for technical advice.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpather.2013.02.035.

References


